Regulation and function of the tumour suppressor 53BP1 at sites of DNA damage

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To my Grand-father,

and my Godmother

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Abbreviations

53BP1: p53 Binding Protein 1

9-1-1: RAD9-RAD1-HUS1

aa: amino acid

ASH1: Absent, Small or Homeotic discs 1

ATM: Ataxia Telangiectasia Mutated

ATR: Ataxia Telangiectasia and RAD3 related

ATRIP: ATR interacting protein

BAT3: HLA-B associated transcript 3

BBAP: B-lymphoma- and BAL-associated protein

BER: Base Excision repair

BLAST: Basic Local Alignment Search Tool

BLM: Bloom syndrome, RecQ helicase-like

bp: base pair

BRCA1: Breast cancer associated gene 1

BRCT: Breast cancer susceptibility protein

BSA: Bovine Serum Albumin

CDC25: cell division cycle 25

cDNA: complementary DNA

CDK: Cyclin-Dependent Kinase

CHK (1/2): checkpoint kinase (1/2)

CPT: camptothecin

CSR: Class Switch Recombination

DAPI: 4',6-diamidino-2-phenylindole

DDR: DNA Damage Response

DMEM: Dulbecco's Modified Eagles Medium

DNA: Deoxyribonucleic Acid

DNA-PK: DNA-dependent protein kinase catalytic subunit

DOT1: Disruptor Of Telomere silencing 1

DSB: double strand break

dsDNA: double stranded DNA

EDTA: Ethylenediaminetetraacetic acid

ES cells: Embryonic Stem cells

EST: expressed sequence tag

EXO1: Exonuclease 1

FACS: Fluorescence-Activated Cell Sorting

FITC: Fluorescein Isothiocyanate

g: gram(s)

G1: Gap phase 1

G2: Gap phase 2

GAR: glycine arginine rich

γH2AX: phosphorylated H2AX (ser139)

Gy: Gray

h: hour

H (3/4): Histone H(3/4)

H3K79me2: Histone H3 lysine 79 di-methylated

H4K20me2: Histone H4 lysine 20 di-methylated

HCl: hydrochloric acid

HMTase : Histone Methyltransferase

HR: Homologous Recombination

IF: Immunofluorescence

IR: Ionizing Radiation

IRIF: Ionizing Radiation-Induced Foci

min: minute

kb: kilo base

kDa: kilodaltons

µFD: microfarad

MDC1: mediator of DNA damage checkpoint 1

MEF: Mouse Embryonic fibroblast

ml: milliliter

mM: millimolar

MMR: mismatch repair

MMSET: multiple myeloma SET domain containing protein type II

MRN: Mre11-Rad50-Nbs1

mRNA: messenger RNA

NaCl: Sodium Chloride

NER: Nucleotide Excision repair

ns: non-significant

NSD(1/2): Nuclear receptor binding SET domain protein (1/2)

N-ter: amino terminus

N-terminal: amino terminus

NHEJ: Non-Homologous End Joining

p53K382me2: p53 lysine 382 di-methylated

PBS: Phosphate Buffered Saline

PCNA: Proliferating Cell Nuclear Antigen

PCR: Polymerase Chain Reaction

PFA: Paraformaldehyde

PI: Propidium Iodide

PIKK: Phosphatidylinositol 3-kinase like kinase

PP2P: protein phosphatase 2p

PRDM6: PR domain containing 6

PTM: Post-Translational Modification

RACE-PCR: rapid amplification of cDNA ends PCR

RAP80: receptor associated protein 80

RFC: Replication Factor C

RNA: Ribonucleic Acid

RNAse: Ribonuclease

RNF (8/168): ring finger protein 8/168

RPA: Replication protein A

RPMI: roswell Park Memorial Institute medium

SDS: Sodium Dodecyl Sulfate

SAM: S-Adenosyl Methionine

SEM: standard Error of the Mean

ser: serine

shRNA: short hairpin RNA

siRNA: small interfering RNA

ssDNA: single stranded DNA

SUV420: Suppressor of Variegation 4-20

TLS: Translesion synthesis

TopBP1: TopoisomeraseII binding protein I

TRITC: tetramethyl-rhodamine isothiocyanate

Triton: t-octylphenoxypolyethoxyethanol

TT domain: Tandem Tudor domain

V: Volt

WRN: Werner syndrome, RecQ helicase-like

WT: wild type

ZWINT: ZW10 interactor protein-1

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Thesis declaration

Declaration of contribution:

I declare that I have not obtained a previous qualification from NUI Galway or elsewhere based upon any of the work contained in this thesis.
I both conducted the experiments presented and wrote the thesis under the supervision of Professor Noel Lowndes.
The knock-out experiments in Figure 2.2.A and 2.6.A were initialized in collaboration with Jennifer Fitzgerald.

Abstract

Cancer is the major cause of death for people in middle age. It results from cell transformation into malignant cells and propagates with normal controls. This process is induced by mutations occurring in DNA through the action of external or internal DNA damaging agents. To protect their genome, cells have evolved mechanisms to respond appropriately to the DNA lesion induced. A signaling cascade is activated, implicating detection of the lesions by "sensor" proteins, amplification of the signal by "mediator" and "transducer" proteins and diversification of this signal by "effector" proteins involved in various pathways including cell cycle arrest, DNA repair, apoptosis or senescence.

53BP1, first identified as a p53 binding partner, is a mediator of the DNA damage response (DDR). It functions in cell cycle checkpoints and DNA repair, promoting non-homologous end-joining (NHEJ) and restraining homologous recombination (HR). Upon DNA damage, 53BP1 proteins relocalize rapidly to DNA double strand breaks (DSBs). It has been shown that this recruitment is dependent on its Tandem Tudor domain recognition and binding to methylated histones. However, some controversy exist in the literature regarding the exact nature of the methylated histone required. It has been defined to be either histone H3 dimethylated on its lysine 79, H3K79me2, or histone H4 dimethylated on its lysine 40, H4K20me2.

Firstly, we investigated the role of H3K79me2 in the recruitment of 53BP1 to DSBs. We generated a DT40 chicken cell line deficient for H3K79me2 and monitored 53BP1 foci formation upon DNA damage. Our data indicated that H3K79me2 is not required for the general recruitment of 53BP1 to DSBs. Technical limitations restrained our investigation on the role of H4K20me2 in 53BP1 relocalisation. However, parallel literature researches lead us to examine the relationship between 53BP1 and p53 upon DNA damage. Our results show that in the absence of p53, the efficiency of 53BP1 localisation to DSBs is reduced while the recruitment of BRCA1, a HR factor, is enhanced. We also found that HR is more efficient in absence of p53. Altogether, our data suggest that p53 acts early in the DDR as a "mediator" protein regulating the balance between HR and NHEJ through the recruitment of 53BP1 and BRCA1.

CHAPTER 1

General introduction on the DNA damage response in the chromatin context

Running title: General introduction

Keywords: DNA damage response, DSBs, NHEJ, HR, histone post-translational modification, p53, 53BP1

1.1 DNA DAMAGE SPECTRUM

Cells are frequently challenged by DNA damaging agents. If unrepaired or repaired inappropriately, the resultant DNA lesions may lead to mutations which can alter the genome integrity of the cell. Amplification and propagations of cells carrying such mutations can be particularly deleterious, potentially generating cancerous cells and promoting tumorigenesis.

DNA damaging agents can be classified by their sources either exogenous or endogenous. Environmental agents include ultra-violet (UV), ionizing radiation (IR) and genotoxic chemicals covering a broad range from pollutants to therapeutic drugs (Hakem, 2008). Endogenous DNA damage can result from by-products generated by cellular metabolism, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), erroneous or abortive topoisomerase activity, replication errors or spontaneous disintegration of chemical bonds in DNA (Lindahl and Barnes, 2000).

The spectrum of DNA lesions generated by these numerous threats is extremely wide. Consequently, cells have developed a large range of DNA repair pathways. Exposure to UV can induce DNA photoproducts including cyclobutane pyrimidine dimers (CDP) pyrimidine (6-4) pyrimidone (6-4PP) and thymine dimers that are mostly repaired by nucleotide excision repair (NER) (Hoeijmakers, 2001; Kojo et al., 2006). The source of alkylating agents can be endogenous, from by-products of the cellular oxidative metabolism, or exogenous, from pollutants or chemotherapy (Lindahl and Barnes, 2000). The nature of DNA lesions generated by those agents is dependant on the alkyl group transferred, the chemical reactivity toward either the oxygen or the nitrogen atoms of DNA bases, or the number of reactive sites within the alkylating agent (Fu et al., 2012). Monofunctional alkylating agents contain one active site and modify one site on DNA whereas bifunctional agents contain two active sites and can generate interstrand crosslinks (McHugh et al., 2001). Due to the diversity of DNA lesions induced by alkylating agents, various DNA repair pathways may intervene. This includes the direct repair by either the methyltransferase O6-methylguanine-DNA methyltransferase (MGMT) (kaina et al., 2007) or AlkB homologue (ALKBH) family of dioxygenase enzymes (Aravind and Koonin, 2001) and the non-direct repair by NER, base excision repair (BER), mismatch repair (MMR) and homologous recombination

(HR) (Hoeijmakers, 2001). Ionizing radiation produces a wide range of DNA lesions including base oxidation and DNA break formation (Mikkelsen and Wardman, 2003). DNA double strand breaks, considered the most harmful DNA lesion, are repaired either by HR or non-homologous end joining (NHEJ). The following Figure 1.1 presents in more detail the various kinds of DNA lesions and the DNA repair pathways involved.

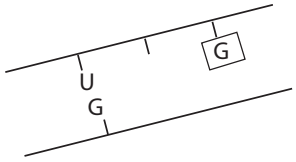
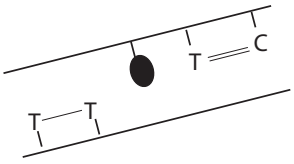
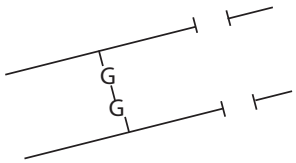
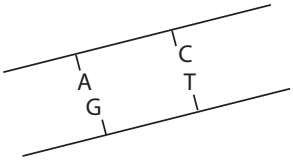
Damaging agent	Form of DNA damage		Repair Pathway
Cytosine deamination	uracil		Base Exision Repair (BER)
Hydrolysis of nucleotide	abasic site		
Oxidation	8-oxoguanine		
UV	thymine dimers 6-4 photoproducts cyclobutane dimers		Nucleotide Exision Repair (NER)
genotoxic aromatic compounds	Bulky adducts		
Anti tumour agents	intrastrand crosslink		Non Homologous End Joining (NHEJ) or Homologous Recombination (HR)
Replication fork stalling	DSB		
V(D)J recombination	DSB		
Topoisomerase inhibitors	DSB		
Ionizing radiation	DSB		
Replication errors	mismatch		Mismatch Repair (MMR)

Figure 1.1: Overview of various form of DNA damage
Examples of DNA lesions induced by DNA damaging agent and their most relevant DNA repair pathway. UV: ultra violet light, DSB: double strand break

1.2 DNA DAMAGE RESPONSE

To preserve their genome integrity, cells have evolved a DNA damage response that consists of a chain of signaling reactions aiming at activating the appropriate response for the specific DNA lesion perceived (Ciccia and Elledge, 2010; Niida and Nakanishi, 2006). “Sensor” proteins detect the DNA damage and relay the signal to “mediator” and “transducer” proteins that amplify and transmit it to the appropriate “effector” proteins. The latter are involved in various pathways including DNA repair but also cell cycle checkpoint allowing cells time to repair the DNA breaks. Effectors can also initiate apoptosis if the damages cannot be repaired.

1.2.1 Initiation of the DNA damage signal

Damage generated by oxidation, deamination or alkylation which does not disturb the DNA helix is mostly recognized by the DNA glycosylase family involved in BER (Krokan et al., 1997) . DNA helix disturbing damage such as intrastrand crosslinks and bulky adducts is recognised by Xeroderma pigmentosum group C (XPC) protein which is a component of the NER repair pathway (Sugasawa et al., 1998). However, helix distorting DNA lesions may obstruct transcription and replication machinery. If such damage encounters transcription machinery before being located by XPC, the stalled RNA polymerase is released by the cockaine syndrome group A and B (CSA and CSB), component of the transcription coupled repair pathway (TCR) (Le Page et al., 2000). If replication machinery encounters helix distorting lesions, two mechanisms of bypass are possible depending on the location of the damage either on the lagging or the leading DNA template strand (Yeeles et al., 2013). If the damage is located on the lagging template strand, a DNA gap is generated, creating a single strand DNA break (SSB). On the other hand, damage located on the leading template strand may arrest the DNA replication fork, leading to its possible collapsing and consequently generating a one end DNA double strand break (DSB).

SSB with naked single strand DNA and DSB signaling involves the activation of kinases belonging to the phosphatidylinositol(PI)3-kinase-like kinases (PIKK) family via their respective “sensor” proteins (Jackson and Bartek, 2009). Ataxia Telangiectasia Related (ATR) is recruited to single stranded DNA via its “sensor” protein RPA whereas Ataxia Telengiectasia Mutated (ATM) and DNA-dependent protein kinase

catalytic subunit (DNA-PK) are recruited to DSBs via the “sensor” protein complexes MRN and Ku respectively.

RPA is a complex containing the three subunits RPA70, RPA32 and RPA14. It is implicated in various genomic events such as DNA replication, DNA recombination and DNA repair. RPA is found associated to single strand DNA protecting it from endonucleases-mediated degradation (Binz et al., 2004). Upon DNA damage, RPA accumulates on newly generated single strand DNA and recruits the RAD17 complex composed of RAD17 and four RFC subunits, RFC 2-5 (Zou and Elledge, 2003). Then, both RPA and the RAD17 complexes facilite the loading of the 9-1-1 complex composed of RAD9, RAD1 and HUS1. The 9.1.1 complex has toroidal structure that is similar to the PCNA clamp (Dore et al., 2009). It is loaded on the 5’junction between the double strand DNA (dsDNA) and the ssDNA coated with RPA (Shiotani and Zou, 2009) via the binding of RAD9 with both RPA70 and RPA32 (Wu et al., 2005; Xu et al., 2008). Independently, the complex Ataxia telagiectasia related protein (ATR)/ATR interacting protein (ATRIP) is recruited on the single strand DNA via the binding of ATRIP with RPA sub-unit RPA70 (Xu et al., 2008; Xu and Leffak, 2010). Dependently of RPA, ATRIP and ATR kinase activity, ATR autophosphorylates on threonine 1989 (Liu et al., 2011). However, the completion of ATR activation requires its association to topoisomerase binding protein (TopBP1). TopBP1 is recruited at the junction between dsDNA and ssDNA. TopBP1 interacts with 9-1-1 complex and connects with phosphorylated ATR via its BRCT domain 7 and 8 to complete ATR stimulation (Lee et al., 2007). Recently, it has been suggested that 9-1-1 complex is not required for the recruitment of TopBP1 but for its function (Duursma et al., 2013). It was proposed that the MRE11/Rad1/Nbs1 complex (MRN) also binds to the ss/ds DNA junction and interacts via Nbs1 to the BRCT domain 1-2 of TopBP1. Then, TopBP1 binds to Rad9 of 9-1-1 complex, changing TopBP1 conformation allowing the exposure of its ATR activated domain. Consequently, TopBP1 is able to interact with the PIKK regulatory domain of ATR, enabling ATR hyper-activation.

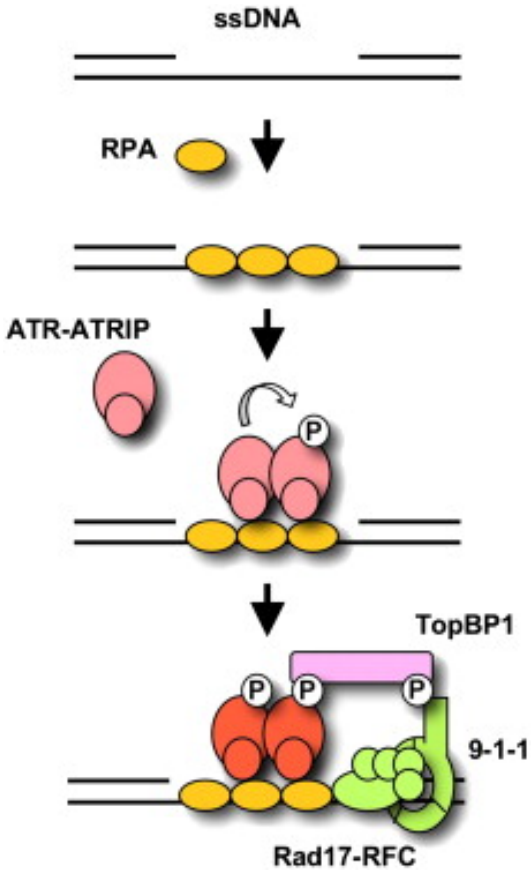


Figure 1.2: Mechanism of ATR activation
Single stranded DNA is first coated with RPA. Accumulation of RPA is recognized by the complex ATR/ATRIP and ATR autophosphoryles on threonine 1989. Accumulation of RPA did also allow the recruitment of Rad17 and RAD9-HUS1-RAD1 complex at the ds-ssDNA junction. Finally, recruitment of TopBP1 and its association with ATR promote the optimal activation of ATR (Liu et al., 2011).

DSB repair by non-homologous end joining involves the activation of DNA-PK via the Ku proteins, Ku70 and Ku80, as "sensor" proteins (NHEJ is discussed in more detail in section 1.4.2). However, DBSs refractory to repair due to a complex chromatin context require the activation of ATM via the localization of the DSB by the MRN complex (Goodarzi et al., 2008).

MRN complex is composed of two subunits each of MRE11 and RAD50 plus one subunit of the NBS1 protein. NBS1 contains on its C-terminus a phosphatidylinositol 3-kinase like kinase (PIKK) binding motif specific to ATM (You et al., 2005). On its inactive form, ATM exists as a dimer in association with protein phosphatase 2A (PP2A). It was also shown that the majority of the soluble fraction of ATM (around 90%) is associated with the acetyltransferase Tip60 independent of DNA damage (Sun et al., 2005). This complex of Tip60/ATM is formed through the FATC domain of

ATM. Upon DNA damage, the MRN complex recruits simultaniously ATM and Tip60 to DSBs. ATM activation is associated with its auto-phosphorylation on various residues, including its serine 367, 1893 and 1981, and subsequently its dimer dissociation into active monomer (Bakkenist and Kastan, 2003; Falck et al., 2005; Goodarzi et al., 2004). Prior to its autophosphorylation, ATM requires the acetylation activity of Tip60 on its lysine 3016 located on a conserved domain termed PIKK regulatory domain (PRD) adjacent to the FATC domain (Sun et al., 2005). Activation of Tip60 is dependent on the direct interaction of its chromodomain to the histone H3 tri-methylated on lysine 9 (H3K9me3) (Sun et al., 2009). Point mutations on this chromodomain abolished both Tip60 activation and ATM autophosphorylation. The overall level of H3K9me3 remains unchanged upon DNA damage treatment. This histone mark, characteristic to the heterochromatin (See section 1.5) but also present in some non-heterochromatin regions, is mostly associated with the heterochromatin protein HP1 (Nielsen et al., 2002). Upon DNA damage, HP1 is phosphorylated by the caseine kinase 2 (CSK2) (Ayoub et al., 2008) and subsequently released from chromatin, leaving H3K9me3 accessible for its association with Tip60 (Sun et al., 2009). Inhibition of CSK2 abolishes HP1 phosphorylation and blocks Tip60 activation. Independently to the MRN complex, ATM may be also able to sense DNA through changes of chromatin conformation (Kitagawa et al., 2004). However, the mechanism of this pathway remains unclear.

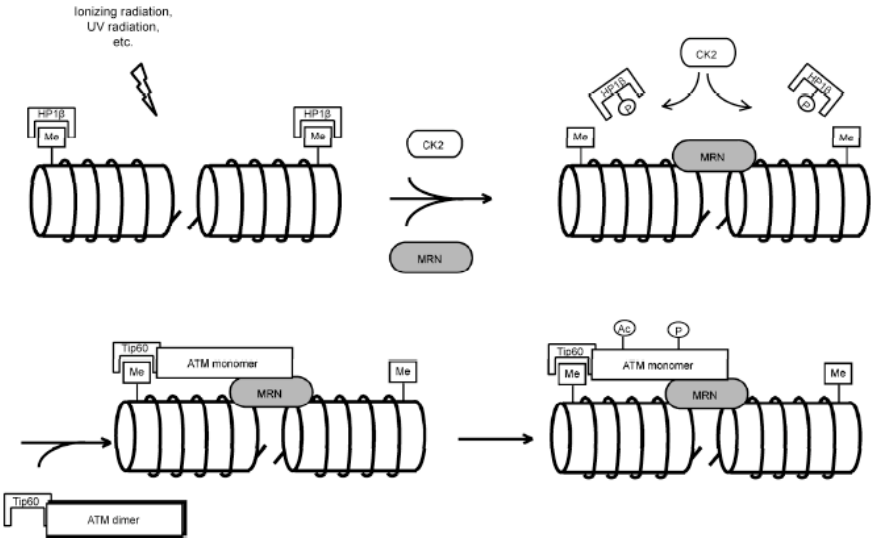


Figure 1.3: Mechanism of ATM activation
Upon DNA DSBs occurrence, HP1 protein is phosphorylated by the kinase CK2 and is released from the chromatin. Free H3K9me3 is accessible for the recruitment of Tip60/ATM complex via the

chromodomain of Tip60. Tip60 is activated by its association with H3K9me3 and acetylate ATM promoting ATM activation (Sun et al., 2009).

1.2.2 ATM and ATR signaling pathways

1.2.2.1 Meditator of the ATR signalling pathway

Claspin and TopBP1 are the main mediators involved in the ATR signaling pathway. Claspin is a ring shaped protein (Sar et al., 2004) that is distributed homogenously throughout the nucleus before and after DNA damage (Liu et al., 2006). After DNA damage induction, Claspin is phosphorylated on its threonine 916 and serine 945, both localised on a CHK1 binding domain. This phosphorylation is dependent on ATR but catalysed by another kinase, CK1γ (casein kinase 1 gamma) (Bennett et al., 2008; Meng et al., 2011). Subsequently, Claspin associates with CHK1 promoting its phosphorylation and activation by ATR. As Claspin association with CHK1 is reduced upon CHK1 phosphorylation, it has been suggested that Claspin recruits CHK1 to ATR promoting its phosphorylation before its release (Jeong et al., 2003; Liu et al., 2006).

TopBP1 is a large protein of 180Kda with eight BRCA-carboxy-terminal (BRCT) domains distributed from the C-terminus to the N terminus of the molecule(Makiniemi et al., 2001). BRCT domains are present in many “mediator” proteins and play a role in protein-protein and protein-phosphoprotein interactions. In unstressed conditions, TopBP1 is also a regulator of DNA replication (Makiniemi et al., 2001). Upon DNA damage, TopBP1 relocalizes to sites of damage, where it forms foci observable by immunofluorescence microscopy. This recruitment requires the fifth of the eight BRCT domain of TopBP1 and colocalizes with ATR foci (Liu et al., 2006; Yamane et al., 2002). TopBP1 facilitates the phosphorylation of a wide range of ATR substrates including CHK1, NBS1 and H2AX (Kumagai et al., 2006; Liu et al., 2006). Indeed, TopBP1 has been shown to be required for the interaction between CHK1 and Claspin upon DNA damage, placing TopBP1 upstream of Claspin in the ATR signaling pathway(Liu et al., 2006).

1.2.2.2 Meditator of the ATM signalling pathway

ATM signaling pathway is mostly mediated by MDC1, 53BP1 and BRCA1 proteins all containing a tandem BRCT domain. MDC1 is first recruited to DNA damage sites via the binding of its BRCT domain with the histone variant H2AX phosphorylated at its

serine 139, also termed γH2AX (Stucki and Jackson, 2006). This phosphorylation, catalyzed by ATM, ATR and DNA-PK, is the earliest event observable by microscopy after DNA damage (Kinner et al., 2008). H2AX phosphorylation is discussed in more detail in the section 1.5.1.

The recruitment of MDC1 to DNA lesions is indispensable for its phosphorylation by ATM. Phosphorylated MDC1 promotes additional accumulation of activated ATM and subsequently additional phosphorylation of H2AX. Thus, a self-reinforcing loop between H2AX phosphorylation and MDC1 recruitment is generated, creating a platform required for the accumulation of many DNA damage response mediators (Bekker-Jensen et al., 2006; Lou et al., 2006; Stucki and Jackson, 2006). Knock-down of MDC1 abrogates recruitment of NBS1, and the two mediator proteins, 53BP1 and BRCA1 (Dimitrova and de Lange, 2006; Mok and Henderson, 2012; Wilson and Stern, 2008).

BRCA1 mutations are associated with breast and ovarian cancer (Rosen et al., 2003). Essential for cell viability, BRCA1 has been shown to be implicated in transcriptional regulation, DNA replication, cell cycle checkpoint, DNA repair, centrosome function and chromosome X inactivation (Deng, 2006). BRCA1 is a large protein of 1863 amino acid that contains two BRCT domains, similar to 53BP1 and MDC1, and a RING domain at its N-terminal. Via its RING domain, BRCA1 associates with BARD1 forming a heterodimer with an ubiquitin E3 ligase activity (Drost et al., 2011; Sankaran et al., 2006; Mezza et al., 1999; Wu et al., 1996). This function does not seem to be required for cell viability however it has been implicated in H2A ubiquitinilation, heterochromatin structure maintenance and heterochromatic silencing (Zhu et al., 2011). The BRCT domain of BRCA1 has been shown to interact directly with the three proteins Abraxa, Bach1 and CtIP forming the BRCA1-A, -B and-C complexes respectively. All BRCA1 complexes are implicated in cell cycle checkpoints, G2-M checkpoint for the complexes A and C (Wang et al., 2007; Yu and Chen, 2004) and the replication checkpoint control for the BRCA1 complex B (Xu et al., 2001; Xu et al., 2002). The BRCA1-C complex composed of BRCA1, CtIP and MRN is involved in DNA end resection (Chen et al., 2008; Huertas and Jackson, 2009; Sartori et al., 2007; Schlegel et al., 2006) (see section 1.4.1). The BRCA1-B complex is composed of phosphorylated S990 and Bach1 and has a role in DNA interstrand crosslink repair (Wang, 2007). Lastly, The BRCA1-A complex is composed of BRCA1, Abraxa,

Rap80, NBA1, BRE and BRCC36. Its recruitment to DSBs is dependent on the ubiquitin E3 ligases RNF8 and RNF168 (Stewart et al., 2003). The interaction platform generated by MDC1 allows the recruitment of two ubiquitin ligases, RNF8 and RNF168. These two E3 ubiquitin ligase catalyses the poly-ubiquitylation of H2A-type histones on lysine 13-15 (Mattiroli et al., 2012). It has been suggested that the recruitment of the BRCA1-A complex results from the interaction of RAP80 ubiquitin-interacting motif (UIM) with the polyubiquitylated chain of the H2A-type histones (Messick and Greenberg, 2009). The role of this complex at DSBs remains unclear. Using a HR assay inducing DSB by I-SceI cleavage, it has been suggested that this complex may promote the HR repair pathway (Wang et al., 2007). However, it has been recently proposed that this complex restricts DNA end resection by limiting nuclease accessibility, including Mre11 and CtIP, in order to prevent excessive DNA end resection (Coleman et al., 2011). RNA interference of either RAP80 or BRCC36, promotes HR and abrogates NHEJ suggesting that BRCA1-A complex would regulate the DSB repair pathway choice between HR and NHEJ.

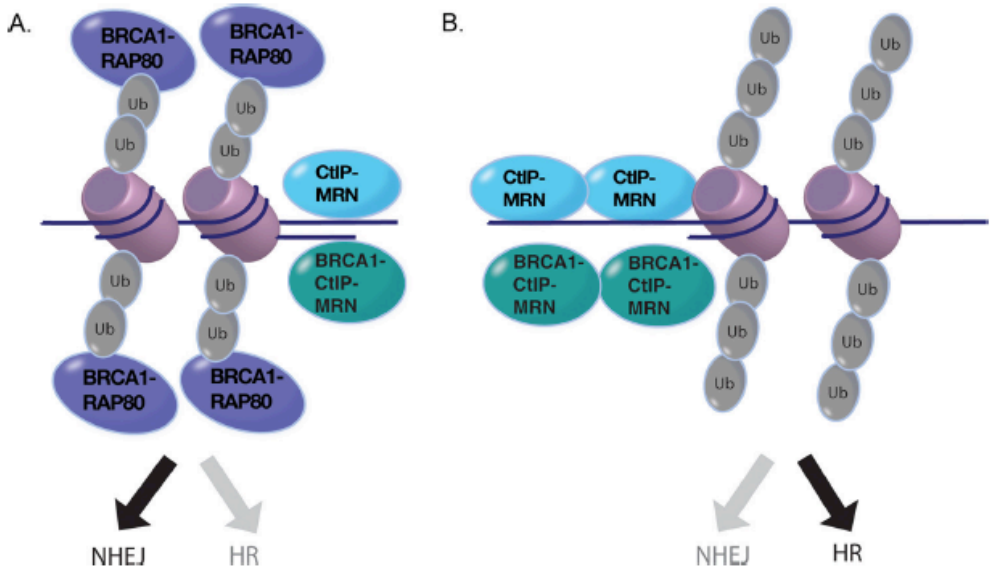


Figure 1.4: Regulation of DNA end resection by BRCA1 complexes
DSB can be repaired either by NHEJ or HR repair pathway. (A) Upon DSB damage, the complex BRCA1-RAP80 is recruited via interaction of the UIM domain of RAP80 with poly-ubiquitynated H2A-type histone. Presence of this complex limits recruitment of BRCA1-CtIP-MRN complex required for DNA end resection. Consequently, DNA end resection is limited and NHEJ repair pathway is privileged. (B) In abence of the BRCA1-RAP80 complex, BRCA1-CtIP-MRN complex recruitment is increased, promoting DNA end resection and subsequently HR (Coleman et al., 2011).

53BP1 was first identified as a binding partner of the tumor suppressor protein p53 (Iwabuchi et al., 1994). Their interaction involves the tandem BRCT domain at the

carboxy-terminus of 53BP1 (Figure 1.5) with the DNA binding domain of p53 and promotes the transcriptional activity of p53 (Iwabuchi et al., 1998). Positioned just upstream its two BRCT domains, 53BP1 also contains an oligomerisation domain and a tandem Tudor domain, both required for its relocalization at DSBs (Botuyan et al., 2006; Huyen et al., 2004; Zgheib et al., 2009). Also, multiple SQ/TQ phosphorylation sites are localized on the amino-terminus of 53BP1. Upon DNA damage, 53BP1 is hyper-phosphorylated by the PIKK proteins ATM, ATR and possibly DNA-PK (Jowsey et al., 2007; Ward et al., 2003a). This hyper-phosphorylation is not required for 53BP1 relocalization to DNA damage sites and reciprocally, 53BP1 relocalization is not required for its hyperphosphorylation (Ward et al., 2003a). The exact process of 53BP1 recruitment to DNA damage sites remains unclear. In unstressed condition, 53BP1 can be visualized by immunofluorescence microscopy either homogenously distributed in the nucleus or accumulated in few but particularly bright foci called "53BP1 nuclear bodies" (Lukas et al., 2011). Upon DNA damage, 53BP1 molecules redistribute at DNA damage sites visualized as foci colocalizing with several other proteins of the DDR including MRN, γH2AX, MDC1 and BRCA1(Bekker-Jensen et al., 2005; Schultz et al., 2000; Stewart et al., 2003; Wang et al., 2002). The mechanism of 53BP1 recruitment has been shown to require two distinct elements: MDC1 signaling cascade involving thus RNF8 and RNF168 (Bohgaki et al., 2011; Stewart et al., 2003) and the association between 53BP1 Tandem Tudor domain with the histone H4 dimethylated on its lysine 20 and/or histone H3 dimethylated on its lysine 79 (Botuyan et al., 2006; Huyen et al., 2004).

Figure 1.5: Diagram illustrating 53BP1 protein domains
Ionizing radiation induced foci (IRIF) domain corresponds to the minimal region required for 53BP1 recruitment to DNA damage sites. GAR stretch: Glycine arginine rich sequence. BRCT: BRCA- carboxy terminal domain.

The precise function of 53BP1 remains to be fully elucidated. Nonetheless, it has been shown that 53BP1 facilitates ATM phosphorylation of various substrates in the DDR including CHK2 (Wang et al., 2002; Ward et al., 2003b), SMC1, RPA2 and BRCA1

(Wang et al., 2002), reflecting 53BP1 role as mediator of the ATM signaling response. 53BP1 knock-out mice are highly sensitive to IR and are tumour prone (Morales et al., 2003; Ward et al., 2003). Also, they present a reduced isotype switching in mature B cells, revealing a defect in chromatid sister exchange (CSR), and in early thymocyte, revealing a defect in VDJ recombination (Manis et al., 2004; Morales et al., 2003; Ward et al., 2003). These two types of recombination require the NHEJ repair pathway, also referred as "long range NHEJ". This pathway is also required for fusion of deprotected telomeres. Further studies of 53BP1 function at deprotected telomeres showed that 53BP1 may contribute to chromatid dynamics, thus promoting fusion of distal DNA ends (Dimitrova et al., 2008). Additionally, a new role for 53BP1 has been identified in DNA end resection for homologous recombination (HR) (Buntibg et al., 2010; Dimitrova et al., 2008; Zimmermann et al., 2013). DSB can be repair by either NHEJ or HR. The balance between these two repair pathways is regulated during DNA end resection where BRCA1 and 53BP1 are competing to either promote or inhibit it. Deletion of 53BP1 in BRCA1 deficient cells rescues the homologous recombination defect (Bunting et al., 2010).

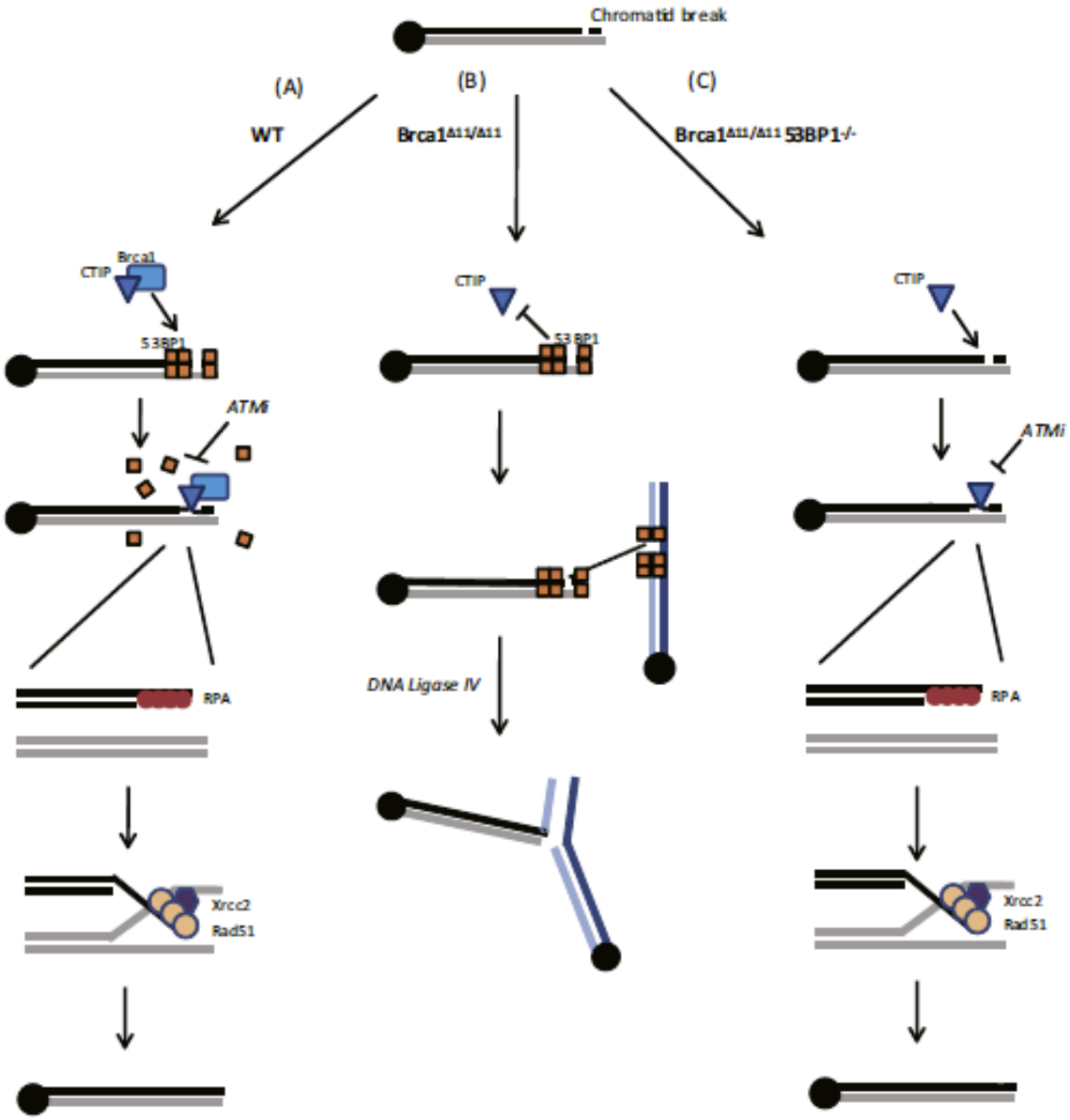


Figure 1.6: Diagram illustrating HR rescue in BRCA1 deficient cells by 53BP1 depletion
(A) In WT cells, 53BP1 is first recruited to DSBs. Then, BRCA1 displaces 53BP1 thus allowing recruitment of CtIP-BRCA1 complex to DSBs and promoting DNA end resection and consequently HR. (B) In absence of BRCA1, 53BP1 is not displaced from DSBs and can potentially promotes DNA end joining with another DNA breaks present in the genome, thus altering genome integrity. (C) In absence of both BRCA1 and 53BP1, CtIP is recruited in a BRCA1-independent manner to DSBs in order to promote DNA end joining and subsequently HR. (Bunting et al., 2010).

1.2.2.3 *"transducer" and "effector" proteins*

ATM and ATR signals are relayed by "transducer" proteins to the "effector" proteins. These "effector" proteins are phosphorylated and consequently regulated to ensure the coordination of the multiple events necessary for cell recovery including cell cycle

checkpoints and DNA repair pathways. If the damage encountered cannot be repaired, cells are driven toward apoptosis or senescence.

CHK1 and CHK2 are the "transducer" proteins of the ATR and ATM signaling pathways respectively (Liu et al., 2000; Matsuoka et al., 2000). Their activation is mediated by ATR phosphorylation on serine 317 and 345 for CHK1 and ATM phosphorylation on tyrosine 68 for CHK2 (Matsuoka et al., 2000; Zhao and Piwnica-Worms, 2001). Once activated, CHK1 and CHK2 rapidly arrest the cell cycle, mainly by inhibiting proteins of the CDC25 family (Chaturvedi et al., 1999; Furnari et al., 1997; Liu et al., 2000; Matsuoka et al., 1998; Sanchez et al., 1997). A slower pathway involving the phosphorylation and activation of p53 by CHK2 sustains the arrest (Iliakis et al., 2003). Both "transducer" proteins have been also implicated in the regulation of DNA repair. CHK1 and CHK2 have been shown to phosphorylate the repair factor RAD51 and BRCA1 respectively, both implicated in homologous recombination repair (HR) (Gibson et al., 2006; Sorensen et al., 2005). However, if the DNA damage is too great, it is thought that p53 is eventually activated by CHK2 and enhances transcription of factors required for apoptosis or senescence (Chehab et al., 2000; Hirao et al., 2000). Cell cycle checkpoint, DNA repair, apoptosis and senescence are discussed in more detail in the following section 1.3.2, 1.4 and 1.6.2 respectively.

1.3 CELL CYCLE CHECKPOINT ACTIVATION

1.3.1 Cell Cycle

Cell proliferation depends on duplication and then segregation of its content including DNA and organelles. The succession of events termed "the cell cycle" consists of four phases: Gap1 (G1), synthesis (S), Gap2 (G2) and mitosis (M) phases (Pollard et al., 2007). In G1, cells grow in size, producing RNA and proteins in preparation for the DNA duplication occurring in S phase. In G2, cells are duplicating organelles and getting ready for the segregations occurring during mitosis. Non-proliferating cells can be found in a fifth stage, Gap 0 (G0). This is a quiescent stage that cells can enter and exit according to mitogenic induction.

The progression from one stage to one other is tightly regulated by cyclin-dependent kinases (CDK)/cyclin complexes (Morgan, 1997). As indicated by their name, CDKs

require cyclins to activate their kinase activity. CDKs can be positively or negatively regulated via phosphorylation of their kinase domain and ATP binding domain respectively (Lees, 1995; Morgan, 1997). The regulation of cyclins mainly occurs via oscillations in their protein levels throughout the cell cycle (Evans et al., 1983; Pines 1991)

1.3.1.1 *The G1/S phase transition*

Under mitogenic stimulation, the protein level of cyclin D progressively increases (Sherr, 2000). To pass the "restriction point", a point where the cells are committed to the duplication/segregation phases without the possibility of entering G0 again before the next cell cycle, the protein level of cyclin D must be sufficiently elevated to bind and activate some CDK4/6 (Morgan, 1997). CDK4/6 phosphorylates the retinoblastoma protein (Rb). It is an antiproliferative protein inhibiting gene transcription required for progression into S phase by interacting with the transcription factor E2F and subsequently inhibiting it, but also by modifying chromatin structure at E2F activated genes via recruitment of histone deacetylase and chromatin remodeling factors (Sherr, 2000; Morgan, 1997). Phosphorylation of Rb allows the dissociation of the Rb/E2F complex and E2F dependent transcription of genes required for DNA synthesis as well as cyclins E and A (Bartek and Lukas, 2001).

The CDK/cyclin E complex regulates the cell cycle transition from G1 to S phase. Above a protein level threshold, Cyclin E can bind and activate CDK2. Following the removal of inhibitory phosphate groups of CDK2 by the phosphatase CDC25A, CDK2 phosphorylates more Rb proteins (Boutros et al., 2006). Activation of Cyclin E/CDK2 is enhanced by a positive feedback loop where Cyclin E/CDK2 complex phosphorylates p27, a CDK2 inhibitor, leading to its degradation (Bartek and Lukas, 2001; Sherr, 2000).

Cyclin E/CDK2 permit the cell to enter in S phase by upregulating histone biosynthesis. Also, Cyclin E/CDK2 complex facilitates firing of origins of replication (ORI) by promoting the loading of CDC45 to preinitiation complexes at ORI required for the recruitment of polymerase α (Bartek and Lukas, 2001, Morgan, 1997). During S phase, Cyclin E is replaced by Cyclin A. CDC45 loading to ORI is promoted, as well as transcription of Cyclin B (Morgan, 1997).

1.3.1.2 *The G2/M phase transition*

Progression from G2 phase to Mitosis requires the activation of the complex CyclinB/CDK1 (Morgan, 1997). This activation is tightly regulated at three different levels: cyclin B protein concentration, an inner feedback loop and an outer feedback loop (Lindqvist et al., 2009).

Transcription of Cyclin B starts in S phase and reaches a peak in G2 phase (Dynlacht et al., 1994; Ziebolt et al., 1997; Saville et Watson, 1998; Laoukili et al., 2008). The Cyclin B-CDK1 complex localises mainly to the cytoplasm (Hagting and al., 1998; Toyoshima et al., 1998; Yang et al., 1998). In mid-G2, CyclinB accumulates at centrosomes where Cyclin B-CDK1 complex autophosphorylation was first detected in late G2 (Jackman et al., 2003). Subsequently, Cyclin B-CDK1 is imported to the nucleus and is associated with a strong and fast activation just before mitosis. For a complete activation of CyclinB-CDK1, the complex also needs to be phosphorylated at tyrosine 161 by a CDK activating kinase composed of Cyclin H-CDK7 (Tassan et al., 1994) and to be dephosphorylated at T14 and Y15 by Cdc25 phosphatase family (O'Farrell et al., 2001).

In the inner loop, Cyclin B-CDK1 regulates its own activation by directly activating its activators and inhibiting its inhibitors. The inhibiting phosphorylations at T14 and Y15 are catalyzed in G2 by Wee1 and Myt1 (O'Farrell et al., 2001). Once Cyclin B-CDK1 is activated, the complex is able to phosphorylate Wee1 and Myt1, promoting their degradation or kinase activity inhibition respectively (Booher et al., 1997; Nakajima et al., 2003; Watanabe et al., 2004). Also, Cyclin B-CDK1 is able to activate the Cdc25 phosphatases (Hoffman et al., 1993; Baldin et al., 2002; Mailand et al., 2002; Boutros et al. 2006; Bouche et al.,2008). In the outer loop, Cyclin B-CDK1 utilizes intermediaries to regulate its activators and inhibitors. Those intermediaries include Polo-like kinase-1 (Plk1) and Aurora. Plk1 can directly inhibit Wee1 and Myt1 by phosphorylation and promote accumulation of CDC25C (Toyoshima-Morimoto et al., 2002; Elia et al., 2003; Nakajima et al., 2003; Watanabee et al., 2004; Watanabee et al., 2005). Plk1 can also activate the transcription factor FoxM1 that enhances the transcription of Cyclin B, CDK1, Plk1 and the Cdc25 phosphatases (Laoukili et al., 2005; Wang et al., 2005; Wierstra and Alves, 2007; Fu et al., 2008). Aurora A, with its cofactor Bora that can be

phosphorylated by CDK1 (Hutterer et al., 2006; Chan et al., 2008), can activate Cdc25B phosphatase and Plk1 (Dutertre et al., 2004; Macurek et al., 2008; Seki et al., 2008).

1.3.2 Cell Cycle Checkpoint

There are three types of DNA damage checkpoints that arrest the cell at either G1/S, intra-S and G2/M phase of the cell cycle.

1.3.2.1 *The G1/S Checkpoint*

G1/S checkpoints, as well as G2/M checkpoint, is mediated by an ATM/ATR-mediated fast response and by a p53 mediated slow response. Cell cycle progression from G1 to S phase is promoted by the interaction of Cyclin Dependent Kinase 2 (CDK2) with Cyclin E (Moroy and Geisen, 2004). Upon DNA damage, CHK1, activated by ATR, and CHK2, activated by ATM, initiate the G1/S checkpoint by phosphorylating the phosphatase CDC25A, an activator of CDK2, targeting it for degradation (Falck et al., 2001). As a result, CDK2 accumulates in an inactive form and cell cycle progression is arrested (Mailand et al., 2000). As late response, if DNA damages remains, p53 maintain the checkpoint by activating the transcription of p21. p21 is an inhibitor of CDK2 by interacting with it, thus preventing its association with Cyclin E and so preventing cell progression to the S phase (Agarwal et al., 1995; Vogelstein et al., 2000).

1.3.2.2 *The intra-S-Checkpoint*

In response to DNA damage, ATM and ATR can activate the intra-S-checkpoint via two pathways. One pathway is dependent on the phosphorylation of Cdc25A by Chk1, Chk2 and ATM on Ser123, 178, 278 and 292 (Lukas et al., 2001; Sorensen et al., 2003). These phosphorylation events lead Cdc25A to its degradation via ubiquitin-mediated proteolysis (Busino et al., 2003). In absence of Cdc25, CDK2 cannot be activated. Subsequently, CDC45 cannot be loaded on the origin of replication in order to recruit DNA polymerase α (Takisawa et al., 2000). The second pathway, dependent on NBS1 and SMC1, is essential for the intra-S-checkpoint but the mechanism remains unclear. In an NBS1 dependent manner, ATM phosphorylates SMC1 at Ser957 and Ser966 after IR. This phosphorylation was shown to be required for the intra-S checkpoint (Kim et al., 2002; Yazdi et al., 2002; Kitagawa et al., 2004). In addition, phosphorylation on SMC3, a component of the cohesin complex with SMC1 (Haering et al., 2002;

Michaelis et al., 1997), are also implicated in the intra-S checkpoint (Luo et al., 2008). Those phosphorylations on SMC3 are located at ser1067 and ser1083 and catalysed by Casein kinase 2 (CK2) and ATM respectively.

1.3.2.3 *The G2/M Checkpoint*

The G2 to M transition is regulated by CDK1-Cyclin B. However, the CDK1-cyclin B complex is prevented from being active by inhibition of CDK1 through its phosphorylation on its tyrosines 14 and Y15 by the kinases Wee1 and Myt1 (Morgan, 1995). Cell cycle progression through mitosis requires the removal of these inhibiting phosphates on CDK1 by the phosphatase CDC25C (Morgan, 1995). In addition, cyclin B is activated by phosphorylation on its serines 126 and 128 by ERK2 and serine 133 by PLK1. To reinforce the activation of CyclinB/CDK1, a positive loop is generated where PLK1 and CyclinB/CDK1 phosphorylate and so activate more CDC25C (Fisher et al., 2012; Yuan et al., 2002). In the early response to DNA damage, ATR and CHK1 phosphorylate and inactivate CDC25c (Chen et al., 2012). As for the G1/S checkpoint, p53 prolongs G2/M arrest, if necessary, by inducing the transcription of p21 and two other targets, GADD45 and 14-3-3 (Agarwal et al., 1995; Taylor and Stark, 2001; Vogelstein et al., 2000). The p21 sustains cell cycle arrest by binding directly to Cyclin B/CDK1 complex and inactivating it, while GADD45 promotes dissociation of Cyclin B/CDK1 complex and 14-3-3 sequesters CDK1 into the cytoplasm.

1.4 DOUBLE STRAND BREAK REPAIR

Double strand breaks (DSB) are the most harmful lesions that a cell can encounter. DSBs can potentially lead to loss of coding material or DNA rearrangement. To repair them, cells have evolved two mechanisms, the homologous recombination (HR) or the non homologous end joining (NHEJ) repair pathways.

1.4.1 Homologous recombination

HR is an error free repair pathway using the homologous sister chromatid as template to repair the lesion (Figure1.7). Consequently, it occurs only in G2 and late S-phase of the cell cycle when the homologous sister chromatid is available (Hendrickson, 1997). After detection of the break, the two DNA ends of this break are subject to resection, resulting in a 3’ single strand overhanging DNA. The resection is initially catalysed by the complex BRCA1-CtIP-MRN (Chen et al., 2008). BRCA1 recruited to CtIP

stimulates the endonuclease activity of the MRN complex. Then, a long-range resection takes place, implicating either the complex exonuclease EXO1-Bloom helicase BLM (Karanja et al., 2012) or the complex nuclease DNA2-BLM (Nimonkar et al., 2011). The newly generated single strand DNA is first coated with RPA proteins that are then exchanged with RAD51 proteins (Sugiyama and Kowalczykowski, 2002). The loading of RAD51 proteins on the ssDNA, promoted by BRCA2, stretches the ssDNA to facilitate homology search (Krejci et al., 2012; Liu et al., 2010). During the synapsis step, the RAD51 filament invades the homologous duplex DNA generating a structure termed a "Holliday junction". It has been suggested that RAD54, which promotes the pairing of the ssDNA with its homologous DNA sequence, also promotes the steady dissociation of RAD51 proteins from the ssDNA (Solinger et al., 2002). This RAD51 dissociation frees space for the DNA polymerases and allows DNA synthesis. Current models for Holliday junction processing involve three possible pathways: a dissolution pathway mediated by BLM-TOPIIIa-RMI1-RMI2 (Wu and Hickson, 2003; Mankouri and Hickson, 2007) and two resolution pathways mediated either by MUS81-EME1 (Chen et al., 2001; Ciccia et al., 2003; Ciccia et al., 2008; Taylor and McGowan, 2008) associated with SLX1-SLX4 (Andersen et al., 2009; Fekairi et al., 2009; Munoz et al., 2009; Svendsen et al., 2009) or by GEN1 (Ip et al., 2008; Rass et al., 2010). The resolution pathways involve endonucleolytic cleavage resulting in cross-over or non-cross over products whereas the dissolution pathway favours non-cross-over products. Strategic depletion of MUS81, SLX4 or GEN1 on Bloom's syndrome cells gave a first indication of the relative contribution of each pathway in Holliday junction processing (Wechsler et al., 2011). In Bloom's syndrome cells, the BLM protein is inactivated. Also, chromatid sister exchange (CSE) and genome instability are particularly elevated. Studies of the effects of these different depletions on CSE suggest the current model where Holliday junctions are predominantly dissolved by the BLM pathway. In the absence of BLM, the two resolution pathways involving MUS81-SLX4 and GEN1 take the relay.

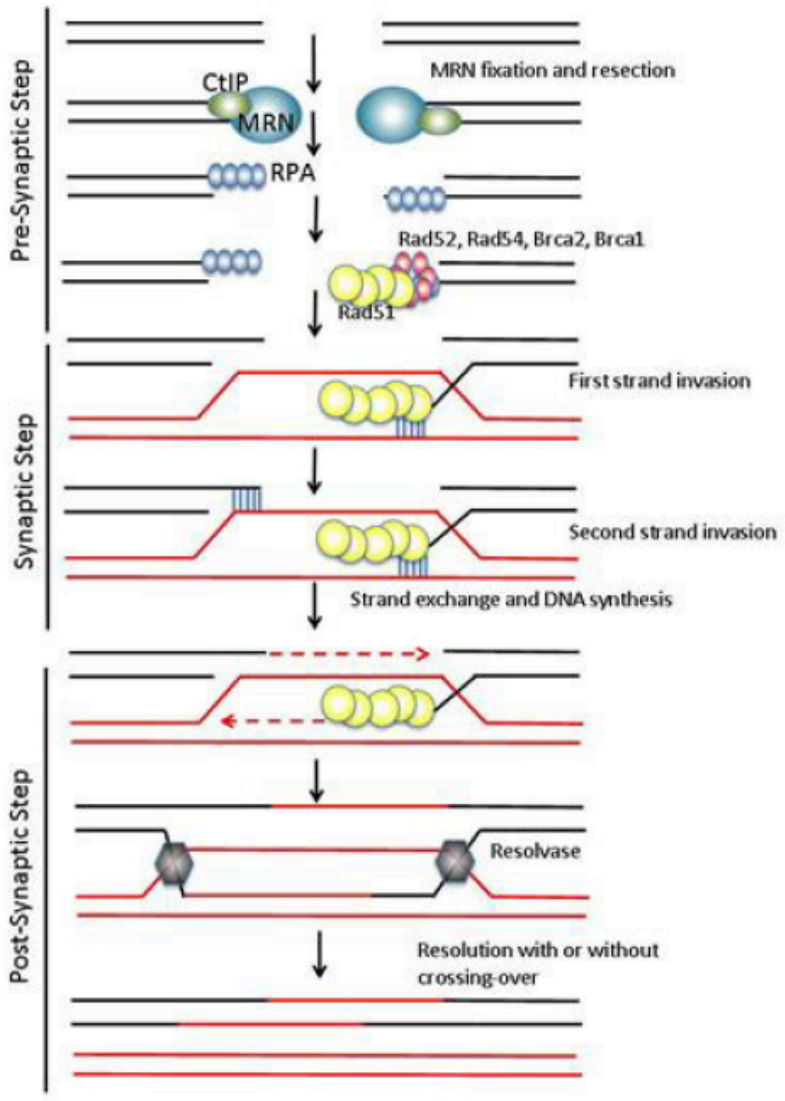


Figure 1.7: Mechanism of DSBs repair by HR
(Renodon-courtiere et al., 2013)

1.4.2 Non-homologous end-joining

NHEJ occurs in all phases of the cell cycle, but is predominant in G0, G1 and early S phase of the cell cycle. This pathway is also required for V(D)J recombination and CSR recombination, specialized processes that generate the diversity of receptors produced by lymphoid cells in order to recognize foreign antigens. NHEJ catalyzes the re-ligation of two broken DNA break ends in three steps: recognition of the break, DNA end processing generating compatible end for re-ligation, the last step of the process (Hoeijmakers, 2001) (Figure 1.8). A DSB is initially detected by the ring shape complex, KU70/80 that is loaded onto each DNA end of the break (Walker et al., 2001).

The KU complexes recruit the PIKK kinase DNA-PK to each end and shift toward the inside of the DNA duplex, allowing interaction between DNA-PK and DNA (Weterings and van Gent, 2004). The crystal structure of DNA-PK and Ku80 fragment complexes revealed that DNA-PK has a head/crown structure where the kinase domain is located, and a ring shape structure with a "gap" where the N'terminal of the protein and the DNA binding domain are likely to be located (Sibanda et al., 2010). Interaction of DNA-PK with DNA results in an autophosphorylation of DNA-PK (Reddy et al., 2004), destabilizing this DNA/DNA-PK interaction. Consequently, each of the two DNA-PK kinases are positioned at the level of a DNA end and can interact with each other spanning the break and forming a "bridge" structure (DeFazio et al., 2002; Spagnolo et al., 2006; Weterings et al., 2003). The conformation change of DNA-PK due to its allow the recruitement to this bridge structure of additional proteins required for successful completion of repair by NHEJ. If necessary, various nuclease proteins including Artemis and PNKP (Ma et al., 2002; Zolner et al., 2011), can be recruited to the DNA ends and process them to generate compatible DNA end for religation (Quennet et al., 2011). If necessary, polymerases recruited to the DSBs, such as pol μ and λ, fill any gaps and a complex of XRCC4/LigaseIV/XLF completes the repair by sealing the DNA end of the break (Hefferin and Tomkinson, 2005).

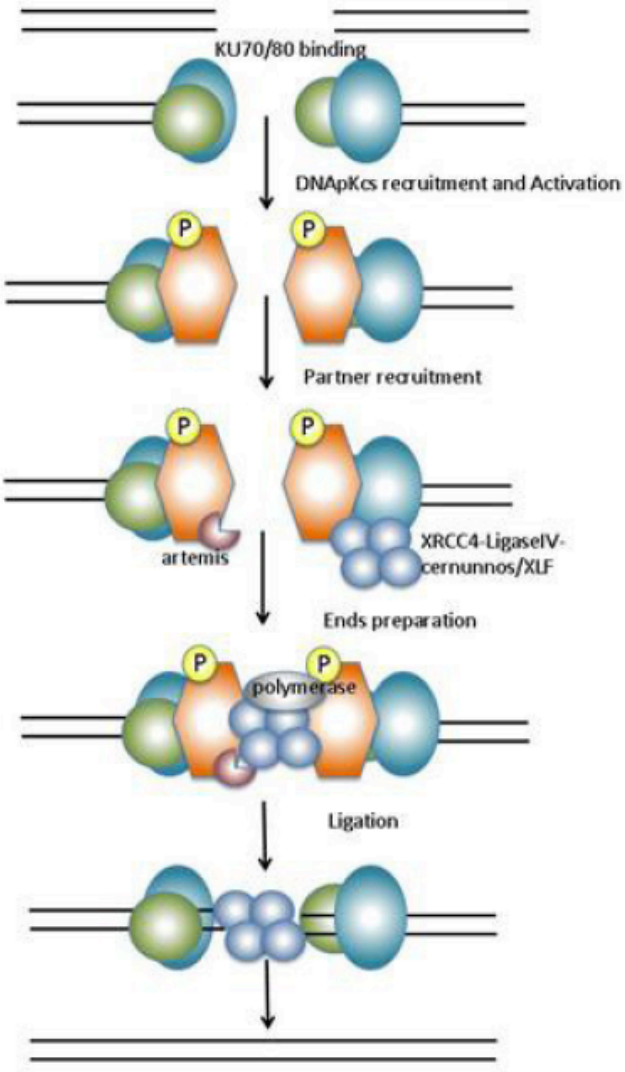


Figure 1.8: Mechanism of DSBs repair by NHEJ
(Renodon-courtiere et al., 2013)

1.5 CHROMATIN ENVIRONMENT

All genomic events, including the DNA damage response, occur in a chromatin environment. DNA is compacted into the nucleus as chromatin. Nucleosomes, the basic units of the chromatin, are composed of 146bp of DNA wrapped around an octamer of histones containing two copies of each of the four core histones H2A, H2B, H3 and H4 (Kornberg and Lorch, 1999; Luger et al., 1997). This first level of compaction forms a 11nm fibre corresponding to the diameter of nucleosomes (Feldenfeld and Groudine 2003; Trojer and Reinberg 2007; Campos and Reinberg 2009). DNA between each nucleosome, termed a DNA linker, is between 10 and 80 bp in length. For each nucleosome, the histone protein H1 binds the DNA linker at the entry and the exit of each nucleosome. This interaction stabilizes nucleosomes and increases the level of compaction to a 30nm fibre (Felsenfeld and Groudine 2003; Richmond and Davey 2003; Campos and Reinberg 2009). The euchromatin is composed of DNA compacted in the 11nm fibre and is associated with transcriptionally active genes. The heterochromatin, formed by DNA compacted in a 30nm fiber and higher, is characterised by transcriptionally silenced genes (Richmond, 2003; Kouzarides 2007; Trojer and Reinberg 2007). The mechanism for 30nm fiber and higher levels of DNA compaction remains unclear. However, the heterochromatin protein HP1 contributes to chromatin compaction by binding to histone H3 trimethylated at lysine 9 (H3K9), a histone mark characteristic of heterochromatin (Campos and Reinberg 2009, Dinant and Luijsterburg 2009).

Upon DNA damage, the alteration and modification of the chromatin structure makes it an important component of the DNA damage response. Three main processes regulate chromatin conformation: incorporation of histone variants, post-translational modifications and nucleosome repositioning (Polo and Jackson, 2011). This section will focus on the post-translational modifications of histones and the DNA damage response in a heterochromatic environment.

1.5.1 Histone post-translational modifications

Histones are globular proteins with a relative long, flexible and charged N-terminal tail that is the principal target for histone modifications. These modifications include methylation, phosphorylation, acetylation, sumoylation or ubiquitination (Lee et al.,

2010) (Figure 1.9). Combinations of these modifications lead to a "histone code" extending the genetic code by recruiting factors regulating the main genetic events like replication, transcription or DNA repair (Jenuwein and Allis, 2001).

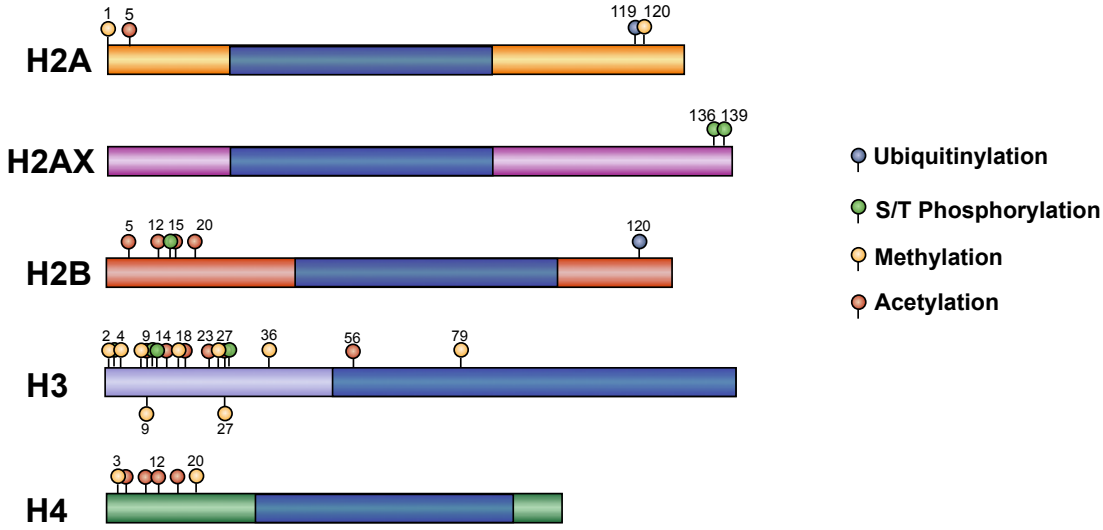


Figure 1.9: Major post-translational modifications on the core histones and H2AX variant.
Blue segments are representative of histone globular regions. Image adapted from (Sarma and Reinberg, 2005).

H2AX is a histone variant that differs from the core histone H2A by its C-terminus tail which can become phosphorylated by the PIKK enzymes ATM, ATR and DNA-PK (Burma et al., 2001; Stiff et al., 2004; Ward and Chen, 2001). Phosphorylation of H2AX on serine 139 in higher cells (termed γH2AX) is one of the most studied histone post-translational modification (PTM) in the DDR. It is catalyzed immediately following DNA damage, within seconds (Rogakou et al., 1998), and spreads for about 2 megabases on each side of the break (Rogakou et al., 1999). γH2AX is the first event of the DDR observable as foci by immunofluorescence microscopy. This histone mark is not required for the recruitment and but for the retention of many factors of the DNA damage response at DNA breaks, such as MDC1, 53BP1 or BRCA1 (Celeste et al., 2003).

Histone acetylation and ubiquitylation also promote the recruitment of proteins upon DNA damage. It is believed that histone acetylation relaxes the chromatin structure allowing accumulation of proteins implicated in DNA damage signalling and DNA repair (Polo and Jackson, 2011). Ubiquitin is a 76 amino acids protein that is transfered

to specific lysine residues of target proteins. This transfer results from a reaction cascade involving three types of enzymes termed E1 activating enzymes, E2 conjugating enzymes and E3 ubiquitin ligase enzymes (Dye and Schulman, 2007). Using ATP, E1 activates ubiquitin, via adenylation of ubiquitin on its C'terninus, and transfers it to E2. The E2 enzyme associates then with E3 ubiquitine ligases that are classified in three groups according to the presence of a HECT, a RING or a U-Box domain (Ciechanover et al., 1982; Hershko et al., 1983). RING and U-Box E3 ligases promote ubiquitination by bringing together E2 and the substrate, whereas HECT E3 ligases contain an ubiquitin active site to transfer the ubiquitin from E2 to the substrate (Passmore et al., 2004). The substrates can be monouquitinated; addition of only one ubiquitin per lysine residus, or polyubiquitinated; addition of multiple ubiquitins forming a chain on a single lysine of the subtrate. Polyubiquitination involves the lysine 48 (K48) or lysine 68 (K63) of ubiquitin (Haglund et al., 2005). K48 linked ubiquitin chains are often associated with protein degradation (Thrower et al., 2000). Whereas K63 linked ubiquitin chains regulate protein-protein interactions implicated in various cellular process such as DNA repair (Huang et al., 2006; Kolas et al., 2007; Doil et al., 2009; Stewart et al., 2009). For example, phosphorylation of MDC1 by ATM upon DNA damage induces the recruitment of the E3 ubiquitin ligases RNF8 and RNF168 that polyubiquitylate H2A-type histones (Mattiroli et al., 2012). This newly generated polyubiquitylated chain has been shown to be crucial for the recruitment of the DNA damage response mediator proteins 53BP1 and BRCA1 (Bohgaki et al., 2011; Stewart et al., 2003).

All PTMs previously cited are formed specifically upon DNA damage. However, certain histone marks implicated in the DNA damage response are constitutively present on the chromatin such as the histone H3 dimethylated on lysine 79 and histone H4 dimethylated on lysine 20. Under unstressed conditions these methylations occur progressively during the cell cycle (Feng et al., 2002; Houston et al., 2008; Huyen et al., 2004; Pesavento et al., 2008; Wakeman et al., 2012). As they are positioned close to or within the globular histone domain, these histone marks remain buried inside the compacted chromatin. Upon DNA damage, the chromatin is relaxed by the damage itself, by chromatin remodelling factors, such as INO80 that have the ability to reposition nucleosomes in close proximity to DSBs, or by histone modifiers that can remove histone post-translational modifications, such as histone deactylase or

demethylase. Once the chromatin is relaxed at the proximity of the DSB, it has been suggested that those two histone marks H3K79me2 amd H4K20me2 become exposed to soluble proteins including 53BP1(Huyen et al., 2004).

1.5.2 DDR in heterochromatin

10 to 25% of the total chromatin is highly condensed, forming heterochromatin (Goodarzi et al., 2009). Associated with transcription inactivation, heterochromatization is promoted by transcriptional repressors and co-repressors such as KAP1(Cann and Dellaire, 2011). They recruit histone deacetylase that catalyze deacetylation of the histones including H3 on its lysine 9. Histone methyltransferases such as Suv39h1 and Suv39h2 bind to deacetylated H3K9 histones and methylate them. In parallel, nucleosome remodeling enzymes enhance further compaction of the chromatin such as the nucleosome remodeling and deacetylase complex (NuRD). Methylated histones H3K9 promote recruitment of a chromodomain protein, HP1, to stabilize the heterochromatin structure (Goodarzi et al., 2010).

KAP1 has been shown to be implicated in the DNA damage response. In unstressed condition, KAP1 is observable by immunofluorescence microscopy as large bright foci corresponding to heterochromatin regions in mouse cells (White et al., 2012). Upon DNA damage, KAP1 is phosphorylated by ATM on serines 824 and 47(White et al., 2012). These modifications reduce the affinity of KAP1 for chromatin resulting in chromatin relaxation (Ziv et al., 2006). In the absence of 53BP1 or ATM, a subset of γH2AX foci overlapping with heterochromatin domains can still be observed at late time points after irradiation (16-24h) (Noon et al., 2010). These data reflect a defect in repair of DSBs within heterochromatin. It has been proposed that 53BP1 promotes the accumulation of the MRN complex and subsequently ATM at heterochromatin facilitating the phosphorylation of KAP1 and consequently chromatin relaxation and DNA repair (Noon et al., 2010).

1.6 THE GUARDIAN OF THE GENOME P53

1.6.1 Regulation of p53

The p53 protein is a transcription factor which plays a fundamental role in the maintenance of genome integrity. In response to a variety of cellular stresses p53

activates several target genes regulating cell cycle progression, activation of DNA repair, senescence or apoptosis (Menendez et al., 2009). The complexity of p53 response relies on the many critical functions it holds. Therefore, a tight regulation, in normal and stress conditions, is crucial for genome integrity. In unstressed cells, p53 protein level is kept low through its N-terminal interaction with MDM2 and MDMX that target p53 for ubiquitylation and subsequent proteasome-dependent degradation (Boyd et al., 2000; Geyer et al., 2000; Kubbutat et al., 1997). p53 interaction with MDM2 also abrogates directly its transcriptional activity (Haines et al., 1994; Oliner et al., 1993). When cells are exposed to DNA damaging agents, a DNA damage response cascade is generated, activating "sensor" and "transducer" proteins such as DNA-PK, ATM, ATR and CHK2. Those proteins promote the accumulation of active p53 in the nucleus by phosphorylating both p53 and MDM2 (Fu and Benchimol, 1997; Kastan et al., 1991; Mosner et al., 1995) which inhibit the p53 and MDM2 interaction and consequently preventing p53 degradation (Chehab et al., 2000; Cheng et al., 2009; Mayo et al., 1997; Shieh et al., 1997).

1.6.2 Cell fate regulation upon DNA damage

One of the main functions of p53 includes deciding the fate of damaged cells in order to eliminate irreparably damaged cells without affecting the integrity of the organism. In presence of DNA damages in cells, p53 arrests cell proliferation by activating cell cycle checkpoints, allowing cells time to repair their genome. But if the damage is too substantial, p53 drives cells in senescence or apoptosis.

How the cell decides between senescence and apoptosis remains unknown. In senescence, cells are arrested irreversibly in G0 phase of the cell cycle. Their morphology change and present a acidic senescence-associated β-galactosidase activity (Kong et al., 2011). Senescence can be activated through two pathways mediated by p53 and Rb. It is not clear whether either or both proteins are activated in parallel or whether p53 activates Rb via p21 (Dimri, 2005). The exact process inducing cell senescence remains unclear. During apoptosis p53 activates mainly the transcription of PUMA and BAX causing permeabilization of the mitochondrial membrane and so releasing Cytochrome C. As a result, the Caspases, cysteine-proteases essential for apoptosis, are activated (Chipuk et al., 2004; Jeffers et al., 2003).

1.6.3 p53 in DNA repair

p53 role in tumor prevention has been mostly associated with its functions in cell cycle checkpoint, apoptosis and senescence. However, it has been recently reported that in the absence of crucial p53-dependent effectors of the G1/S checkpoint, apoptosis and senescence, p53 tumor suppressor functions remains (Valente et al., 2013). These data suggest an additional key role of p53, enabling its tumor stability activity in the earliest stage of the DNA damage response, likely DNA repair.

Base excision repair (BER) and nucleotide excision repair (NER) repair DNA lesions present on one of the DNA strands (Hoeijmakers, 2001). p53 has been shown to regulate these DNA repair pathways through either its transactivation activity or through protein-protein binding. The BER pathway is involved in damaged base repair such as 8-oxoguanine. In brief, DNA glycosylases are the enzymes intervening first in this pathway. They recognize and remove the damaged bases. The AP endonuclease APE1 incises the DNA phosphodiester bond at the 5' of the abasic site and the polymerase β completes the gap by adding one nucleotide. Finally, DNA is sealed by DNA ligaseI or the complex XRCC1/LigaseIV (Hoeijmakers, 2001). p53 has been shown to stimulate the BER pathway in vitro. Its direct interaction with polymerase β , in the presence of APE1, stabilizes the polymerase to the abasic site (Zhou et al. 2001). Strikingly, the role of p53 in BER could be cell cycle specific as it has been shown to enhance BER in G0/G1 phases and inhibit it in G2/M phase of the cell cycle (Helton and Chen, 2007)

NER comprises two pathways: Transcription coupled repair (TCR) in the transcribed region, and global genomic repair (GGR) in the rest of the genome. In GGR, the damaged nucleotide is recognized by XPA and XPC proteins. In TCR, transcription machinery collides with the damaged nucleotide. CSA and CSB are required to displace the stalled polymerase. Then, the following steps are common for both pathways. The helicases XPB and XPD open the DNA around the damage. RPA binds and stabilizes the structure. The endonucleases XPD and XPF incise the DNA on each side of the damaged strand leaving a single strand gap of 24 to 32 nucleotides that is filled by regular DNA polymerase machinery (Hoeijmakers, 2001). Wang et al. suggested that p53 enhanced NER by direct binding with the helicases XPB and XPD (Wang et al.,

1995). P53 may also regulate NER through transcription activation as it has been shown that XPC mRNA increase in p53-dependant manner (adimoolan et al., 2002).

Double strand breaks can be repaired either by non-homologous end joining (NHEJ) or homologous recombination (HR). The role of p53 was assessed in both pathways but conflicting results were shown. Some studies suggest a promoting role of p53 in NHEJ Using an episomal plasmid rejoining assay in mouse embryonic fibroblasts, Tang et al. showed an enhanced DNA joining of short complementary ends (Tang et al., 1999). However, other studies suggest that p53 down regulates NHEJ. Repair of I-Sce1 cleavage sites by NHEJ has been shown to be reduced in presence of p53 (Akyuz et al., 2002). Also, using cellular extracts on linearized plasmid, Bill et al. showed that end rejoining was enhanced in absence of p53 (Bill et al., 1997). On the homologous recombination side, some data suggest that p53 suppresses HR repair either through transcription repression of RAD51 and BRCA1 or through direct protein-protein binding with RPA, RAD51 and RecQ helicases (Helton and Chen, 2007; Yang et al., 2002; Romanova et al., 2004). However, other contradictory studies did not observe any defect in the HR pathway in absence of p53 (Wiktor-Brown et al., 2011; Willers et al., 2001). Finally, a model has been recently proposed where a HR-NHEJ crosstalk could be regulated by the PIKK kinases ATR, ATM and DNA-PK phosphorylation on the P53-RPA complex (Serano et al., 2013). They suggested that a low level of p53 is associated to RPA in non-stressed conditions. Upon DNA damage, RPA is phosphorylated by DNA-PK while P53 is phosphorylated in an ATR-ATM dependent manner. The complex RPA-p53 dissociates and frees both RPA and p53 for their DDR functions, RPA being involved in HR.

1.7 PROJECT AIMS AND OBJECTIVES

Mice deficient in 53BP1 present defective phenotypes such as growth defect, genomic instability, IR sensitivity, and immune deficient (Morales et al., 2003; Ward et al., 2003b). Indeed, 53BP1 functions as mediator of the DNA damage response, generating a recruitment and phosphorylation platform for other proteins of the DDR. It also has direct role in the DSBs repair pathways, HR and NHEJ (Bunting et al., 2010; Dimitrova et al., 2008). Upon DNA damage, 53BP1 relocalizes to sites of DNA damage but the mechanism of this recruitment, as well as its function, remains uncertain. This research aimed to investigate the process of 53BP1 relocalization and its biological relevance.

We first investigated the recruitment of 53BP1 to DSBs as a function of the histone post-translational modifications H3K79me2 and H4K20me2. This involved the generation of DT40 cell lines deficient in the histone methyltransferases responsible for each of these histone marks.

Then, we examined on the relationship between p53 and 53BP1 upon DNA damage. Both proteins have been previously found interacting together and the function of such a complex was associated with p53 transcriptional activity. Recently, it has been suggested that 53BP1 promotes the recruitment and stabilization of p53 at DSBs. We hypothesized a positive feedback loop, where p53 at sites of DNA damage reinforces 53BP1 recruitment at these DSBs location. To investigate this, we mainly used the human cell lines HCT116 either WT or defective for p53. Using immunofluorescence microscopy, we analysed 53BP1 recruitment to DSBs, as well as the recruitment of other factors upstream and downstream of 53BP1 in the DDR.

Our findings lead us to investigate the impact of p53 on the balance between HR and NHEJ repair. In order to achieve this, we followed by immunofluorescence microscopy the relocalisation of HR markers to DSBs in presence or absence of p53. Also, DNA DSB repair was directly monitored by comet assay following DNA damage treatments specific to HR repair.

HR is an error free mechanism, while NHEJ is more error prone. Therefore, we could anticipate that HR is a more suitable repair system for the maintenance of genome integrity compared to NHEJ. However, uncontrolled HR occurring in inappropriate genome contexts, as repetitive sequences, or during inappropriate phases of the cell cycle when the homologous sister chromatid is absent, can results in deletion, insertion, mutation or rearrangement of genomic sequences thus altering genome integrity. Indeed, several human diseases with an over-activated HR have been identified.

In conclusion, this study improves our understanding of the p53-53BP1 complex and its functions after DNA damage. We identified a novel role for p53 in the event leading to the crucial decision to channel DSBs into either repair HR or NHEJ.

CHAPTER 2

Role of the dimethylated H4K20 and H3K79 histone marks in the recruitment of 53BP1 at DNA double strand breaks

Running title: role of H3K79me and H4K20me in 53BP1 IRIF

Keywords: 53BP1, H3K79, H4K20, IRIF, histone marks, DOT1, SUV420

2.1 SUMMARY

Following DNA damage, the tumor suppressor protein 53BP1 is recruited to double-strand breaks leading to activation of DNA damage checkpoints and DNA repair. The tandem Tudor domain of 53BP1, necessary for its relocalisation after damage, is known to interact with methylated histone residues. It is well established that the 53BP1 orthologs Rad9, in budding yeast, and Crb2, in fission yeast, recognize histone H3 methylated at lysine 79 (H3K79me) and histone H4 methylated at lysine 20 (H4K20me) respectively, upon DNA double strand breaks (DSBs). In higher cells, despite being widely investigated, the mechanism of 53BP1 recruitment to DNA lesions remains elusive and subject to controversy.

Here we show that the budding yeast pathway has not been conserved through evolution. We successfully generated a knock-out in chicken and a knock-down in human cells of DOT1, the unique histone methyltransferase known to catalyze H3K79 methylation. The resulting Dot1 deficient cell lines did not show any defects in the recruitment of 53BP1 after IR. Those results suggest that H3K79me does not play a role in the general recruitment of 53BP1 to DNA DSBs. Also, in an attempt to generate a H4K20me deficient chicken cell line, we identified the sequence of a new gene in the chicken genome encoding for a H4K20me histone methyltransferase.

2.2 HIGHLIGHTS

- Generation of a *Dot1* knock-out cell line in chicken presenting a H3K79 methylation deficiency
- Generation of a stable U2OS cell line deficient for DOT1 and H3K79 methylation
- H3K79me is not required for the general recruitment of 53BP1 to DNA damage sites after IR in chicken and human cells
- Generation of a *Suv420* knock-out cell line in chicken.
- *Suv420h1* homologous gene, *Suv420h2*, is present in the chicken genome

2.3 INTRODUCTION

Maintenance of genome stability is critical for cells that are constantly exposed to endogenous and exogenous DNA damaging agents. It is imperative that DNA lesion are detected and repaired before a cell divides, to prevent propagation of mutations that could lead to cancer. DNA damage is initially detected by sensor proteins. This signal is amplified by mediator and transducer proteins that ultimately activate appropriate effector proteins implicated in a variety of pathways such as DNA repair, cell cycle control, transcription and apoptosis (Rouse and Jackson, 2002).

53BP1 is a mediator of the DNA damage response, first identified in a yeast two-hybrid screen as a p53 binding protein (Iwabuchi et al., 1994). Its function was initially associated with p53-mediated transcriptional regulation (Schultz et al., 2000). Later on, it was observed that in response to double strand breaks (DSBs) 53BP1 rapidly relocates to the sites of DNA damage (Rappold et al., 2001; Schultz et al., 2000) where it has a role in the early stage of the DNA damage response and in DNA repair. Mice deficient in 53BP1 present immune deficiencies, high sensitivity to IR and genome instability with a tendency to develop tumours (Morales et al., 2003; Ward et al., 2003b). 53BP1 has also been implicated in intra-S and G2-M checkpoints, although the observed defects were subtle (Fernandez-Capetillo et al., 2002; Morales et al., 2003; Ward et al., 2003b). With respect to DNA DSBs repair, 53BP1 was shown to facilitate the NHEJ repair pathway by regulating long range end joining for CSR (Manis et al., 2004; Nakamura et al., 2006; Ward et al., 2004) and some limited V(D)J recombination (Difilippantonio et al., 2008). 53BP1 increases the mobility of the broken chromatin and enhances their repair (Dimitrova et al., 2008). 53BP1 also down-regulate HR by inhibiting DNA end resection (Bunting et al., 2010).

53BP1 relocation after IR treatment can be easily detected following the appearance of ionizing radiation-induced foci (IRIF) in the nucleus of the cell, by fluorescence microscopy. 53BP1 IRIF formation requires an oligomerisation domain and a tandem tudor domain (TT domain) known for binding methylated histones (Huyen et al., 2004; Zgheib et al., 2009). Histones are components of the nucleosome, the basic unit of chromatin. They are subject to post-translational modifications (PTM) such as acetylation, methylation and phosphorylation, that are known to regulate gene transcription, chromatin condensation, DNA replication and the DNA damage response

(Kouzarides, 2007). In particular the tandem tudor domain of 53BP1 shows high affinity for histone H4 dimethylated at its lysine 20 and histone H3 dimethylated at its lysine 79 (Botuyan et al., 2006; Huyen et al., 2004). It has been proposed that histone marks H4K20me2 and H3K79me2, which are constitutively present on the chromatin, become exposed after DNA damage thus becoming accessible for interaction with proteins involved in DNA repair including 53BP1 (Huyen et al., 2004).

Despite numerous studies, the precise mechanism of 53BP1 recruitment to DNA damage sites and its function remain elusive. In 2004, the Halazonetis group published data characterizing the role of H3K79me2 in 53BP1 recruitment. This histone mark is catalysed by the histone methyltransferase (HMTase) DOT1. Following *DOT1* siRNA treatment of the human U2OS cell line, 53BP1 foci formation was completely abolished after IR treatment (Huyen et al., 2004). Two years later, Mer and collaborators carried out similar experiments but obtained contradictory results. They performed siRNA knock-down of *DOT1* in HeLa cells and *Dot1* knock-out in mouse ES cells. It was found that in *DOT1*-deficient cells, 53BP1 foci formation after IR was similar to that observed in wild type (WT) cells (Botuyan et al., 2006). Moreover, knock-down of another HMTase termed SET8 in HeLa cells was also performed. SET8 catalyzes the monomethylation of H4K20 (Couture et al., 2005; Xiao et al., 2005; Yin et al., 2005), whereas the di- and tri- methylation of H4K20 are mainly catalysed by the SUV420 enzymes (Schotta et al., 2004; Yang et al., 2008). The Mer group showed that *SET8* knock-down not only affected the monomethylation of H4K20 but also abrogated the dimethylation of H4K20. Under this deficiency, 53BP1 foci were not observed at DNA lesions after IR (Botuyan et al., 2006). Furthermore, additional publications reported contradictory results with 53BP1 being recruited to DSBs preferentially by either H4K20me2 or H3K79me2 (Schotta et al., 2008; Yan et al., 2009; Yang et al., 2008).

By contrast, in yeast, the mechanisms of recruitment to DNA damage sites is well established for the 53BP1 orthologs, Rad9 in budding yeast and Crb2 in fission yeast (Willson et al., 1997). As for 53BP1, Rad9 and Crb2 relocalisation to DNA damage is mediated by recognition of methylated histones through their tandem Tudor domain. In budding yeast, where H4K20me2 does not occur, the formation of Rad9 foci at DNA lesions is dependent on H3K79me2 (Grenon et al., 2007; Wysocki et al., 2005). In fission yeast, where H3K79 methylation does not occur, the recruitment of Crb2 to DNA damage sites is dependent on H4K20me2 (Sanders et al., 2004).

As both methylated histones H4K20 and H3K79 exist in higher cells, we hypothesized that both yeast pathways have been conserved throughout evolution and exhibit some overlapping or redundant functions. For this work, we successfully generated a new chicken DT40 cell line deficient in the methylation of H3K79. In absence of this histone mark, no defect in 53BP1 IRIF were detected at any tested doses or time post-irradiation. Also, a *Suv420* knock-out in chicken cells was generated in an attempt to establish a new H4K20me2/3 deficient cell line. This knock-out allowed us to identify the presence of a new gene, *Suv420h2*, in the chicken genome which has not yet been sequenced.

2.4 MATERIALS AND METHODS

2.4.1 Plasmid generation

Dot1 gene targeting strategy was designed in order to delete a 3.4Kb sequence containing exons coding for the SAM motif of Dot1. For that purpose, two targeting vectors, pLoxNeoDot1L and pBSHygroDot1L, were generated. An upstream sequence of 3.8Kb was amplified from the genomic DNA by PCR using the following primers 5'-GAC GGT ACC TGA CTA GCT AAA TCC CAG ATC TCA AGC TTG CTA TGG-3' and 5'-GAC GTC GAC TTA GCT AGT CAG TGT TCA GCT TCA TCG GTT GGG TG-3' which contained a KpnI and a SalI restriction sites respectively. Similarly, a downstream sequence of 5.8Kb was amplified from genomic DNA by PCR using the following primers 5'-GAC ACT AGT TGA CTA GCT AAC CGC AGC CAC GAA CTG CAA ACA TC-3' and 5'-GAC GCG GCC GCT GAC TAG CTA AGC ACG GCG ATG CCC ATT ACT GC-3', containing respectively a SpeI and a NotI restriction site respectively. The 3.4Kb PCR product of the 5'arm was inserted into the targeting vectors pLoxNeo and pBSHygro upstream of the resistance cassette using the KpnI and SalI restriction sites. Similarly, the 5.8Kb PCR product of the 3'arm was inserted into the targeting vector downstream from the resistance cassette using the SpeI and NotI restriction sites.

Suv420h gene targeting was designed to remove the entire gene, resulting in a deletion of a 21.1Kb sequence. Again, two targeting vectors were generated: pLoxBlastSuv420h and ploxNeoSuv420h. An upstream sequence of 4.3Kb was amplified from the genomic DNA by PCR using the following primers 5'- GTC GGT ACC GCT ATG CAA AAG TCC TGC ATA ACA AAA GTA G -3' and 5'- GAC GTC GAC GAA TAA TTT CTG CTC CAA TAC CTA CTG GAA G -3' which contained a KpnI and a SalI restriction sites respectively. Similarly, a downstream sequence of 5.6Kb was amplified from genomic DNA by PCR using the following primers 5'- GTC GCG GCC GCG AAG TCG CAT CAT ATC TTT CCA TGC AGG -3' and 5'-GTC GAG CTC GAA CAC TGT ACC AGC TCA CCT CCT TAG-3', containing a NotI and a SacI restriction site respectively. The 4.3Kb PCR product of the 5'arm was inserted into the targeting vectors pLoxNeo and pBSBlast upstream of the resistance cassette using the KpnI and SalI restriction sites. Similarly, the 5.6Kb PCR product of the 3'arm was inserted in the

targeting vector downstream from the resistance cassette using the NotII and SacI restriction sites.

2.4.2 Culture and transfection of chicken cells

DT40 chicken cell lines were grown in RPMI media complemented with 10% fetal calf serum, 1% chicken serum and 1% of antibiotics (10,000 units of penicillin and 10mg/ml of streptomycin). The cells were always kept growing below $1x10^6$/ml. For stable transfection, $1x10^7$ cells were washed with PBS and then resuspended in PBS at $2x10^7$cells per ml. After 10min of incubation at room temperature in the presence of 30 μg of DNA, cells were electroporated (250 V, 950 μFD). Cells were incubated at room temperature for another 10min before plating in RPMI media. The following day, cells were diluted 8 times in RPMI media with the appropriate antibiotic and plated in 96 well plates for an average of 11 days.

2.4.3 Culture and transfection of mammalian cells

U2OS cells were grown in DMEM media with 10% fetal calf serum and 1% antibiotics (10,000 units of penicillin and 10mg/ml of streptomycin). U2OS cells were stably transfected using lipofectamine 2000 system following instructions from the manufacturer (Invitrogen). The plasmid encoding the *DOT1* short hairpin siRNA as well as the control vector were a generous gift from Prof. Guo-Liang Xu (Lin et al., 2009). The coding sequence for this *DOT1* shRNA are 5'-GAT CCC CGG ATG AAA TGG TAT GGA AAT TCA AGA GAT TTC CAT ACC ATT TCA TCC TTT TTA-3' for the sense strand and 5'-AGC TTA AAA AGG ATG AAA TGG TAT GGA AAT CTC TTG AAT TTC CAT ACC ATT TCA TCC GGG-3' for the antisense strand.

2.4.4 Total RNA extraction and reverse transcription PCR

RNA was extracted from cells using TriReagent (Invitrogen,Paisley, UK). A cDNA pool was generated by reverse transcription using the Superscript First-Strand Synthesis kit (Invitrogen, Paisley, UK). *Suv420h* reverse transcription was performed following manufacturer's instruction using a first pair of primers: the forward primer 5'-ATG AAG TGG TTG GGA GAA TCC AAG AAC ATG-3' and the reverse primer 5'-TTA TGC ATT GAG CCT TAA AGA CTG ATC TTC CC-3' and a second pair of primers: the forward primer 5'-GCA GGG CAT CTA ACG ACC AC-3' and the reverse primer 5'-TTG CTA CTG CTA TCA TGA CGC C-3'. Amplification of *Centrin1* was used as

positive control using 5'- ATC ATT CCT TAA ATT CCA GTC ATT GT-3' as sense primer and 5'- GCC ACC CTG TTT TCC TTC AA-3' as anti-sense primer.

2.4.5 Genomic DNA extraction and Southern blotting

$1.5x10^6$ DT40 cells were collected and lysed with Tail buffer (50 mM Tris-HCl, pH 8.8, 100 mM EDTA, 100 mM NaCl, 1% SDS) and proteinase K at 120μg/ml. After an overnight incubation at 37 ^{O}C, the genomic DNA was precipitated with saturated NaCl (6M) and isopropanol. All of the genomic DNA extracted was digested by the appropriate enzymes for Southern blotting. Half of the digested sample was loaded on the gel. Southern blotting was performed using a non-radioactive Dig-system (Roche). The probe used to screen clones from the *Dot1* gene targeting was first amplified from genomic DNA using the primer 5'-GAG CCT ATA CCC TTC TGA CAC TTG-3' and 5'-GCA CTG CAA TCA CGC TTG TAA GAC-3' and inserted in a Topo vector (Topo cloning kit, Invitrogen). Similarly, the probe used to screen clones from the *Suv420h* gene targeting was first amplified from genomic DNA using the primers 5'- GCT CCT GCA TGG ATA TGA GTG GAA G-3' and 5'- CAG GCA TCT GGG TAT GTT TGT GTG C-3' and inserted in a Topo vector (Topo cloning kit, Invitrogen). Then the *Dot1* and *Suv420h* probes were amplified respectively from these pTopoDot1Pb and pTopoSuv420hPb vectors and labeled with digoxigenin by PCR (PCR Digprobe synthesis kit, Roche).

2.4.6 Cell extracts and Western blotting.

The collected cells were washed in cold PBS and resuspended in 1x SDS-PAGE sample loading buffer ($2x10^5$ cells for 5μl of sample buffer). The lysed cells were then incubated for 10 min at 95^{O}C, sonicated (40% amplitude for 10 seconds) and incubated again for 10min at 95^{O}C. The Western blotting membranes were blocked with 4%milk, incubated overnight with the primary antibody, washed in PBS and incubated again with the secondary antibody for 1h at room temperature. The antibodies used were rabbit anti-53BP1 (Novus biological for extracts from human cell lines and generous gift for Prof. Thanos D Halazonetis for the extracts from DT40 cell lines), mouse γH2AX (Millipore), Dot1 (Santa Cruz), all related H3 and H4 antibodies (Abcam).

2.4.7 Immunofluorescence and microscopy

Cells were fixed with 4% v/v paraformaldehyde and permeabilized with 0.125% v/v of Triton-100. After a blocking step using a 4% BSA solution, cells were incubated for 1h at 37^{O}C with primary antibody, washed, and incubated again for 1h at 37^{O}C with the secondary antibody. Slides were mounted with vectashield media with DAPI. Microscopy imaging was performed on a Deltavision microscope using softworx software (Applied Precision). 0.5μm Z-stacks were collected, deconvolved and merged. The microscopy analyses were performed using Image-Pro Analyser software (MediaCybernetics). Antibodies used for immunofluorescence assay were 53BP1 (Novus biological for extracts fron human cell lines and generous gift for Prof. Thanos D Halazonetis for the extracts from DT40 cell lines), mouse γH2AX (Millipore), FITC-labelled Goat anti-rabbit (Jackson Immunoresearch), TRITC-labelled Goat anti-mouse (Jackson Immunoresearch).

2.5 RESULTS

2.5.1 H3K79me is not required for 53Bp1 IRIF in chicken

To investigate whether H3K79me is required for the recruitment of 53BP1 to DNA damage foci, a cell line deficient in the histone mark H3K79me was generated. The chicken lymphoma B-cell line DT40 was used as model system. Despite a certain evolutionary distance with the chicken and human systems, the DNA damage response appears to be well conserved in chicken (Winding and Berchtold, 2001). In addition, the ratio between targeted and random integration make this cell line a very powerful tool for any gene targeting study (Buerstedde and Takeda, 1991).

DOT1 is the unique histone methyltransferase (HMTase) known to catalyse the mono-, di- and tri-methylation states of the histone H3 at its lysine 79 (Feng et al., 2002; Frederiks et al., 2008; Ng et al., 2002). Well conserved throughout the evolution, DOT1 is the only lysine HMTase characterized, that does not possess a catalytic domain called "SET domain" typical of the histone-lysine methyltransferases. Instead, a different catalytic core domain was identified at the N-terminus of the protein. It contains eight motifs essential for DOT1 activity that are perfectly conserved from chicken to human (Figure 2.1) (Feng et al., 2002). Studies conducted to characterize the functions of these motifs showed that point mutations on motif I, suppressed the interaction with the methyl donor S-adenosyl methionine (SAM) abolishing DOT1 activity on the histone H3 (Feng et al., 2002). Also, a point mutation introduced on motif IV, necessary for the catalysis of methyl transfer, abolished the HMTase activity of DOT1 on the histone H3 (Min et al., 2003). According to these data, motifs I and IV, coded respectively by exons 5-6 and 9, are pivotal for DOT1 function and therefore a gene targeting strategy was designed to partially knock-out exon 5 and exon 6. The resulting mRNA is missing a sequence of 75 aa which includes the motif I. Also, a stop codon was introduced between those two exons to prevent expression of the further C-ter of the chicken Dot1 protein (Figure 2.2A).

DT40 wild-type cells were first transfected with a targeting vector containing a blasticidin resistance cassette flanked by two *Dot1* homologous arms. 36 clones were screened by Southern Blotting and 16 clones were positive for gene targeting at a first *Dot1* allele. Two of the heterozygous DT40 clones *Dot1*$^{+/-}$ identified were then

transfected with a second targeting vector containing a neomycin resistance cassette flanked by two *Dot1* homologous arms in order to target the second *Dot1* allele. 84 and 72 clones isolated from each heterozygous *Dot1*$^{+/-}$ transfection were screened by Southern-blotting and positive clones with both *Dot1* alleles targeted were identified (Figure 2.2B). Western blotting showed the absence of the histone mark H3K79 in the new *Dot1*$^{-/-}$ cell lines (Figure 2.2D). This result was also confirmed by mass spectrometry, where no H3K79me2 was detected (FitzGerald et al., 2011).

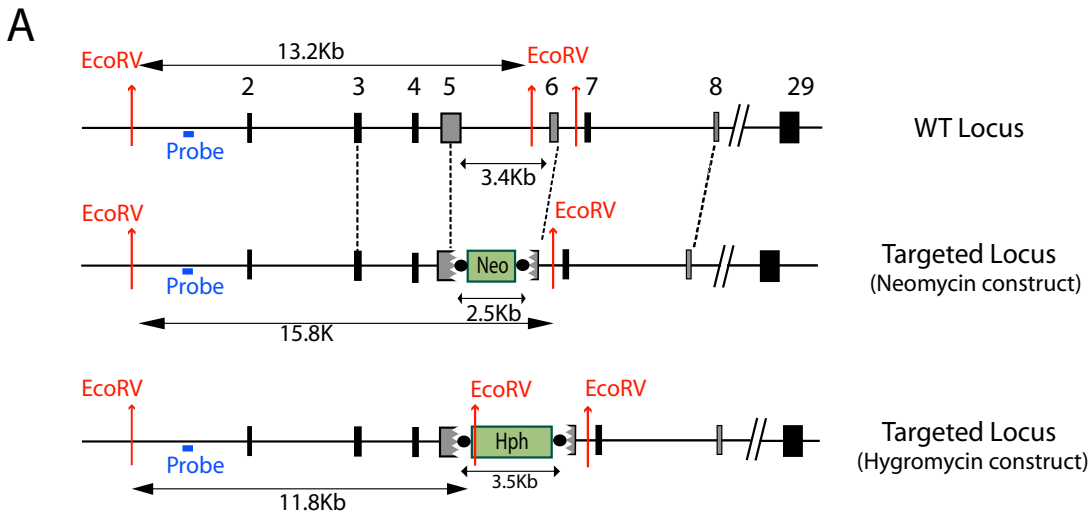
A

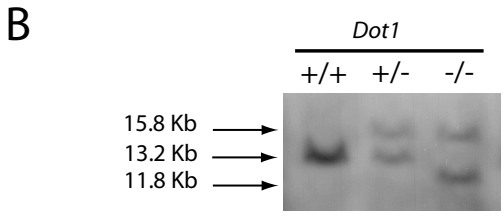
B

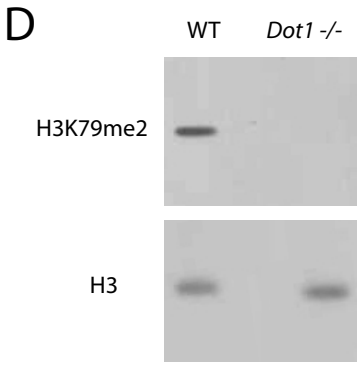
	Identity	Conservation
full Dot1 protein	75.1 %	92.6%
catalytic domain (1-345)	93.3%	95.4%
non-catalytic domain (346-1537)	69.9%	91.9%

Figure 2.1: Comparison of Dot1 protein between chicken and human.
(A) Clustal alignment of the catalytic domain of DOT1 of human (hDOT1) and chicken (ggDOT1). Amino acid in alternative blue and green font correspond to *Dot1* exons. In the red boxes are the conserved sequences motif assembled at the active site. Amino acids highlighted in grey correspond to

the residues knocked-out after gene targeting. (B) amino acid homology between human and chicken of DOT1 protein.







C

Cell line transfected	clone screened	targeted events	retargeted events	targeting efficiency
Dot1 +/+	36	16	ND	44.4%
Dot1 +/- clone1	84	1	4	1.2%
Dot1 +/- clone 2	72	1	8	1.4%

Figure2.2: Generation of a *Dot1* knockout cell line.
(A) Schematic representation of the chicken *Dot1* locus. Two targeting vectors containing either a neomycin or a hygromycin cassette were used to disrupt *Dot1* gene in both alleles. Exons in grey contain conserved motifs clustered at the active site of Dot1 (shown in the Sup Fig1). Circles represent stop codons. Black arrows indicate the expected sizes of bands in Southern blot after digestion of the genomic DNA by EcoRV. Neo:Neomycin, Hph:hygromycin. (B) Southern blot analysis of the Dot1 locus using the probe indicated in (A). (C) Targeted integration efficiency at the *Dot1* locus. (D) Dimethylation of H3K79 is undetectable by Western Blot in absence of active Dot1.

The two newly-generated *Dot1*$^{-/-}$ cell lines were treated with IR at high, medium and low doses, and 53Bp1 foci were monitored by immunofluorencence assay after 1h recovery. As shown in the Figure 2.3A, the ability to recruit 53Bp1 to DNA damage sites remained unaffected in H3K79me-deficient DT40 cell lines. The quantification of 53Bp1 IRIF did not reveal any defect. On the contrary, 53Bp1 foci were even more numerous and intense in the *Dot1*$^{-/-}$ cells (Figure 2.3B and C) compared to WT before and after IR.

Numerous publications showed that DOT1 is involved in transcription regulation through its methylation activity on H3K79, as H3K79me2 is often associated with active genes (Steger et al., 2008; Wang et al., 2008). Moreover, it was shown that DOT1 itself is part of complexes implicated in the regulation of the RNA polymerase II (Bitoun et al., 2007; Mueller et al., 2009). A microarray assay was performed on these DT40 *Dot1*$^{-/-}$ cell lines to detect any transcription pattern variation (FitzGerald et al., 2011) and the results showed an increase of *53Bp1* RNA of 1.7 fold (Data not shown) compared to WT cells. An elevation of 53Bp1 protein expression could explain the increase of 53Bp1 foci signal observed before and after IR.

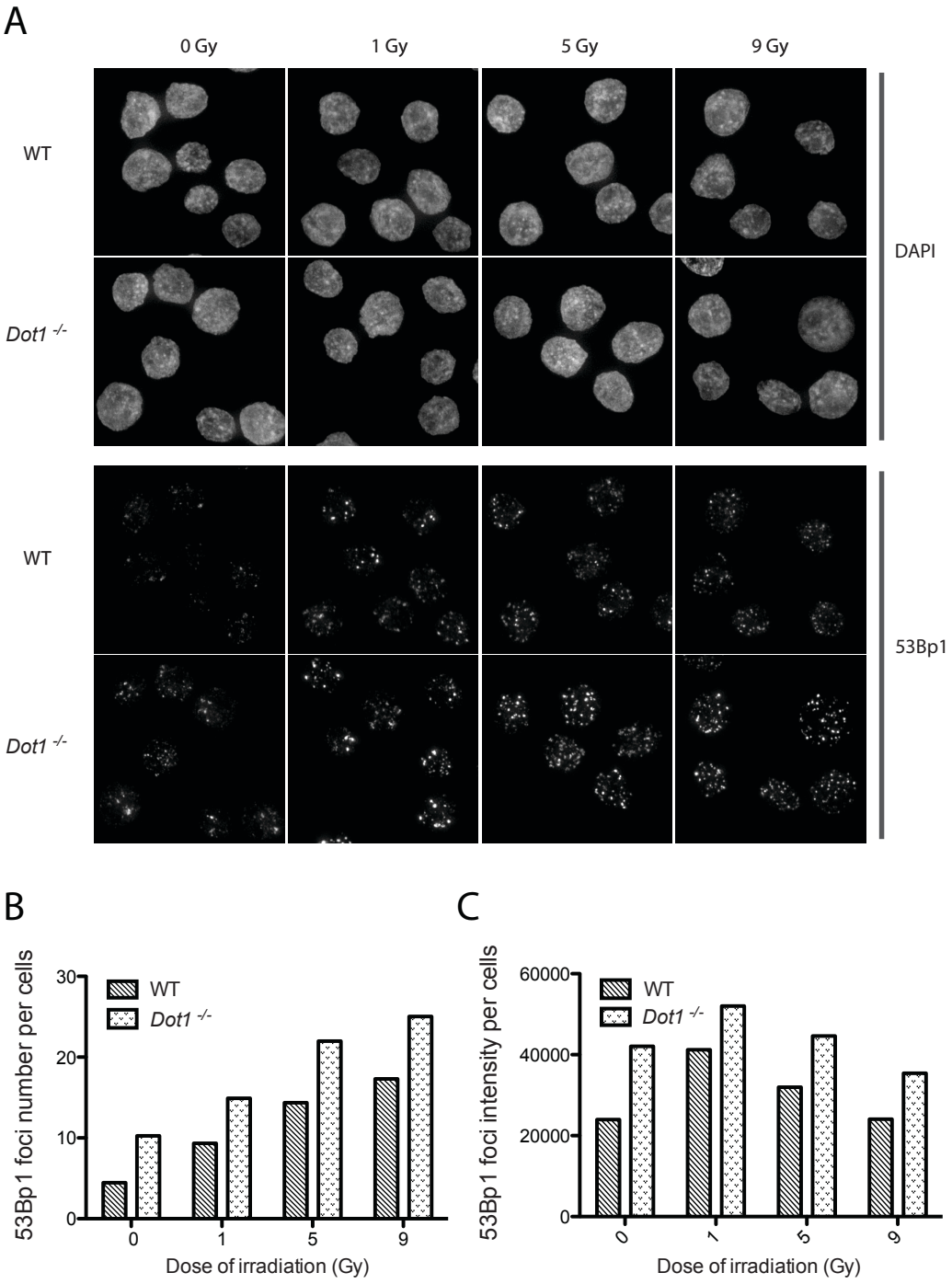


Figure2.3: H3K79me is not required for 53Bp1 IRIF in DT40.
(A) Detection of endogenous 53Bp1 by immunofluorescence. Cells were irradiated with the indicated doses of IR, fixed 1h after IR and then stained with 53Bp1 antibody (B) Quantification of the number of 53Bp1 foci in (A). (C) Quantification of 53Bp1 foci intensity in (A).

2.5.2 H3K79me2 may be required for 53BP1 IRIF in mammalian cells

Our previous results showed that H3K79 is not implicated in the general recruitment of 53Bp1 to DNA lesions in chicken DT40 cells. As the DNA damage response pathways in chicken cells and mammalian cells present high degree of fuctional overlap (Winding and Berchtold, 2001), we can hypothesize that H3K79me2 is most likely not required for 53BP1 IRIF in human cells. However, the lack of functional p53 in DT40 cells limit extrapolations from DT40 to mammalian cells (Takao et al., 1999). A study by Huyen *et al* was the first published work describing a H3K79me dependency for 53BP1 IRIF. That work was performed on U2OS cells using a siRNA targeting *DOT1* (Huyen et al., 2004). All the other studies were carried out on other cell lines and using different methods of DOT1 disruption (Figure 2.4) (Botuyan et al., 2006; FitzGerald et al., 2011; Wakeman et al., 2012).

Sources	Cell lines	Targets (methods)	IR treatments (recovery time)	53BP1 IRIF formation
Huyen et al. (Nature, 2004)	U2OS	*DOT1** (siRNA)	9Gy (15 min)	deficient
Botuyan et al. (Cell, 2006)	MEF	*Dot1* (K.O)	3Gy (5 min)	proficient
	A549	*DOT1* (shRNA)	1Gy (1h)	proficient
Fitzgerald et al. (Plos One, 2009)	DT40	*Dot1* (K.O)	2-15 Gy (time course)	proficient
Wakeman et al. (EMBO J., 2012)	U2OS	*BAT3* (siRNA)	5Gy (1 h)	deficient
		BAT3 (shRNA)	5Gy (10 min)	deficient
		*DOT1** (siRNA)	5Gy (1 h)	deficient

Figure 2.4: Literature research on 53BP1 foci formation in absence of H3K79me.
Studies published about the recruitment of 53BP1 at IR-induced DNA damage sites in absence of the histone mark H3K79 dimethylated and the experimental conditions used by each study. The asterisk indicates that the same *Dot1* siRNA sequence was utilized in both experiments.

Our approach consisted in using similar experimental conditions (same cell line and IR treatment settings) to the study of Huyen *et al.* We generated two stable U2OS cell lines constitutively expressing a shRNA targeting *DOT1*. However, the *DOT1* shRNA sequence used here was different from the sequence used by Huyen et *al* and Wakeman *et al*.. Clones selected by antibiotic resistance were first screened by Western blot. Two clones displayed a 91-97% reduction of methylation levels on the lysine 79 of the histone H3 (Figure 2.5A and B). Also, DOT1 protein levels in those two clones were not detectable by Western blot (Figure 2.5C). Those two newly generated cell lines were irradiated with 9 Gy of IR and 53BP1 IRIF formation was assessed by IF after 15min of recovery.

The experiment was repeated 4 times with both *DOT1* shRNA U2OS clones and unfortunately two contradictory results were obtained. In two experiments, the two H3K79me-deficient U2OS cell lines, C1 and C3, were able to induce the recruitment of 53BP1 foci after DNA damage similarly to WT cells. This suggests that DOT1 and H3K79me2 deficiency did not seem to affect the recruitment of 53BP1 to DNA damage sites. The quantification of 53BP1 IRIF supported this conclusion (Figure 2.5G and data not shown). This data agree with our previous results in chicken cells as well as the work conducted by Botuyan *et al.* (Botuyan et al., 2006). However, in two further experiments, a partial defect in 53BP1 recruitment to DNA damage sites was detected in cell lines deficient for H3K79 methylation (Figure 2.5D). Quantification of one of these experiments confirmed our observations. *DOT1* shRNA U2OS cell lines presented fewer cells positive for 53BP1 IRIF compared to WT (Figure 2.5E). Also, those positive cells exhibited a lower number of 53BP1 foci in the *Dot1* knock-down cell lines compared to WT (Figure 2.5F). The contradictory results open the possibility to a potential role for H3K79me2 in 53BP1 recruitment in mammalian cells, although, only under specific conditions yet to be characterized. However, this immunofluorescence assay may present some limitation for such a possible subtle defect. Recruitment of 53BP1 to DSBs in Dot1 deficient cell lines should be assessed in a more specific context, such as cell cycle phase or chromatin context.

9% 3%

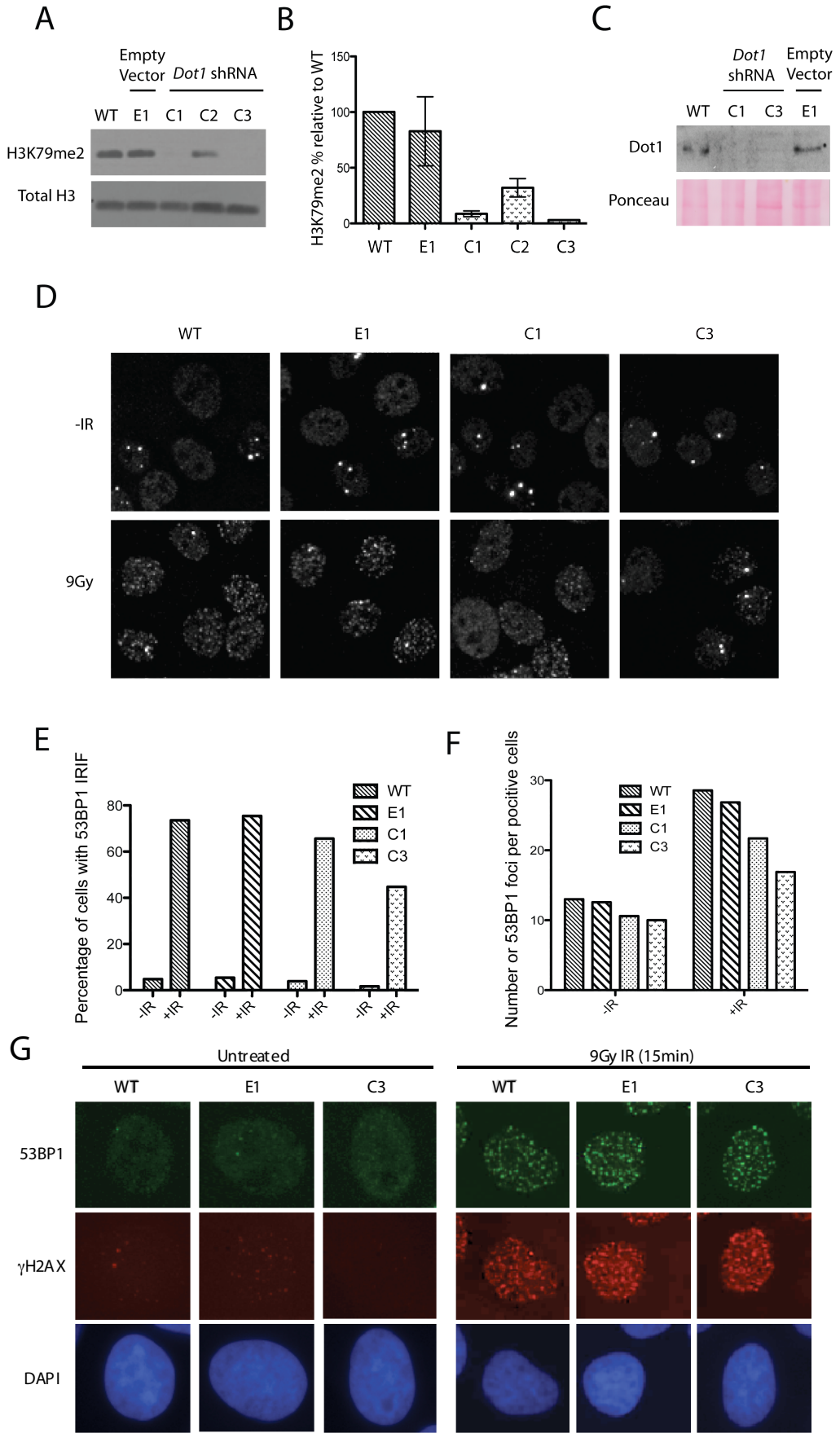


Figure 2.5: H3K79me may be not required for 53BP1 IRIF in human cells.

(A) Western blot analysis of H3K790 methylation status in *DOT1* knock-down U2OS cell lines. (B) Quantifications of the Western blot analysis performed in (A). (C) Western Blot Analysis of DOT1 status in the *DOT1* knock-down U2OS cell lines. (D) Detection of endogenous 53BP1 IRIF by immunofluorescence (pictures representative of two replicates). Cells were fixed 15min after 9Gy IR and then stained with a 53BP1 antibody. Quantification of 53BP1 foci induction in (E) and intensity in (F). (G) Detection of endogenous 53BP1 IRIF by immunofluorescence. Cells were fixed 15min after 9Gy IR and then stained with a 53BP1(in green) and γH2AX antibody (in red) (pictures representative of two other replicates)

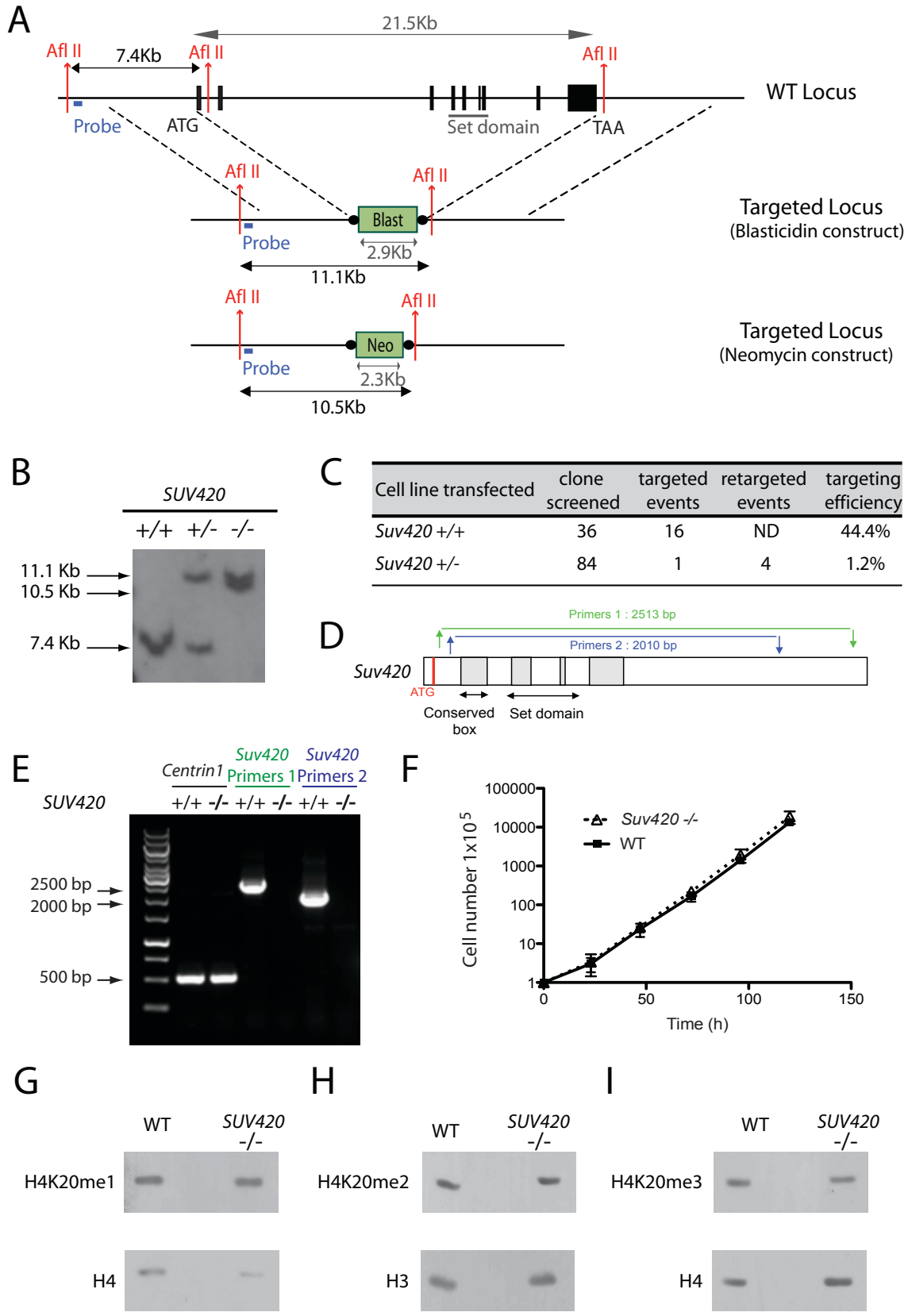
2.5.3 Generation and characterisation of *Suv420-/-* DT40 cell lines

It has been suggested that H3K79me2 is not the main histone mark regulating the formation of 53BP1 foci, but that H4K20me2 could also be implicated (Botuyan et al., 2006). This histone post-translation modification is more complicated to study as 7 potential histone methyltransferase enzymes have been identified either *in vitro* or *in vivo*– ASH1 (Beisel et al., 2002), NSD1 (Rayasam et al., 2003), NSD2/MMSET (Marango et al., 2008; Pei et al., 2011), PRDM6 (Wu et al., 2008), SET8 (Fang et al., 2002; Nishioka et al., 2002), SUV420H1 and SUV420H2 (Yang et al., 2008). *In vivo*, siRNA knock-down of the only three last cited HTMases, SET8, SUV420H1 and SUV420H2 affect the H4K20 methylation pattern in absence of DSBs (Yang et al., 2008). Knock-down of Ash1 and NSD1 in *drosophila* S2 does not affect the methylation pattern of H4K20 (Yang et al., 2008). Recently, it has also been suggested that the MMSET enzyme is capable of H4K20 dimethylation specifically after DNA damage (Pei et al., 2011). PRDM6 knock-down attempts are, for the moment, inconclusive (Wu et al., 2008). The three methyl-transferases SET8, SUV420H1 and SUV420H2 are responsible for fluctuations observed in H4K20 methylations levels during the cell cycle (Houston et al., 2008). During G2 and M phases, SET8 catalyzes the mono-methylation of lysine 20 on H4. In the following G1 phase, monomethylation levels decrease progressively while di- and tri-methylation of H4K20 appear (Houston et al., 2008). In S phase, *de novo* histones are displayed on the chromatin and the total level of H4K20 methylation is reduced. The methyl-transferases SUV420H1 and SUV420H2 are responsible for H4K20me2 and H4K20me3. They preferentially catalyze the addition of one or occasionally two methyl groups on H4K20 monomethylated but may also be able to use H4K20 unmethylated as a substrate in absence of SET8 (Yang et al., 2008).

To investigate the possible involvement of H4K20me2 in 53BP1 IRIF formation, we decided to generate a new DT40 cell line deficient in this histone mark. Knocking out

Set8 in DT40 is unlikely to be possible as *Set8* appears to be an essential gene. Knock-out of *Set8* in mice and drosophila was shown to be lethal (Karachentsev et al., 2005; Nishioka et al., 2002; Oda et al., 2009). Indeed, SET8 was shown to be required for S phase progression (Huen et al., 2008; Jorgensen et al., 2007; Tardat et al., 2010; Tardat et al., 2007) and mitosis (Houston et al., 2008; Wu et al., 2010). Cells lacking Set8, and subsequently H4K20me1, arrest their cell cycle and are driven to apoptosis (Houston et al., 2008). Therefore, we designed a targeting strategy to knock-out the entire coding region of *Suv420*. In chickens, as in *Drosophila*, only one Suv420 gene had been identified. Two targeting vector, one with a resistance cassette for blasticidin and another one for neomycin, were successively used (Figure 2.6A). By Southern blot screening, a DT40 clone with both *Suv420* alleles targeted was identified (Figure 2.6B). As we lack an antibody that recognizes the Suv420 chicken protein, a reverse transcription PCR assay was designed to confirm the absence of *Suv420* mRNA in the newly generated *Suv420*$^{-/-}$ cell line. Two sets of primers were used to amplify *Suv420* mRNA in WT and *Suv420*$^{-/-}$ cell lines. Corresponding fragments were successively amplified in WT but not in *Suv420*$^{-/-}$ cell line (Figure 2.6D and E). To confirm the quality of both WT and *Suv420*$^{-/-}$ cDNA samples, we performed as positive control a PCR reaction to amplify the *Centrin1* cDNA. A PCR product of the right size was amplified from both sample, therefore, we concluded that Suv420 was no longer expressed in the newly generated *Suv420*$^{-/-}$ cell line.

In 2008, Shotta et al. generated single and double knock-out mice mutated for *Suv420h1* and *Suv420h2*. Whereas *Suv420h2* knock-out mice did not display any phenotypic defect, *Suv420h1*$^{-/-}$ mice were reported to present perinatal lethality and smaller dimensions. *Suv420h* double knock-down mice present a similar phenotype to *Suv420h1*$^{-/-}$ mice. Also, MEF cells issued from *SUV420h* double knock-down mice display proliferation defects with an S-phase entry delay (Schotta et al., 2008). To assess a possible growth defect in *Suv420*$^{-/-}$ DT40 cell lines, we monitored their proliferation in parallel with DT40 WT cells. The doubling time calculated from the growing curves was similar for both WT and *Suv420*$^{-/-}$ cell lines with 9.08h and 8.72h respectively (Figure 2.6F).



Cell line transfected	clone screened	targeted events	retargeted events	targeting efficiency
Suv420 +/+	36	16	ND	44.4%
Suv420 +/-	84	1	4	1.2%

Figure 2.6: Generation of *Suv420* knock-out in DT40 and its characterization.
(A) Schematic representation of the chicken *Suv420* locus. Two targeting vectors containing either a blasticidin or a neomycin cassette were used to disrupt the *Suv420* gene in both alleles. Black circles represent stop codons. Black arrows indicate the expected size of bands in Southern blot after digestion of the genomic DNA by AflII. Blast: blasticidin, Neo: neomycin. (B) Southern blot analysis of the *Suv420*

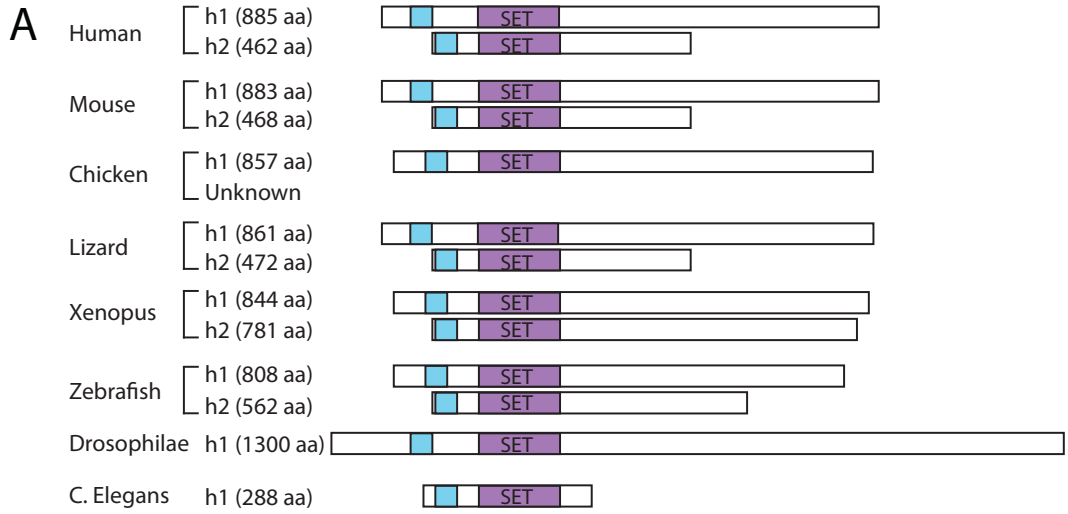
locus using the probe indicated in (A). (C) Targeted integration efficiency at the *Suv420* locus. (D) Reverse transcription PCR strategy used to confirm *Suv420* knock-out in the new cell line generated. (E) Reverse transcription PCR analysis of *Suv420* mRNA. Centrin1 primers were used as positive control. (F) Growth curve analysis of *Suv420*$^{-/-}$ cell line compared to WT. Seeding was originally at $1x10^4$ cells/ml and counted every 24 hours. Results are from one experiment performed in triplicate. (G), (H) and (I) Western blot analysis of H4K20 methylation status in *Suv420*$^{-/-}$ cell lines compared to WT. Antibody against mono-, di- and tri- methylated H4K20 were used respectively.

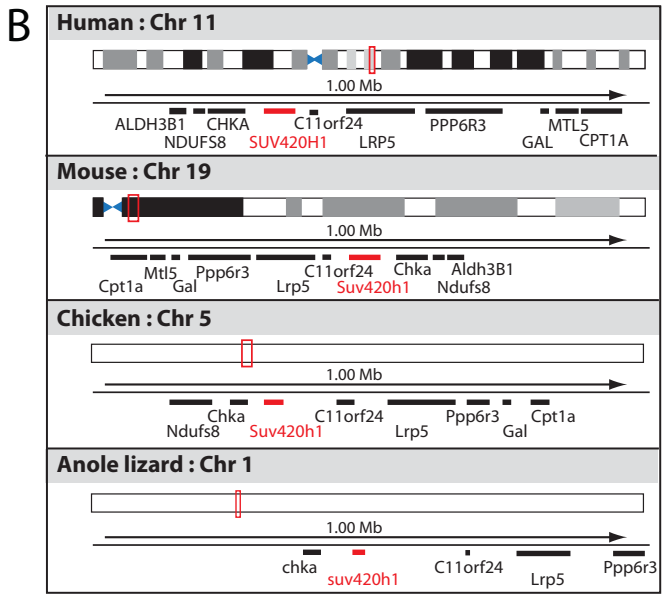
By western blotting, we analyzed the levels of each H4K20 methylation form in *Suv420*$^{-/-}$cells. Compared to the wild-type cell line, H4K20me1 level seems slightly higher in absence of Suv420 (Figure 2.6G) as expected. Also, we were still able to detect both di- and tri- methylations of H4K20 in the *Suv420*$^{-/-}$ cell line. Compared to WT, the di-methylation levels of H4K20 in the *Suv420*$^{-/-}$ cell lines appeared unchanged while H4K20me3 levels seemed only slightly lower (Figure 2.6H and I). This could be evidence for the existence of at least a second enzyme able to di- and tri-methylate the histone H4 at its lysine 20 in chicken.

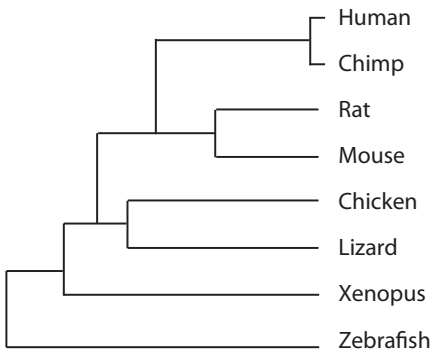
2.5.4 *Suv420h1* and *Suv420h2* are present in the chicken genome

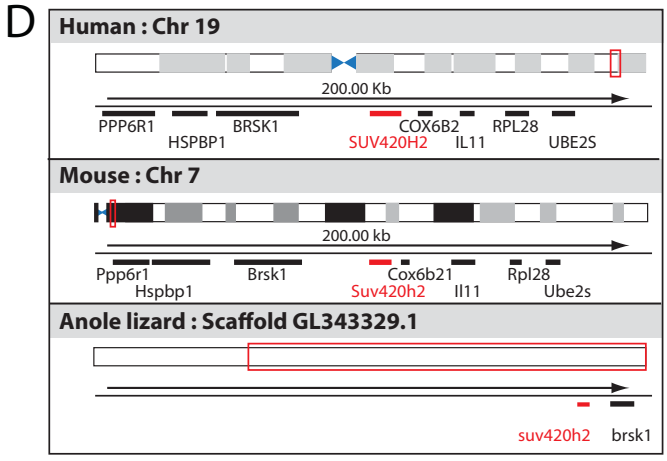
The two homologous proteins SUV420H1 and SUV420H2, coded by two homologous genes, were first identified in mouse while only one corresponding gene, coding Suv420, was identified in *Drosophila* (Schotta et al., 2004). Analysis of the proteomic and genomic databases of a variety of species showed that the two homologous proteins, SUV420H1 and *SUV420H2* are present in zebra fish and conserved throughout evolution to humans (Figure 2.7A). As analysis of genomic relationships among vertebrate species suggests that the chicken genome displays close homology to the lizard genome (Miller et al., 2007) (Figure 2.7C), which possesses genes corresponding to *Suv420h1* and *Suv420h2*, therefore we can expect that both genes *Suv420h1* and *Suv420h2* should also be present in the chicken genome. As only 90% of the chicken genome has been sequenced so far, it is conceivable that the chicken *Suv420h2* gene is localized in the 10% of the genome that has not yet been sequenced.

Genomic context analysis of *SUV420H1* showed that the genes flanking the *SUV420H1* gene in human and mouse also flank the *Suv420h1* gene in chicken (Figure 2.7B). However, a BLAST analysis in the chicken genome of the genes flanking *SUV420H2* in the human and mouse genome did not permit their identifications (Fig 2.7D). We hypothesise that this whole segment of the chicken genome may not be sequenced yet.









E

SUV420 H1 vs H2 - full length				
	hu	ms	xe	zf
% cDNA ident.	48	51	49.7	49.2
% aa ident.	47.9	48.6	31	37.5
% aa cons.	78.7	75.6	94.4	80.5
SUV420 H1 vs H2 N-Ter conserved Region				
	hu	ms	xe	zf
% cDNA ident.	60	64.8	67.6	64.5
% aa ident.	72.2	72.2	69.4	63.9
% aa cons.	94.4	88.8	94.4	80.5
SUV420 H1 vs H2 Set Domain				
	hu	ms	xe	zf
% cDNA ident.	62.2	64.3	67.6	62.6
% aa ident.	70.2	70.2	72.3	64.5
% aa cons.	90.1	88.6	92.2	85.8

Figure 2.7: SUV40H1 and SUV420H2 sequence analysis.
(A) Protein alignment of SUV420H1 and SUV420H2 in various species. Blue box: N-Ter conserved region. Purple box: SET Domain. (B) Genomic context of *SUV420H1* in the indicated species. (C) Dendrogram representing genetic evolutionary relationship. Figure adapted from Miler W and al., 2007 (D) Genomic context of *SUV420H2* in the indicated species (E) Amino acids similarities between

SUV420H1 and SUV420H2 entire protein or indicated region or domain. Hu: human, Ms: mouse, Xe: xenopus, zf: zebrafish.

The bioinformatic analysis of SUV420H1 and SUV420H2 revealed two very well conserved sequences between species at both proteomic and genomic level. The first sequence, termed SET Domain, is the catalytic domain that is present in all lysine histone methyltransferase with the exception of DOT1. The second sequence, located at the N-terminal of the protein, does not have a known function but is specific to SUV420 proteins (Figure 2.7E). A BLAST analysis conducted against the conserved N-terminal region in the human protein database, recognized only the two different isoforms of SUV420H1 and the unique isoform known of SUV420H2, confirming the specificity of this region (data not shown). In order to identify the chicken Suv420h2 protein, we used the nucleotide sequence coding for the N-terminal conserved region to scan the chicken ESTs (expressed sequence tag) database using BLAST. A transcript from normalized chicken breast muscle, leg muscle and epiphyseal growth plate cDNA library (BM489727.1) provides a significant alignment with 98% coverage and 82% identity (data not shown). The alignment of the full sequence of this newly-identified transcript (603 nucleotides) with the human and mouse *SUV420H2* cDNA showed that this new sequence also covers a portion of the genomic sequence coding for the set domain. Using the 3' end of the newly identified mRNA sequence, we scanned again the chicken ESTs database using BLAST. This second search permitted the identification of a second transcript of 410 nucleotides length (AJ393751.1). The alignment showed 100% coverage and 100% identity (data not shown). This second sequence codes for the SET domain and a few amino acids downstream. A third round of BLAST analysis using the 3' end sequence of the second transcript did not recognize any product with strong similarity. Nevertheless, the two partial mRNAs obtained by the first two BLAST screens allowed the identification of a portion of Suv420h2 in Gallus Gallus, starting from the first methionine of the protein and ending a few amino acids downstream of the SET domain (Figure 2.8). A reverse transcription PCR using this partial mRNA sequence would confirm the existence of the SUV420H2 in chicken.

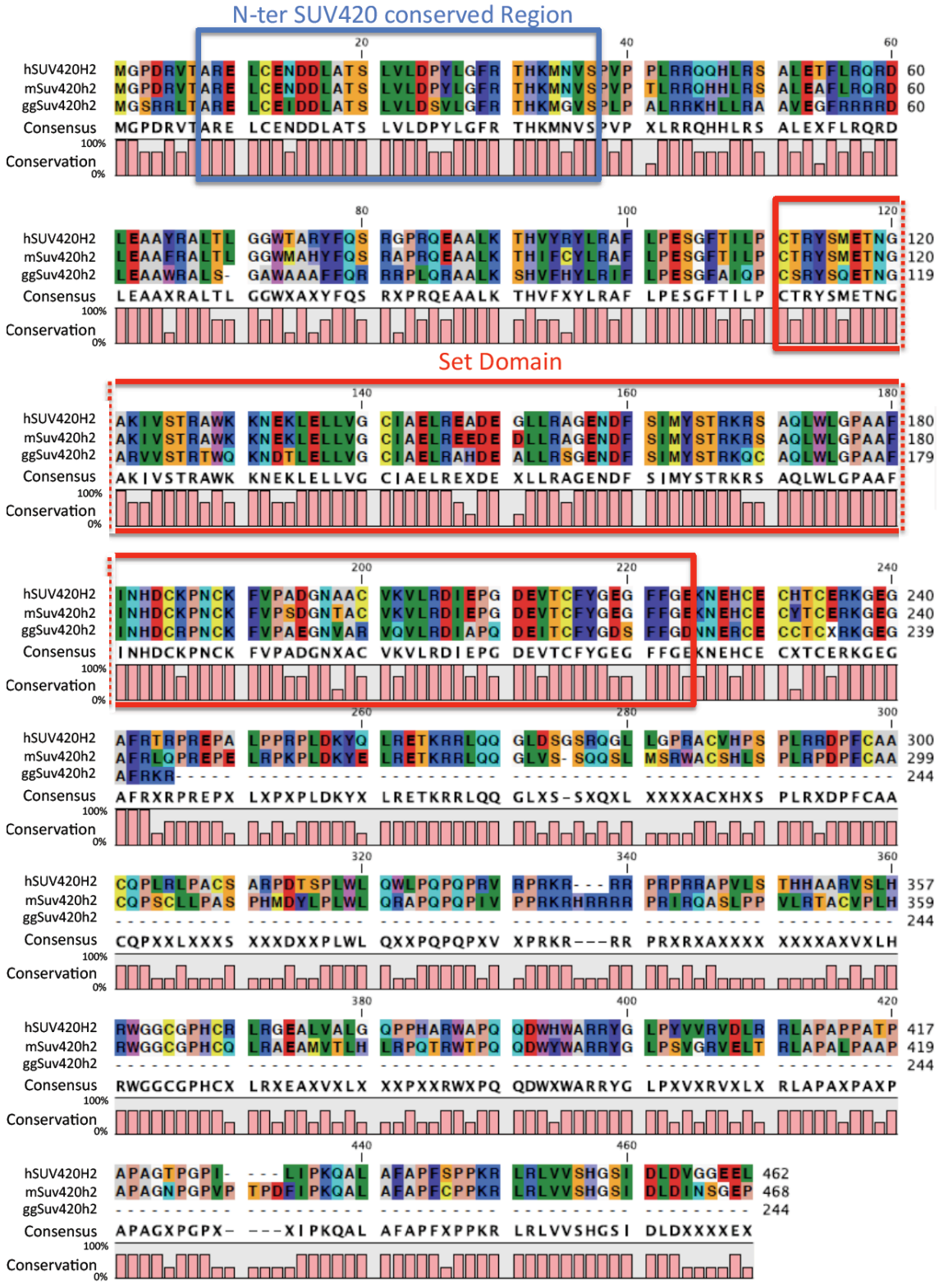


Figure 2.8: SUV420H2 sequence analysis.
Protein alignment of SUV420H2 in human and mouse compared with the partially known protein sequence of Suv420h2 in chicken. h: Human, m: Mouse, gg: Gallus Gallus. SET domain according to SMART Protein.

2.6 DISCUSSION

53BP1, a mediator of the DNA damage response, functions in various pathways such as DNA damage signaling and DNA repair. It assembles at DNA double strand breaks within minutes of IR treatment. Despite numerous studies, the mechanism of 53BP1 recruitment to DSBs in higher organisms remains ambiguous. In yeast, however, the recruitment mechanisms of 53BP1 orthologs at DNA lesions are well defined. The yeast orthologues of 53BP1 are Rad9 in budding yeast and Crb2 in fission yeast (Willson et al., 1997). All three proteins display two BRCT domains and a tandem Tudor domain. The association of Rad9 and Crb2 Tudor domains with methylated H3K79 and methylated H4K20 respectively is essential for their foci formation (Grenon et al., 2007; Sanders et al., 2004; Wysocki et al., 2005). In vitro, the tandem Tudor domain of 53BP1 is able to bind both histone marks H3K79me2 and H4K20me2 (Botuyan et al., 2006; Huyen et al., 2004; Kim et al., 2006). However the relative contributions of these histone marks to 53BP1 recruitment has yet to be resolved. Here we hypothesized that these modifications have overlapping or redundant roles in the recruitment of 53BP1.

It was first suggested that the histone mark H3K79me is necessary for the focal recruitment of 53BP1 upon DNA DSBs (Huyen et al., 2004). However, this observation was later contradicted in another studies (Botuyan et al., 2006; Schotta et al., 2008; Wakeman et al., 2012; Yan et al., 2009; Yang et al., 2008). We speculated that the different conclusions regarding a H3K79me2 role for 53BP1 IRIF might be a consequence of experimental conditions. DOT1 is the unique histone methyltransferase responsible for H3K79 methylation (Feng et al., 2002; Frederiks et al., 2008; Ng et al., 2002). Huyen et al. used a single DOT1 siRNA sequence at high dosage and for three successive transfections. On the other side, Botuyan et al., who observed a contradictive result, have chosen to knock down Dot1 using shRNA. We hypothesized that their contradictive observations may result from the RNAi sequences used, one of the two potentially producing some off-target effect. .

In this report, we successfully generated two cell lines deficient in H3K79 methylation by targeting DOT1. We first established a chicken DT40 cell line partially knocked out for the *Dot1* gene. The newly generated cell line expresses a deficient Dot1 protein. As western blotting and mass spectrometry show near to complete absence of H3K79 dimethylation, we named the newly generate cell lines *Dot1*$^{-/-}$. We monitored the

formation of 53Bp1 foci at DNA damage sites after IR in the *Dot1*$^{-/-}$ cells and no defect was observed in comparison with the WT cells.

The second cell line was generated from U2OS, expressing stably and constitutively a *DOT1* shRNA. We identified two independent clones displaying a particularly low level of H3K79me2 with reductions of 91% and 97% as measured by Western blot. The recruitment of 53BP1 in those two U2OS DOT1 deficient cell lines was analyzed after IR treatment and repeated four times. Two contradictory results were observed. In two cases, no defect in 53BP1 IRIF was observed in both independent clones. On the contrary, in two additional experiments, a partial defect in 53BP1 IRIF formation was detected in absence of DOT1. This defect was particularly subtle in the clones with 9% of H3K79me2 remaining and greater in the clone with only 3% of H3K79me2 remaining. This result suggests that H3K79me may have none or only a limited role in the recruitment of 53BP1 at DNA DSBs. Also, a potential role for H3K79me2 in 53BP1 recruitment to DSBs can be masked by redundancies with other histone marks such as H4K20me2. The poor reproducibility of this result may reveal a limitation of the technique and experimental approach used. The role of H3K79me2 in 53BP1 IRIF formation may need to be analysed under a specific context, such as DSBs chromatin localization or phases of the cell cycle.

Recently, new published data has suggested that H3K79me2 facilitates the recruitment of 53BP1 during specific phases of the cell cycle. They showed that a protein termed BAT3 associates with DOT1 and is required for the DOT1 function. In the absence of BAT3, H3K79 is not methylated and the recruitment of 53BP1 to DNA damage sites is partially defective with a percentage of cells positive for 53BP1 foci and an average of 53BP1 foci number per cell reduced. They suggested that H3K79me2 is required for 53BP1 IRIF when the global level of H4K20me is reduced during G2 until the early-G1 phase of the next cell cycle (Wakeman et al., 2012). Indeed, Wakeman et al. observed a stable level of H3K79me2 in all phases of the cell cycle. This last result remains to be confirmed as a conflicting report published by Feng et al described H3K79me2 levels decreasing during S phase and returning to its basal level in mitosis similarly to H4K20me2 (Feng et al., 2002; Houston et al., 2008; Pesavento et al., 2008).

Besides, we cannot exclude the possibility that H3K79me2 role in 53BP1 recruitment to DSBs may be restricted to specific genomic locations such as heterochromatin. Indeed,

it has been shown that Dot1 is required for heterochromatin structure (Jones et al., 2008). In absence of DOT1, mouse ES cells show a reduced abundance of H4K20me3, a histone mark specific for heterochromatin. Also, 53BP1 is required for ATM-dependent KAP1 phosphorylation (noon et al., 2010; Ziv et al., 2006). KAP1 is a heterochromatin protein required for heterochromatin formation. Phosphorylation of KAP1 releases KAP1 from the chromatin, resulting in its relaxation and faciliting DNA repair.

In mammalian cells, both histone marks H4K20me2 and H3K79me2 are present in the genome. The dimethylated form of H3K79 represent only 3% of the overall H3K79 population while the dimethylated form of H4K20 represent over 80% of the overall H4K20 population (FitzGerald et al., 2011; Jones et al., 2008; Pesavento et al., 2008; Schotta et al., 2008). No correlation has been identified yet between these two histone marks regarding their locations in the genome. Therefore, some nucleosomes could exhibit exclusively H3K79me2 or H4K20me2, while other could have both histone marks. We can hypothesize that if a DSB occurs at proximity to a nucleosome presenting exclusively H3K79me2, it is this later mark that is recognized by the tandem tudor domain of 53BP1. We can also speculate about a redundant role between both histone marks when they are simultaneously present on the nucleosome involved around the DNA breaks.

Unlike H3K79me2, the involvement of H4K20me2 in the recruitment of 53BP1 is well established but the degree of its impact on the DDR still needs to be defined. H4K20 is first mono-methylated by SET8 and then di-methylated by the two homologous enzymes SUV420H1 and SUV420H2 (Fang et al., 2002; Nishioka et al., 2002; Yang et al., 2008). Also, the mono-ubiquitylation of H4K91, catalysed by BBAP, seems to be required for H4K20 methylation (Yan et al., 2009). At first, Botuyan *et al.* showed an almost complete loss of 53BP1 foci formation in the absence of H4K20me2 (using *SET8* siRNA) one hour post-IR in HeLa cells (Botuyan et al., 2006). Later on, additional studies using siRNA knock-down targeting *SUV420H1/H2* or *BBAP* observed a similar defect also using HeLa cells (Yan et al., 2009; Yang et al., 2008). However, in 2008, Shotta et al. generated single and double knock-out mutant mice for Suv420h1 and Suv420h2 and used the resulting MEFs to follow 53BP1 foci formation. They observed that, in absence of H4K20me2, the abrogation of 53BP1 IRIF was modest, being only apparent during the first five minutes after IR (Schotta et al., 2008).

Here, we designed a knock-out strategy to generate a DT40 chicken cell line deficient for H4K20me2. Unlike other vertebrates but similarly to *Drosophila*, only one *Suv420* gene had been identified in chicken at the commencement of this work. However, knock-out of *Suv420* did not abrogate the dimethylation of H4K20 suggesting the existence of a second HMTase catalyzing this same methylation. In fact, in all other vertebrate species examined there were two conserved copies of both genes SUV420H1 and SUV420H2 in their genome. A more detailed analysis of the primary structure of SUV420 proteins in various species highlighted a well conserved region at the N-terminus of the proteins. A nucleotide BLAST search in the chicken ESTs database using the sequence corresponding to this N-terminal conserved region allowed the partial identification of a second *Suv420* cDNA coding Suv420h2 protein in chicken cells.

Sequence alignment showed that this partial *Suv420h2* cDNA sequence in chicken corresponds to the first seven of the nine exons of *SUV420H2* in human and mouse. RACE-PCR experiments using this partial Suv420h2 cDNA sequence may allow the completion of that sequence. As exon-intron organization is relatively conserved across chicken and mammalian species, PCR on the chicken genome using the chicken *Suv420h2* cDNA sequence may allow the identification of the introns positioned between each known exons. Such sequencing could give enough information to generate a knock-out of *Suv420h2* in the *Suv420h1*$^{-/-}$ chicken cell line generated in this work. This could allow the establishment of a chicken DT40 cell line deficient for H4K20me2 and H4K20me3. Such a double *Suv420h1* and *Suv420h2* DT40 knock-out cell line would be valuable to determine the degree of H4K20me2 required for the recruitment of 53Bp1 upon DNA DSBs. Also, such a cell line would be a precious tool to study or highlight additional pathways independent of H4K20me2 for 53Bp1 IRIF such as H3K79me2.

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CHAPTER 3

The p53 tumor suppressor is a key regulator of the balance between DNA double strand break repair pathways.

Running title: p53 regulates the balance between DSBs repair

Keywords: 53BP1, BRCA1, IRIF, p53, HR, NHEJ, DNA repair

3.1 SUMMARY

Following DNA damage, the 53BP1 mediator of the DNA damage response is recruited to double-strand breaks (DSBs) promoting DNA damage checkpoint activation and DNA repair. Despite numerous studies, the mechanism of this recruitment remains unclear. Nevertheless recent studies have published that 53BP1 requires a functional oligomerisation domain and Tandem Tudor domain (TT domain) to form ionizing radiation-induced foci (IRIF). Here, we propose that p53 is implicated in 53BP1 relocation after IR. Indeed, recent publications reported an in vitro binding affinity of 53BP1 Tandem Tudor domain for p53 dimethylated at lysine 382 and showed that this methylation is enhanced after DNA damage. In addition, chromatin immunoprecipitation analysis revealed the recruitment of both 53BP1 and p53 at DSBs. Finally, careful comparison of several publications exploring 53BP1 IRIF suggested that in the absence of H4K20me2 there is a possible correlation between the p53 status of the cell lines used and the time delay of 53BP1 foci formation. In this report, we highlighted for the first time a defect in 53BP1 IRIF formation in absence of p53. This defect is associated with an increase in BRCA1 and RAD51 foci formation to sites of DNA damage. Using in vivo DNA repair assays we showed an increase in homologous recombination efficiency in cells depleted for p53. Overall, we propose that in addition to its traditional role as an "effector" protein in the DNA damage response, p53 plays an important role as a regulator of the mediators 53BP1 and BRCA1.

3.2 HIGHLIGHTS

- p53 enhances 53BP1recruitment to DNA DSBs sites in the early stage of the DNA damage response.
- In absence of p53, the relocalisation of 53BP1 at DSBs is particularly reduced in the early phases of the cell cycle: G1 and early S phase
- p53 limits BRCA1 and RAD51 accumulation at DNA lesions.
- p53 down-regulates the homologous recombination repair pathway.
- p53 up-regulate Non-homologous end-joining repair pathway.

3.3 INTRODUCTION

Double strand breaks (DSBs) are the most threatening DNA lesions to genome integrity. By interrupting the continuity of the DNA and producing DNA ends, which are potentially vulnerable, DSBs can lead to loss of substantial genetic informations. Also, DSBs repair is particularly delicate as, if incorrect, it can lead to dramatic large-scale DNA rearrangement. Therefore, cells have evolved two distinct repair pathways: non- homologous end joining (NHEJ) and homologous recombination (HR). NHEJ occurs predominantly in the early phase of the cell cycle: G0, G1 and early S-phase. It is an error prone repair pathway that catalyses the re-ligation of the two DNA ends of the break. 53BP1, a mediator of the DNA damage response, has been shown to promote specifically long range NHEJ (Difilippantonio et al., 2008). 53BP1 knock-out mice display an impaired thymocyte development, reflecting a defect in V(D)J recombination, and a reduced isotype switching in mature B cells, reflecting a defect in class switch recombination (CSR) (Manis et al., 2004; Morales et al., 2003; Ward et al., 2003b). Recently, data suggests that 53BP1 enhances long range NHEJ by increasing chromatin mobility at each end of the break and therefore stimulating their re-ligation (Dimitrova et al., 2008)

HR occurs predominantly during G2 and late S-phase of the cell cycle. It is an error free pathway using the homologous sister chromatin as a template to repair the break. Once the break is detected, the extremities are subject to DNA end resection. The resulting single strand is coated by RAD51 to form the RAD51 filament that promotes the search for the homologous sequence on the sister chromatid. It has been shown that the regulation of DNA end resection results from a balance between 53BP1 and BRCA1 mediators. In cells with unprotected telomeres, lack of 53BP1 is associated with an increase of 3’ overhang ssDNA at telomere sites, revealing an inhibitory role for 53BP1 in DNA end resection (Bunting et al., 2010; Dimitrova et al., 2008; Zimmermann et al., 2013). On the other hand, BRCA1 recruitment to DNA damage sites enhances DNA break processing (Schlegel et al., 2006; Yun and Hiom, 2009). Hence, BRCA1 knock-out mouse cells present reduced HR levels, genomic instability and cancer predisposition as DNA end resection is suppressed. In these mice, an additional knock-out of 53BP1 rescues markedly the BRCA1 phenotype as HR is restored (Bunting et al., 2010).

53BP1 and BRCA1 relocate rapidly to the sites of DSBs in response to DNA damage. These accumulations are easily detected using fluorescence microscopy by monitoring the appearance of ionizing radiation-induced foci (IRIF) in the nucleus of the cell. Upon DNA damage, the histone variant H2AX is phosphorylated at its serine 139 (γH2AX). MDC1 binds directly to γH2AX and facilitates the recruitment of numerous components of the DNA damage response including the E3-ubiquitin ligases, RNF8 and RNF168. H2A-type histones surrounding the lesion, first ubiquitylated by RNF8 then poly-ubiquitylated by RNF168, recruit BRCA1-containing complexes by direct interaction (Bohgaki et al., 2011; Pinato et al., 2009). Interestingly, MDC1 recruitment and poly-ubiquitylation of H2A-type histones by RNF8 and RNF168 are also required for 53BP1 recruitment. However, it also requires the binding of 53BP1 Tandem Tudor domain with the histone H4 dimethylated on its lysine 20. Lack of H4K20me2 in HeLa cells resulted in nearly complete abrogation of 53BP1 foci formation for at least an hour after DNA damage induction (Botuyan et al., 2006; Yan et al., 2009; Yang et al., 2008). Controversially, another study showed that lack of H4K20me2 in MEF resulted in a partial defect of 53BP1 IRIF exclusively during the first 5 minutes after DNA damage (Schotta et al., 2008). We noticed that an important and overlooked difference between such works was the different p53 status of the cell lines under investigation, suggesting that p53 may influence the recruitment of 53BP1 at DSBs. Indeed, recent studies have demonstrated that p53 dimethylated on its lysine 382 (p53K382me2) has increased affinity for 53BP1 Tandem Tudor domain (Kachirskaia et al., 2008; Roy et al., 2010). Importantly, p53 is specifically dimethylated on its lysine 382 upon DNA damage induction (Kachirskaia et al., 2008).

Here, using human and primary mouse cell lines, we showed that p53 regulates the recruitment of the mediator 53BP1. Our data reveal that in the absence of p53, 53BP1 is recruited less efficiently, especially in G1 and early S phase of the cell cycle, whereas BRCA1 recruitment to DNA damage is promoted by lack of p53. Consistent with this result, the recruitment of Rad51 to DSBs is also increased, while recruitment of MDC1, which is upstream of BRCA1 recruitment, is not affected. Finally, we showed that cells lacking p53 repair DNA DSB generated by camptotecin, which are typically repaired by HR, more efficiently. While on the contrary they are less efficient in repairing DSBs generated by etoposide, which are mostly repaired by NHEJ. Overall, this study highlighted for the first time a key role for p53 in regulating the balance between

53BP1/BRCA1 at a very early stage of the DNA damage response, in order to choose the most appropriate DSBs repair pathway: HR or NHEJ.

3.4 MATERIALS AND METHODS

3.4.1 Cell culture and transfection

HCT116 and p53-null derivative cells were supplied by Dr. B. Vogelstein (Bunz et al., 1998). HCT116 p53-/- were generated by gene targeting in order to knock-out p53 using two targeting vectors that contained either neomycin or hygromycin resistance. Both HCT116 cell lines were grown in DMEM media with 10% foetal calf serum and 1% antibiotics (10,000 units of penicillin and 10mg/ml of streptomycin). Mouse embryonic fibroblasts (MEFs) and p53-null derivative cells were a gift from Dr. S. Jones. Cells were grown in DMEM media with 15% foetal calf serum and 1% antibiotics. SiRNA transfections were performed with oligofectamine following manufacturers instructions. TopBP1siRNA is a siGenome siRNA SMART pool (Dharmacon)

3.4.2 Cell extracts and Western blotting

The collected cells were washed in cold PBS and resuspended in sample buffer ($2x10^5$ cells for 5ul of sample buffer). The lysed cells were then incubated for 10 min at 95^OC, sonicated (40% amplitude for 10 seconds) and incubated again for 10min at 95^OC. The nitrocellulose western blotting membranes were blocked with 4% milk, incubated overnight with the primary antibody, washed in PBS and incubated again with the secondary antibody for 1h at room temperature. The antibodies used were rabbit anti-53BP1 (Novus biological), mouse anti-γH2AX (Millipore), anti-BRCA1 (Santa Cruz), anti-p53 (Cell signaling), anti-p53 DO1 (Santa Cruz), anti-ATR (Santa Cruz), anti-TopBP1 (Abcam), anti-H4 and anti-H4K20me2 (Abcam).

3.4.3 Immunofluorescence and microscopy

Cells were fixed with 4% paraformaldehyde and permeabilized with 0.125% of Triton-100. After a blocking step using a 4% BSA solution, cells were incubated for 1h at 37^OC with primary antibody, washed, and incubated again 1h at 37^OC with the secondary antibody. Slides were mounted using vectashield media with DAPI. Microscopy imaging was performed on a Deltavision microscope using softworx software (Applied Precision, Issaquah). 0.5μm Z-stacks were collected, deconvolved and merged. The microscopy analyses were performed using Image-Pro Analyser

software (MediaCybernetics). Antibodies used for immunofluorescence assays were anti-53BP1 (Novus biological), mouse anti-γH2AX (Millipore), anti-BRCA1 (Santa Cruz), anti-PCNA (Kevin sullivan), anti-ZWINT (Kevin Sullivan, CCB), anti-RAD51 (Abcam), FITC or TRITC Goat anti-rabbit (Jackson Immunoresearch), FITC or TRITC Goat anti-mouse (Jackson Immunoresearch), Cy5 Goat anti-rabbit (Jackson Immunoresearch), TRITC Goat anti-human (Jackson Immunoresearch).

3.4.4 Fluorescence-activated cell sorting

Cells were washed with PBS, resuspended at $5x10^6$ cells/ml in PBS and then fixed in 70% ice-cold ethanol. Cells were washed again with PBS before a 30min Propidium iodide (PI) staining (40μg/ml of PI (Sigma) and 250μl/ml of RNAse A (Qiagen) in PBS). The analysis was performed using the FACS Calibur Platform and CellQuest software (BD biosciences). To detect γH2AX signal, $1.0x10^6$ cells were collected, washed in PBS 0.1%BSA and then fixed in PFA-fixation buffer (25mM Tris-HCl, 1% Triton-X100, 1mM EDTA, 0.1g/l BSA, phosphatase inhibitors and 0.2% PFA). Cells were then washed in PBS 0.1%BSA in resuspended in Block-9 buffer (1X PBS, 1g/l BSA, 8% chicken serum, 0.1g/l RNaseA, phosphatase inhibitor, 0.25g/l salmon sperm DNA, 0.1% triton-X100, 5mM EDTA and 0.05% sodium azide) containing anti-gamma-H2AX antibody for 2 hours. Cells were washed again with PBS 0.1% BSA and resuspended in Block-9 buffer containing FITC-anti-mouse antibody for 30 min. Finally, cells were washed with PBS 0.1% BSA and resuspended in PBS 0.1% BSA containing 40μg/ml propidium iodide for 30min before analyses of the samples.

3.4.5 Comet assay

Cells were treated with 1.25μM of camptothecin for 1h, washed with PBS then collected at the indicated time of recovery. The neutral comet assay method was adapted from the manufacturors instructions (Trevigen). Cells were harvested, combined with LMAgarose at a concentration of $1x10^5$cells/ml and loaded on polylysine slides. The slides were incubated at 4°C in the dark for 30min to allow the LMAgarose to set. Cell lysis was performed by placing the slides in ice-cold lysis buffer overnight and neutralized in neutral electrophoresis buffer for 30min. Electrophoresis was realized by immersing slides in an elecrophoresis unit filled with neutral electrophoresis buffer and conducted for 1h at 24V corresponding to 1V/cm between electrodes. DNA of the cells trapped in agarose was treated with a

precipitation buffer, washed with 70% ethanol. Slides were allowed to dry at 37'C before staining DNA with cyber-green and visualization of the comets by microscopy. COMET analysis was performed using the software CometScore from Tritek corporation.

3.5 RESULTS

3.5.1 p53 is required for optimal 53BP1 IRIF

53BP1 was first identified as a p53 binding partner by yeast two-hybrid screen technique (Iwabuchi et al., 1994). It was shown that 53BP1 interacts with the DNA binding domain of p53 through its BRCT domain, enhancing p53 transcriptional activity (Derbyshire et al., 2002; Iwabuchi et al., 1998; Joo et al., 2002). Recently a second interaction between these two proteins was identified involving the Tudor domain of 53BP1 and a dimethylated lysine on the C-terminal of p53 (p53K382me2) (Kachirskaia et al., 2008; Roy et al., 2010). It has been suggested that when 53BP1 relocates to DSBs, it binds and stabilizes p53 at DNA damage sites through this second interaction (Kachirskaia et al., 2008).

The 53BP1 Tudor domain was originally shown to be essential for 53BP1 recruitment to DSBs (Botuyan et al., 2006; Huyen et al., 2004). We therefore asked if the involvement of 53BP1 in the recruitment and stabilization of p53 at DSBs could be reciprocal. Using the HCT116 WT human cell line and its p53 null derivative, we monitored 53BP1 IRIF formation after irradiation treatment. As shown in figure 3.1A and B, we detected significantly fewer 53BP1 foci formed after IR in the HCT116 cell line deficient for p53 compared to those formed in HCT116 wild type cells. The number of γH2AX foci formed after IR was similar in both cell lines, suggesting equal amounts of DSBs in both cell lines (Figure 3.1C and 3.2). In addition, we also quantified the average foci intensity per cell. We calculated a ratio between average foci intensity per cell in HCT116 WT over HCT116 deficient for p53. Thus, a ratio over 1 represented stronger 53BP1 foci intensity in WT HCT116 cell line and a ratio below 1 represented stronger 53BP1 foci intensity in HCT116 p53-/-. As show in the figure 3.4.1D, the intensity of 53BP1 foci was statistically significantly higher in HCT116 WT cells compared to HCT116 cells deficient for p53. In γH2AX-stained control samples, no significant difference in foci intensity was observed between both cell lines with the exception of 15 minutes after DNA damage, when γH2AX foci were more intense in HCT116 p53-/- cells (Figure 3.1D and 3.2).

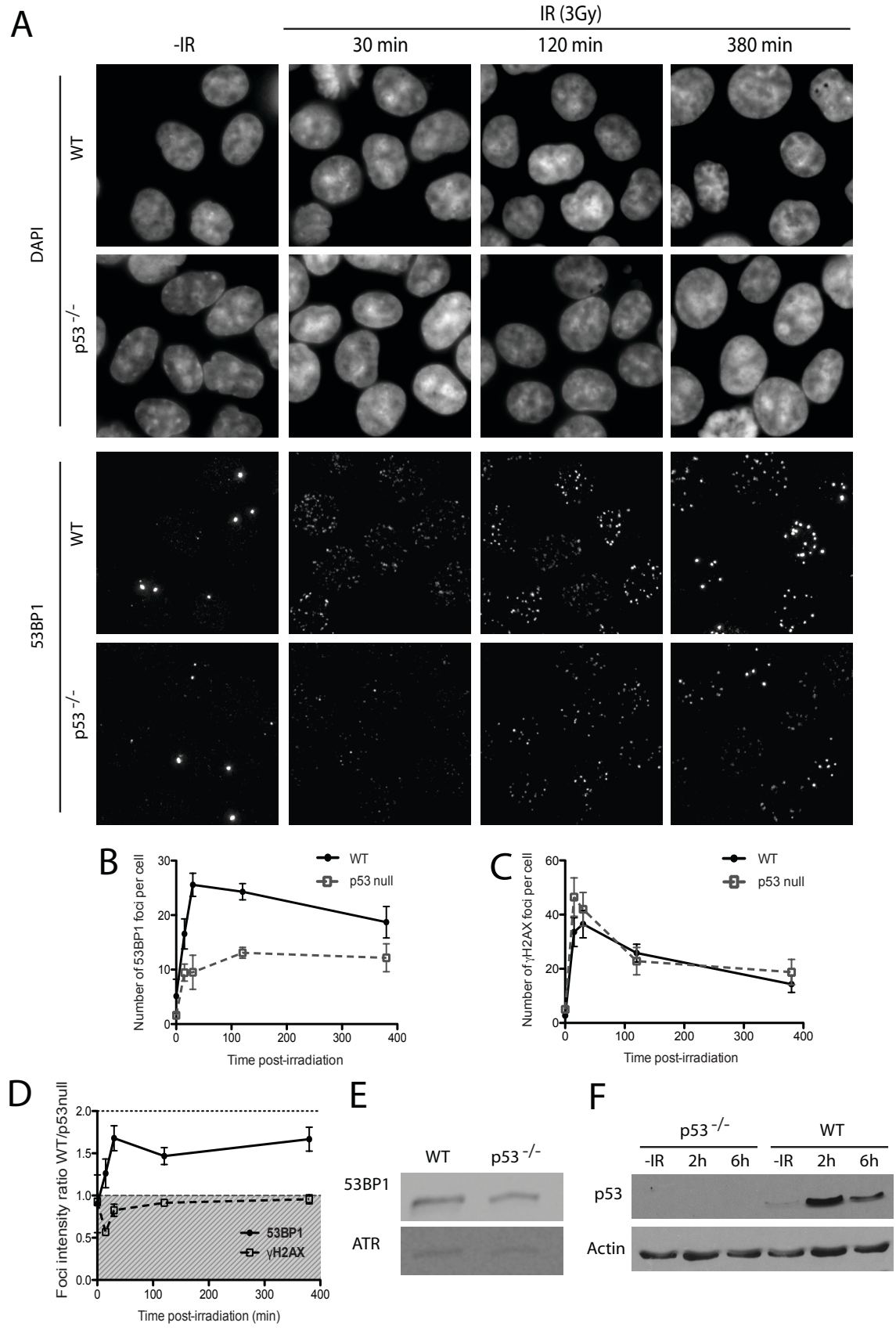


Figure 3.1: p53 promotes 53BP1 recruitment to DNA damage sites.
(A) Detection of endogenous 53BP1 by immunofluorescence. Cells were irradiated with 3Gy, fixed at the indicated time and then stained with 53BP1 antibody. (B) Quantification 53BP1 foci number in (A). (C) Quantification of of γH2AX foci number in (A). (D) Ratio of 53BP1 and γH2AX foci intensity in WT

cells over p53 null cells (E) 53BP1 protein level in WT and p53 null cells analyzed by Western blot from whole cell extract. (F) p53 protein level in HCT116 WT and p53 null analyzed by western blot from whole cell extract before or after IR exposure (5Gy) at the time indicated.

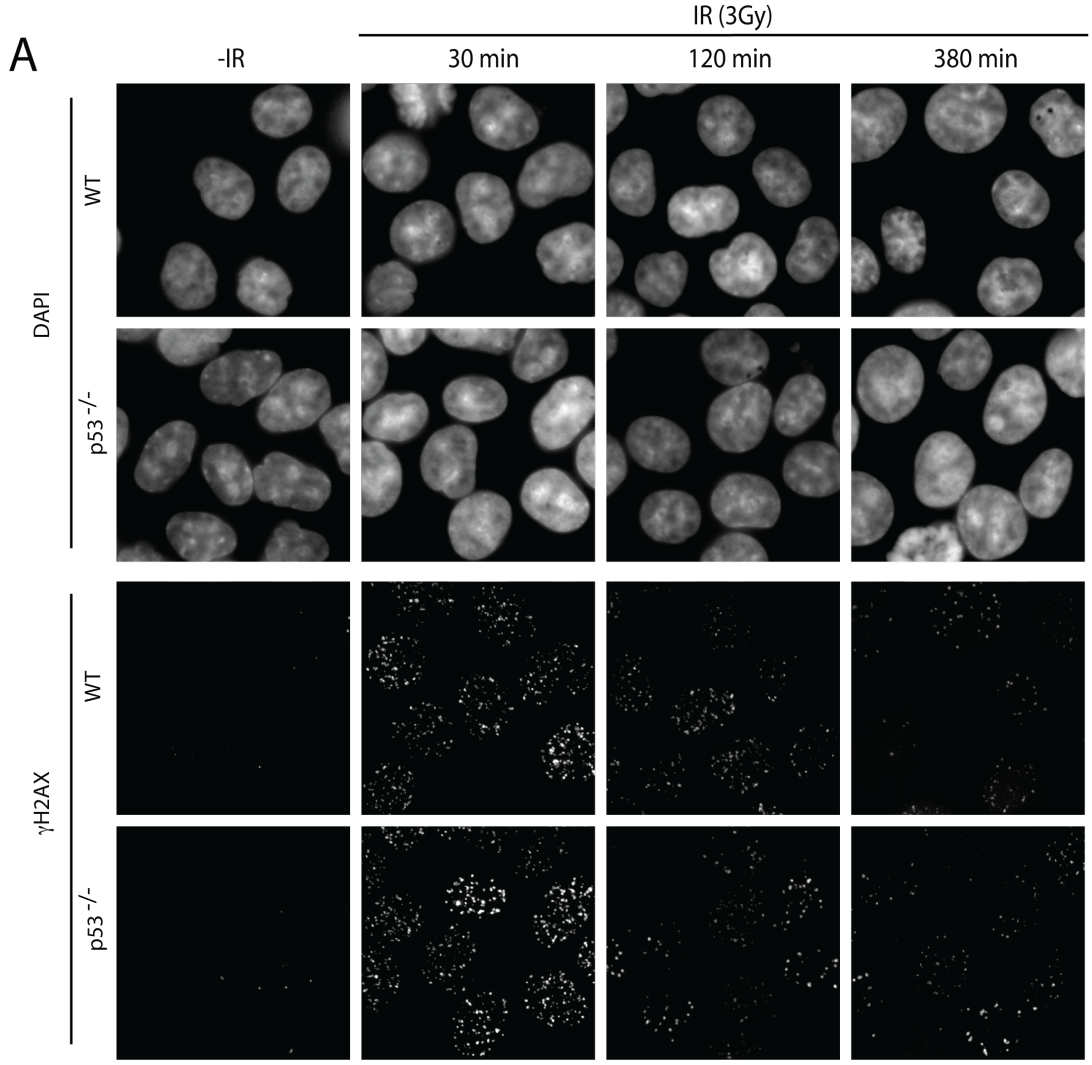


Figure 3.2: p53 does not regulate γH2AX to DNA damage sites in HCT116.
(A) Detection of endogenous 53BP1 by immunofluorescence. Cells were irradiated with 3Gy, fixed at the indicated time and then stained with γH2AX antibody.

To confirm this result, we decided to repeat this experiment using mouse embryonic fibroblast (MEF) WT cell line and its p53-null derivative at early passage (passages 2 to 5). As previously demonstrated in HCT116 cells, MEF deficient for p53 presented less 53BP1 foci after irradiation compared to WT cells within the first hour recovery after IR (Figure 3.3A and B). Also, 53BP1 foci intensity was weaker in MEF deficient for p53 compared to WT cells, in the first two hours after IR (Figure 3.3D).

As the primary function of p53 is to regulate transcription of its target genes, we asked whether p53 could enhance the concentration of 53BP1 at DSBs by promoting its

expression. Western blot analysis showed that 53BP1 protein levels were similar in both WT and p53-/- HCT116 and MEF (Figure 3.1E and 3.3F). Altogether, these data indicate that p53 contributes to the recruitment of 53BP1 to DNA damage sites.

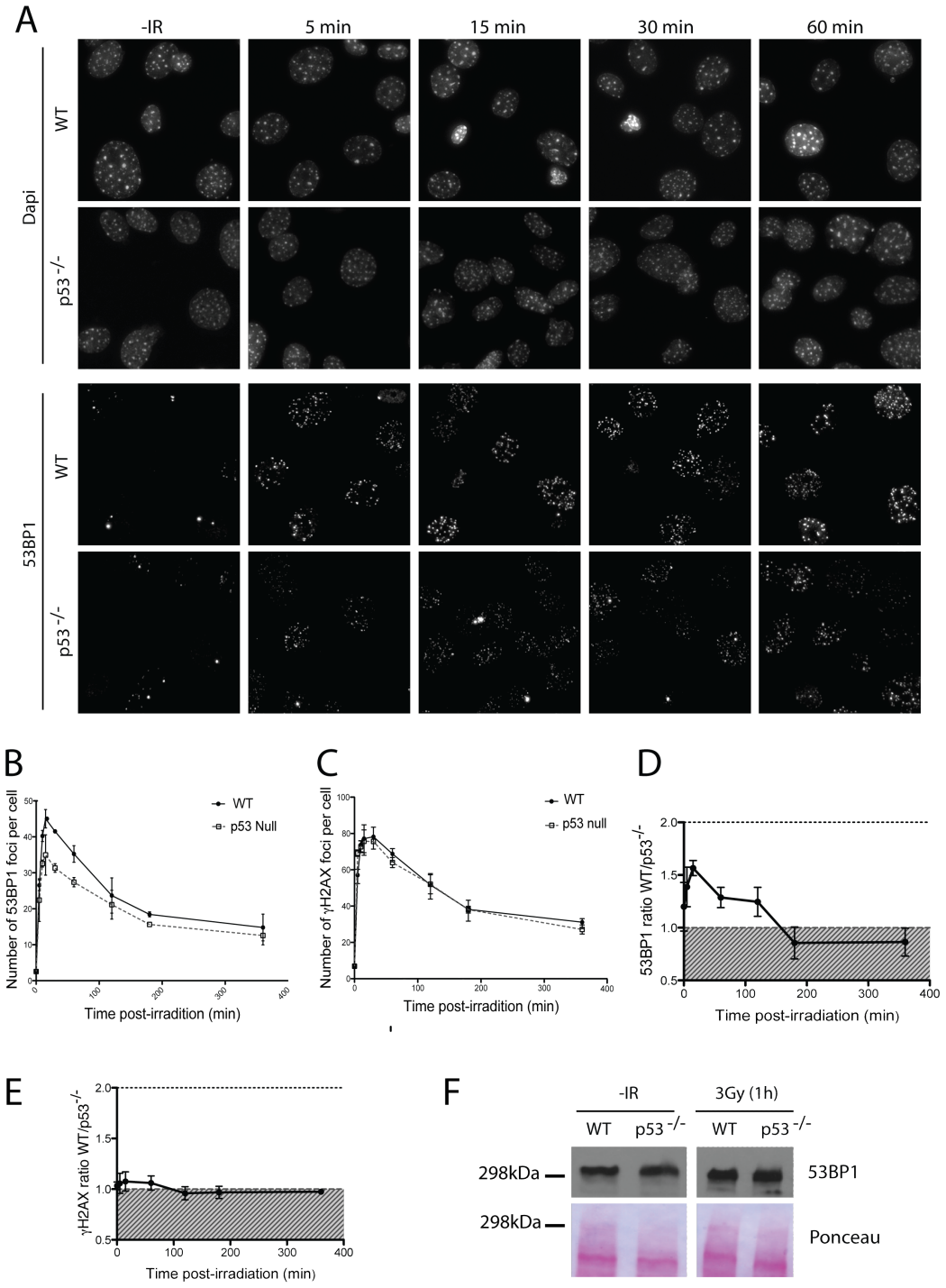


Figure 3.3: p53 promotes 53BP1 recruitment to DNA damage sites in MEF.
(A) Detection of endogenous 53BP1 by immunofluorescence. Cells were irradiated with 3Gy, fixed at the indicated time and stained with 53Bp1 antibody. (B) Quantification of 53BP1 foci number in (A). (C)

Quantification of γH2AX foci number in (A). (D) and (E) ratio of 53Bp1 and gH2AX foci intensity in WT cells over p53 null cells respectively. (F) 53BP1 protein level in WT and p53-null MEF cells by Western blot from whole cell extracts. (G) p53 protein level in WT and p53-null MEF cells by western blot from whole cell extracts before or after IR exposure (5Gy) at the time indicated.

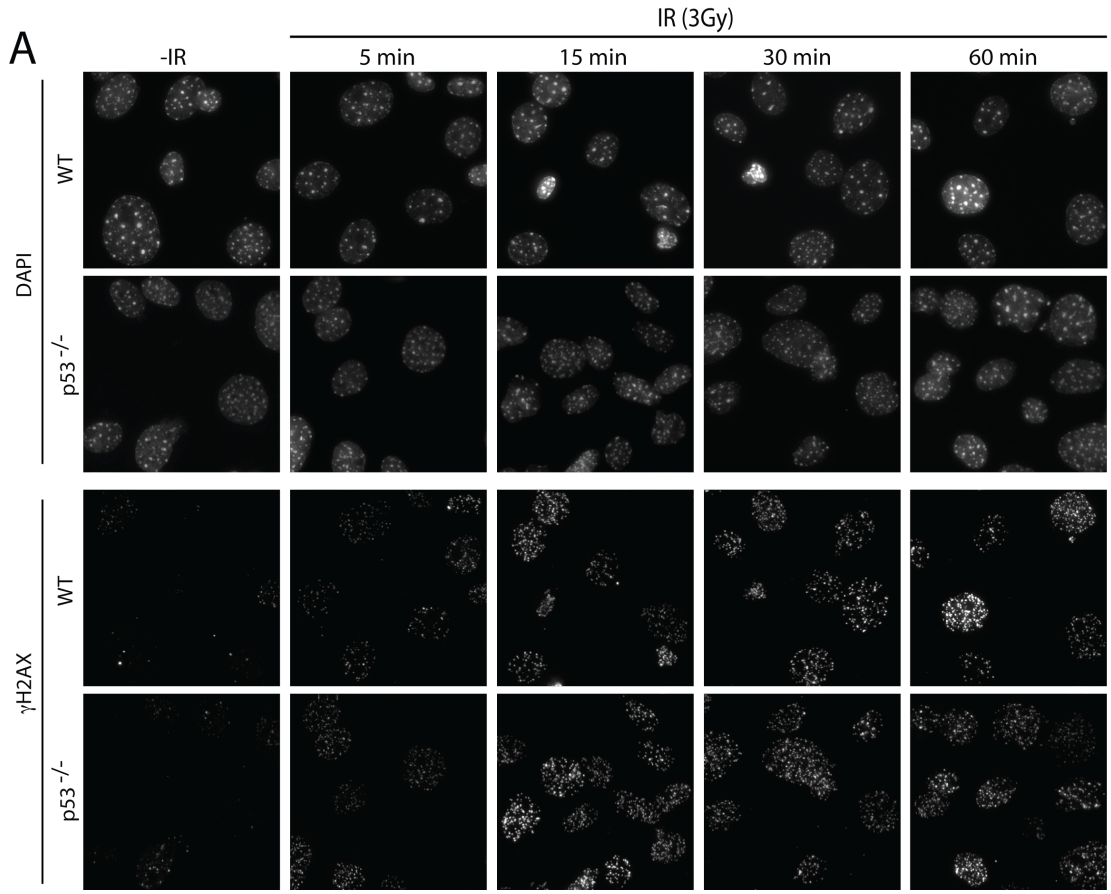


Figure 3.4: p53 does not regulate γH2AX at DNA damage sites in MEF.
(A) Detection of endogenous 53BP1 by immunofluorescence. Cells were irradiated with the 3Gy, fixed after the indicated time of recovery and stained with γH2AX antibody.

3.5.2 p53 is required for optimal 53BP1 IRIF from low to high IR doses

The function of 53BP1 and p53 varie depending the degree of DNA damage inflicted on the cells. In the presence of DNA damage, p53 arrests cell proliferation by activating a either G1/S or G2/M cell cycle checkpoint to give cells time to repair their genome. However, if the damage is too substantial, p53 drives cells into senescence or apoptosis. 53BP1 was shown to facilitate the phosphorylation of CHK2 specifically below 5Gy IR dose (Ward et al., 2003b). Clonogenic survival of chicken DT40 cell lines deficient for 53BP1 showed IR sensitivity only below 4Gy IR (FitzGerald et al., 2011; Nakamura et al., 2006). Also, 53BP1 knock-down cells display a defect in the G2/M checkpoint at low (3Gy) but not high (10Gy) IR doses (Fernandez-Capetillo et al., 2002). Taking this data into consideration, we decided to follow the recruitment of 53BP1 with respect to

p53 status at different IR dose treatments. In each condition tested, from low dose (1Gy) to high dose (10Gy) of IR, p53 knock-out HCT116 cells present a lower number of 53BP1 foci and weaker 53BP1 foci intensity compared to WT cells (Figure 3.5). As a side note, for both cell lines, 53BP1 foci are less intense at the higher the dose of IR. It has been observed previously that no 53BP1 de novo synthesis occurs upon DNA DSBs (huyen et al., 2004). Thus we can suppose that higher is the dose of IR, more DNA damages is induced per cell, consequently reducing the number of 53BP1 molecules per foci.

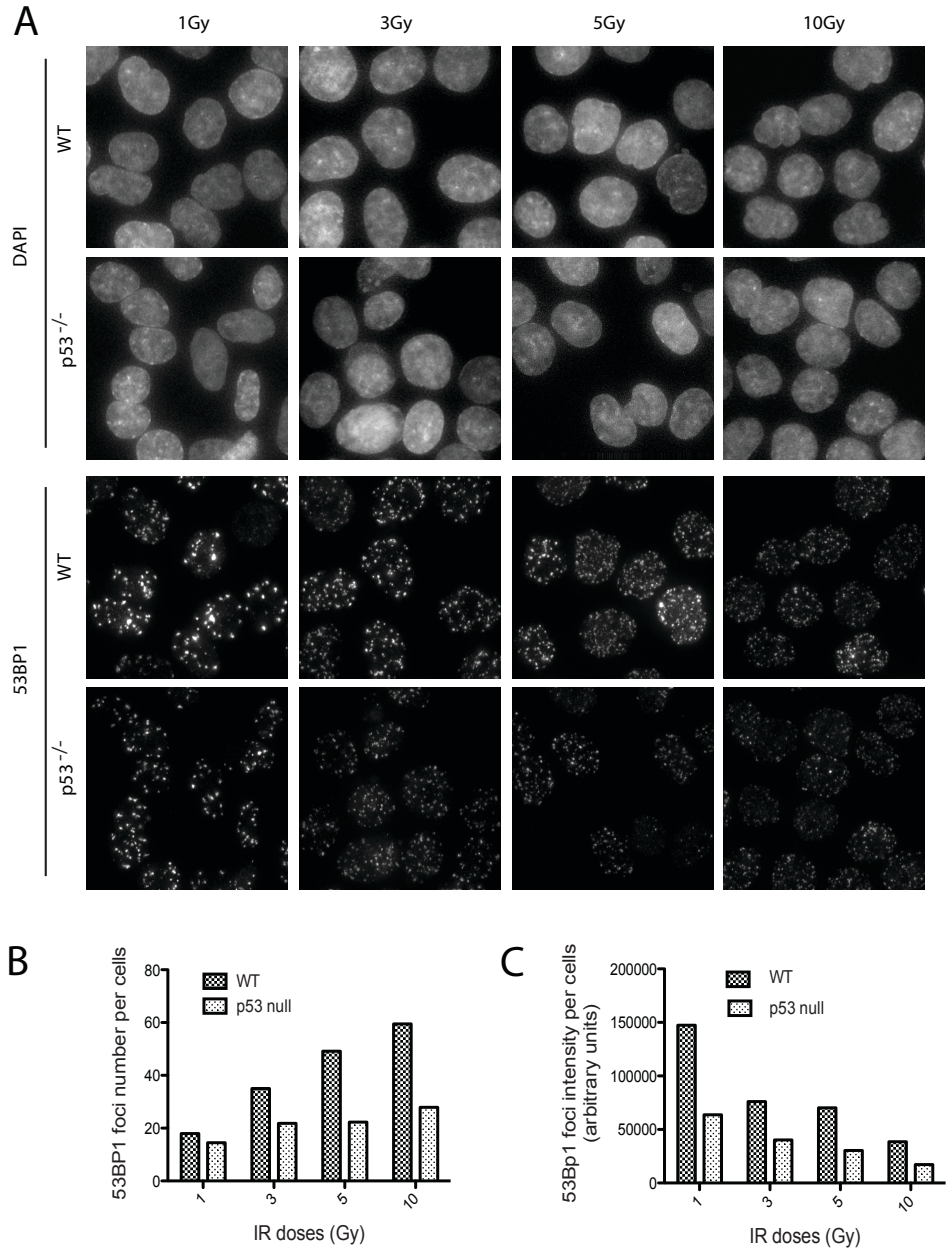


Figure 3.5: p53 promotes 53BP1 recruitment to DNA damage sites from low to high IR doses.
(A) Detection of endogenous 53Bp1 by immunofluorescence. Cells were irradiated at the indicated doses, fixed after 30min of recovery and then stained with 53Bp1 antibody. Assay was performed in triplicates.

(B) Quantification of 53BP1 foci number in (A). (C) Quantification of 53BP1 foci intensity in (A). Quantifications were performed on a replicate representative of all replicates.

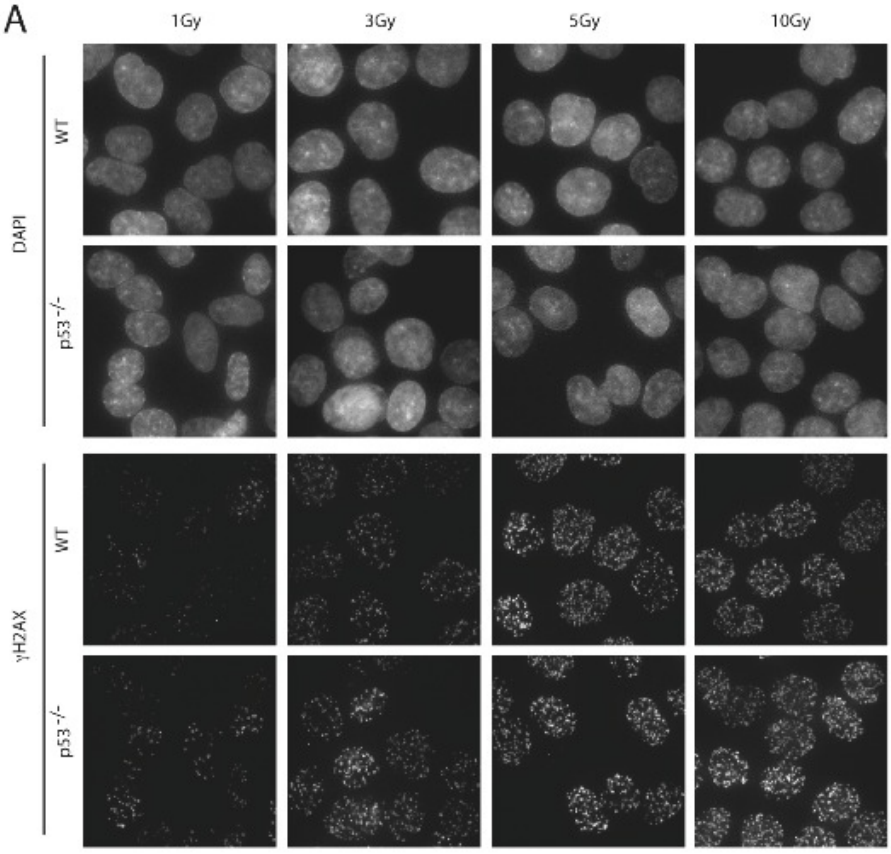


Figure 3.6: γH2AX foci at various IR doses in function of p53 status of cells.
(A) Detection of γH2AX foci by immunofluorescence. Cells were irradiated at the indicated doses, fixed 30min after DNA danage and stained with γH2AX antibody.

3.5.3 p53 does not regulate 53BP1 via MDC1

The recruitment of 53BP1 to DNA damage is the result a complex cascade of events still not fully understood. The accumulation at DSBs of MDC1, a mediator of the DNA damage response is crucial for the recruitment of 53BP1. In the absence of MDC1, 53BP1 IRIF formation is strongly abrogated (stewart et al., 2003; bekker-jensen et al., 2005). To examine the possibility that p53 regulates 53BP1 recruitment by interfering with an upstream component of the DDR, we monitored MDC1 recruitment after irradiation in HCT116 WT and p53-null cells by immunofluorescence. As shown in the Figure 3.7A and B, depletion of p53 did not affect the recruitment of MDC1 to DNA lesions. MDC1 foci formed similarly in number and intensity in p53 knock-out cells compared to wild-type cells upon IR treatment. This data led to the hypothesis that p53

may regulate 53BP1 either directly or by interfering on a component of the DDR cascade between MDC1 and 53BP1.

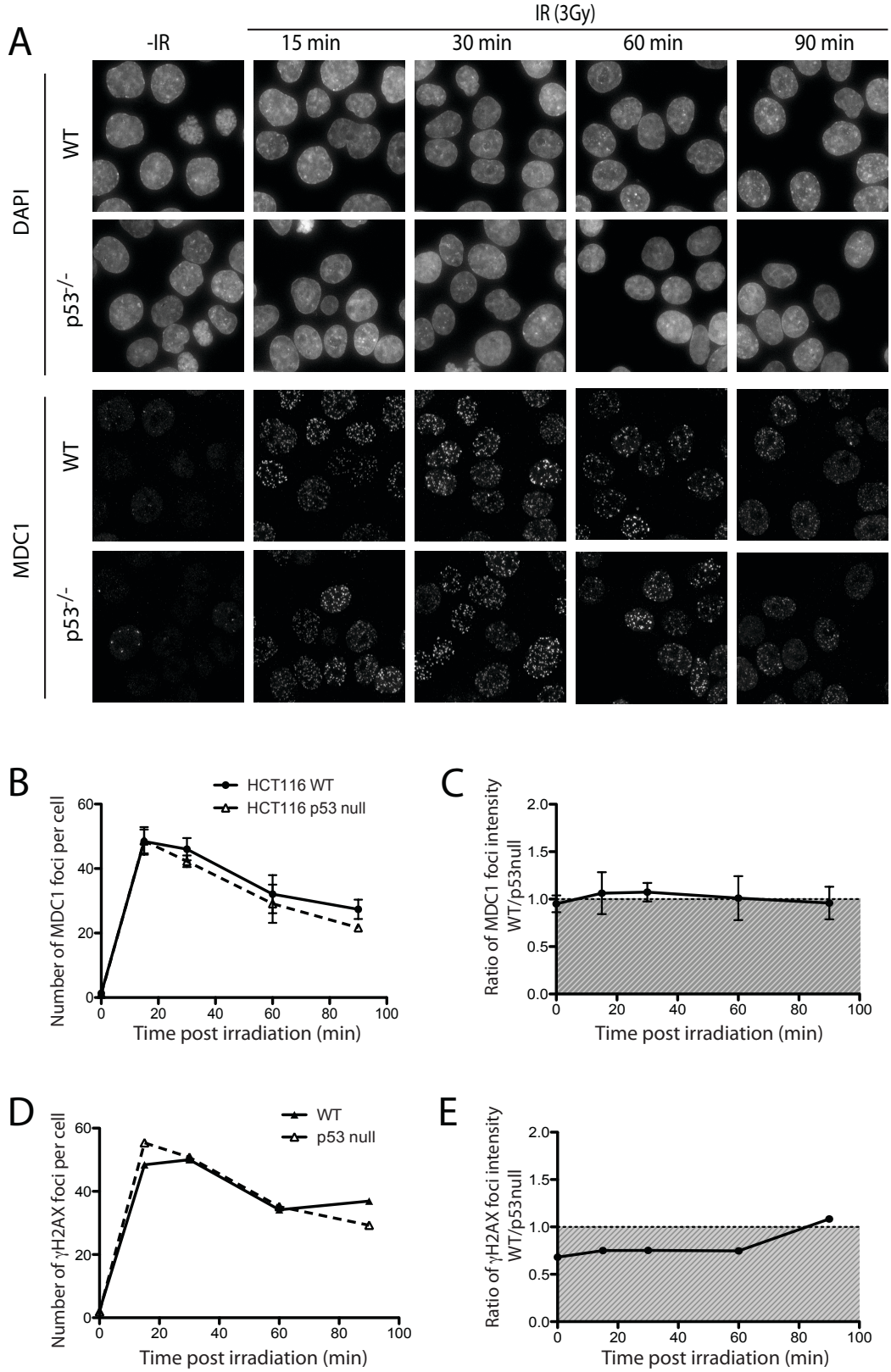


Figure 3.7: p53 does not regulate MDC1 recruitment to DNA damage sites.
(A) Detection of endogenous MDC1 by immunofluorescence. Cells were irradiated with 3Gy, fixed at the indicated time and stained with MDC1 antibody. (B) and (D) Quantification of the number of MDC1 and γH2AX foci in (A) respectively. (C) and (D) ratio of 53BP1 and γH2AX foci intensity in WT cells over p53 null cells.

3.5.4 p53 regulate 53BP1 IRIF in a cell cycle-dependent manner

53BP1 was shown to have a role in promoting NHEJ (Nakamura et al., 2006), which occurs mainly in the early phases of the cell cycle. Consistent with this observation, upon DNA damage, volumes of 53BP1 foci were observed higher in the G0-G1 phase of the cell cycle, phases in which NHEJ repair is most efficient. At later stages of the cell cycle, 53BP1 focal volumes progressively decreases until reaching its lowest level in G2 (Chapman et al., 2012). We decided to investigate whether p53-dependent 53BP1 IRIF formation was associated to a specific phase of the cell cycle. Wild-type and p53-depleted HCT116 asynchronous cells were irradiated and allowed to recover for 1h. Cells were fixed and stained for 53BP1 and two cell cycle markers ZWINT and PCNA, in addition to DAPI staining. ZWINT is required for kinetochore assembly and can be observed as foci from G2 until mitosis (Kasuboski et al., 2011). PCNA, proliferating cell nuclear antigen, is necessary for DNA replication and can be observed during the S phase in distinct focal staining patterns. Cells stained only with DAPI (decondensed chromatin) but not with ZWINT or PCNA were in G1 phase.

As observed by Chapman and collaborators, 53BP1 recruitment in WT cells was more efficient at an early stage of the cell cycle, in G1 and early S phase. Then, the average intensity of 53BP1 foci decreased steadily, reaching a minimum at G2 (Figure 3.8B and C). In the absence of p53, 53BP1 focal intensity also reached a maximum in early S phase and then decreased to a minimum in G2, as observed in WT cells. However, the intensity of 53BP1 foci was overall reduced in all phases of the cell cycle, except in G2, in p53-null cells. This reduction of 53BP1 foci intensity was particularly significant in the G1 and early S phases of the cell cycle (Figure 3.8C).

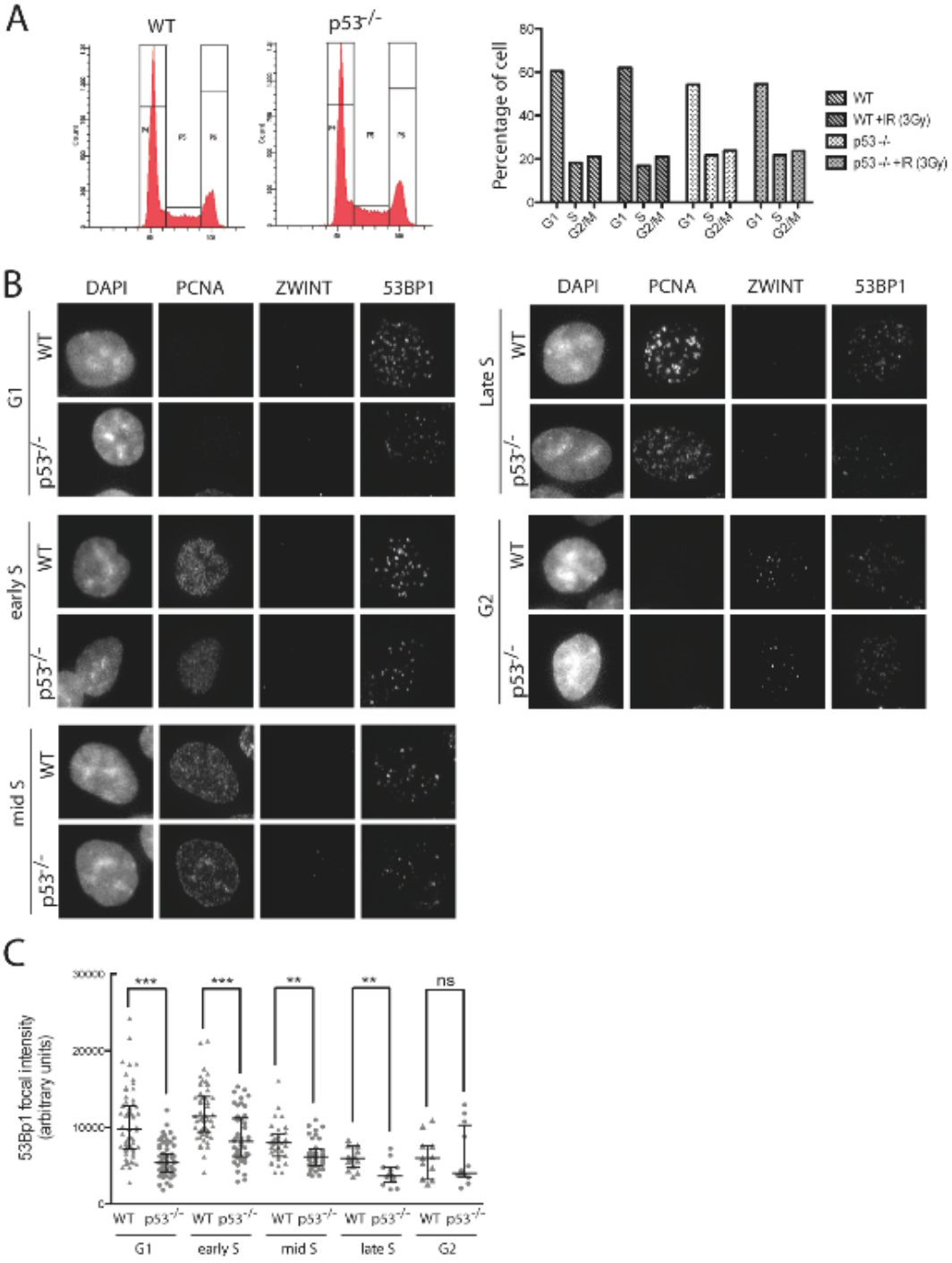


Figure 3.8: p53 regulates 53BP1 IRIF intensity at early stages of the cell cycle.
(A) Asynchronous wild-type and p53-null HCT116 cell cycle profiles analysed by FACS before and 1hour after IR exposure. Cells were stained with propidium iodide. (B) Quantification of the proportion of cells in each phase of the cell cycle before and after IR performed by FACS after PI staining (C) Detection of endogenous 53BP1 and classification in function of the phase of the cell cycle for each cell analyzed - S phase (PCNA positive staining), G2 (ZWINT positive staining) and G1 (PCNA and ZWINT negative staining. (C) Quantification of 53BP1 foci intensity for each cell analysed in (B) in function its phase in the cell cycle. 175 cells were scored for each cell line. Each dot represents one single cell. ***P<0.0001, **P<0.001, Mann-Whitney test.

3.5.5 BRCA1 recruitment to DSBs is restrained by p53

Apart from its role in promoting NHEJ, 53BP1 is also known to negatively regulate homologous recombination by inhibiting DNA end resection in opposition to BRCA1 (Bunting et al., 2010). It has been shown that 53BP1 and BRCA1 localization to DNA DSB is reciprocal. The recruitment of 53BP1 to DNA DSBs was associated with an exclusion of BRCA1 at those specific DNA damage sites (Chapman et al., 2012).

To investigate whether p53 status had an impact on BRCA1 IRIF formation as previously shown for 53BP1, we analyzed the focal formation of BRCA1 in the absence of p53. HCT116 cells were irradiated and BRCA1 recruitment was analysed by immunofluorescence (Figure 3.9A). In WT cells, BRCA1 foci started to appear four hour after IR, with about 50% of the cells displaying BRCA1 foci. In absence of p53, nearly 60% of cells presented BRCA1 IRIF only two hours after IR, reaching a maximum of 70% of cells displaying BRCA1 IRIF four hours after IR (Figure 3.9B).

p53 is known to regulate the expression of BRCA1 as late response to DNA damage (Arizti et al., 2000). It has been observed that BRCA1 mRNA and protein level decrease 12h to 24h after irradiation in p53 positive cells. We analysed BRCA1 protein levels after IR in function of the p53 status of the cells by western blot at early timepoints (1 to 2 hour) and we did not observe a reduction of BRCA1 protein level in both HCT116 WT and p53-null cell lines (Figure 3.9E). Therefore, the enhanced recruitment of BRCA1 in p53-null cells cannot result from a higher BRCA1 protein level.

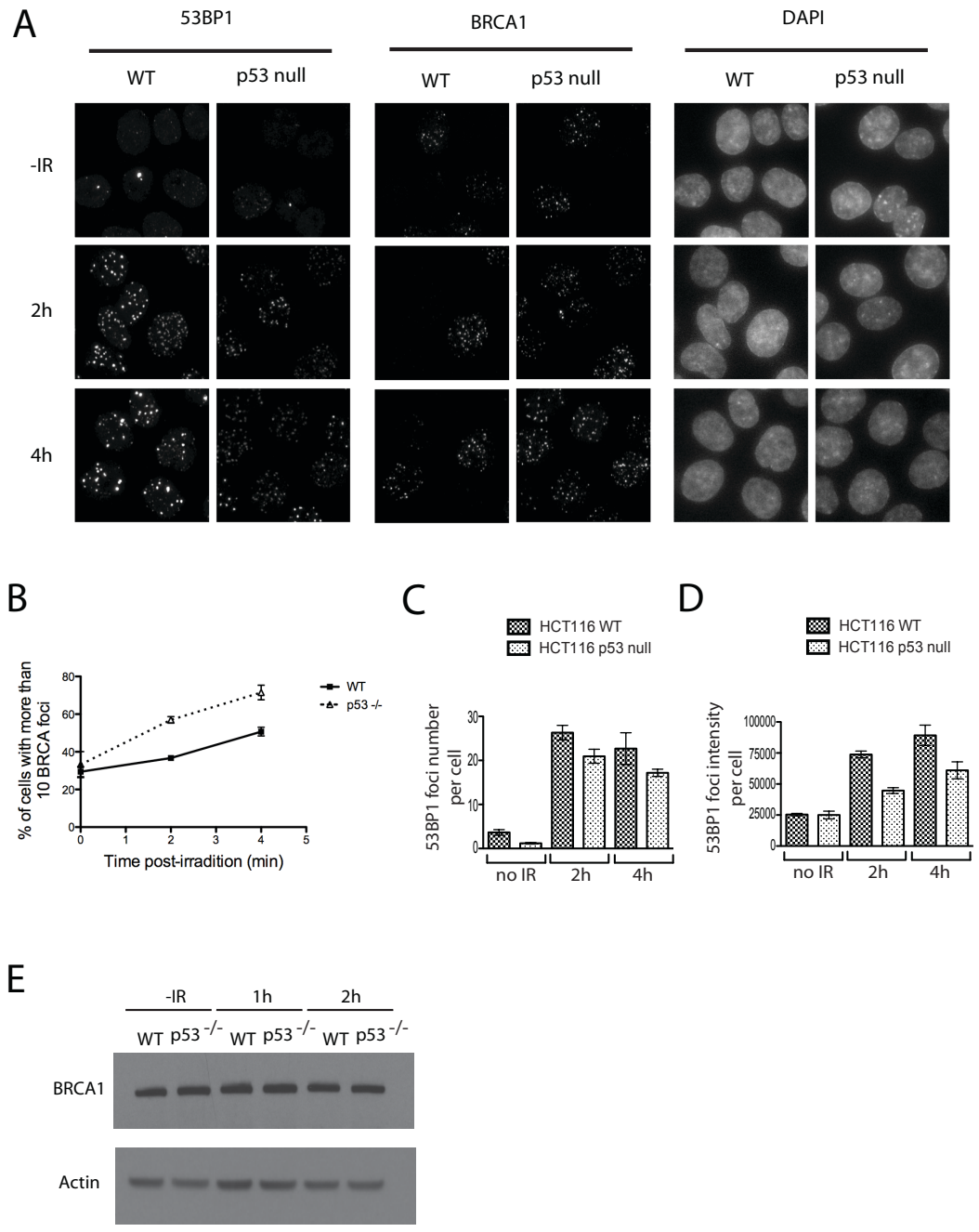


Figure 3.9: p53 negatively regulates BRCA1 recruitment to DSBs.
(A) Detection of endogenous 53BP1 and BRCA1 by immunofluorecence, HCT116 WT and p53-null cell lines were irradiated with 3Gy, fixed after 2h or 4h and stained with BRCA1 and 53BP1 antibodies. (B) Quantification of BRCA1 foci average number per cell in (A) (C) and (D) Quantification of the number and intensity of 53BP1 foci per cell in (A) respectively. (E) Western blot analysis of BRCA1 protein level in WT and p53-null HCT116 cells, 30 min after IR (3Gy)

3.5.6 p53 negatively regulates HR repair

Our previous data indicated that p53 promotes the 53BP1 recruitment to DSBs, promoting HR inhibition, while it restricts the accumulation of BRCA1, an essential component of the HR response, To verify if p53 does indeed negatively regulate HR as our previous results suggested, we first analyzed the focal formation of Rad51 by immunoflurescence assay. As shown in figure 3.10A and B, the percentage of cells presenting Rad51 foci and the number of Rad51 foci per cell increased in absence of p53.

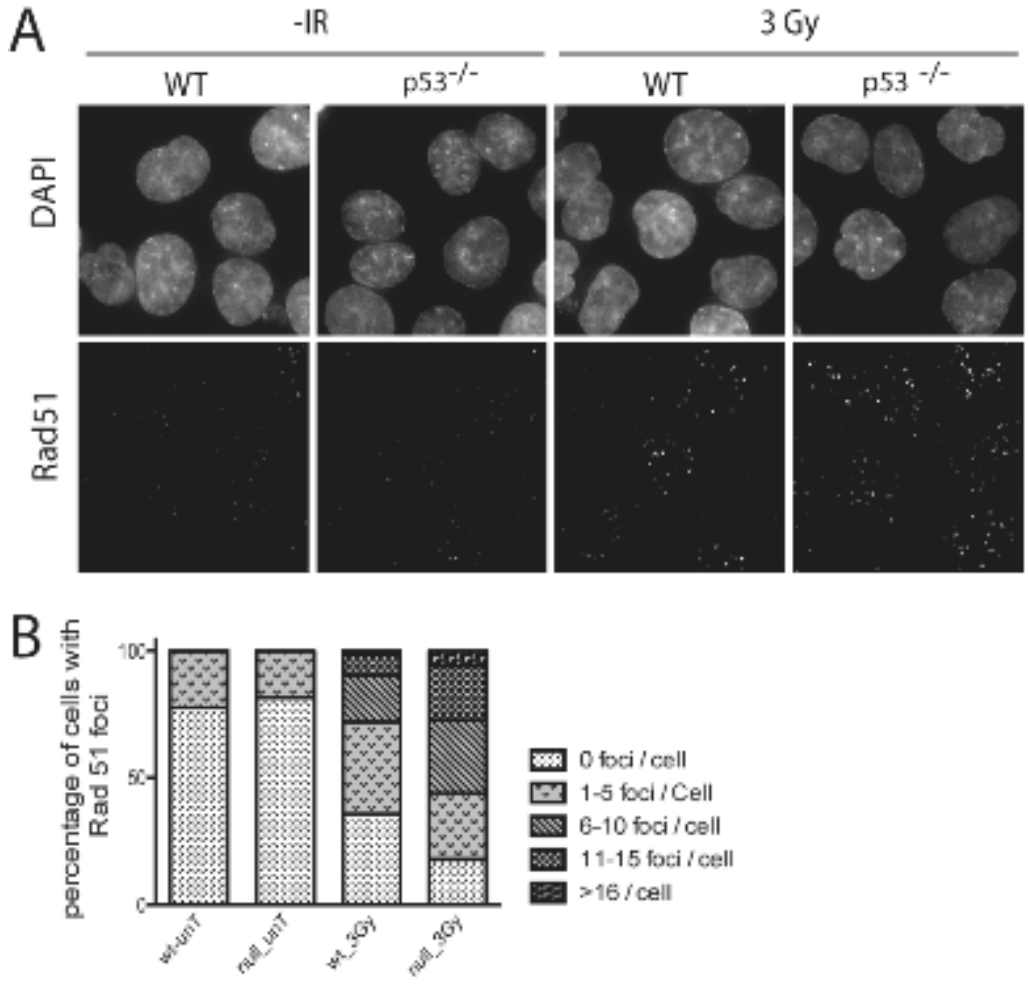
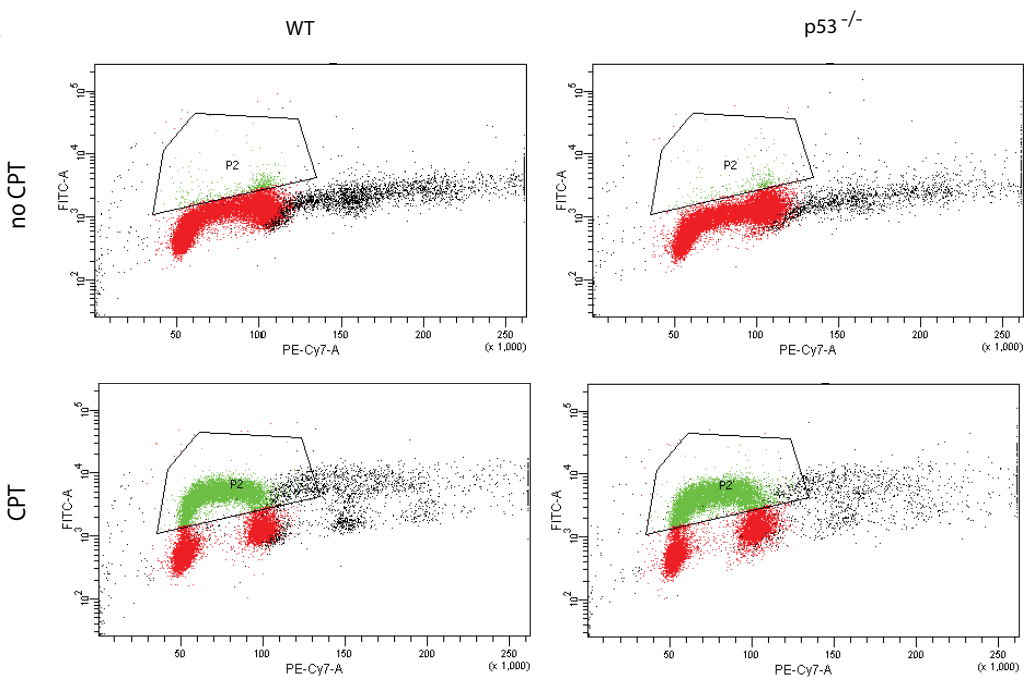


Figure 3.10: RAD51 foci are enhanced in absence of p53.
(A) Detection of endogenous RAD51 by immunofluorecence,. Cells were irradiated with 3Gy, fixed after 5h recovery and stained with RAD51 antibody. (B) Quantification of the number of RAD51 foci per cell in (A).

Immunofluorescence assays are an indirect method of observing DNA repair. Consequently we decided to perform a neutral comet assay to visualise DSBs in cells treated for 1h with CPT, a drug that generates DSBs specifically repaired by HR. CPT is an inhibitor of the DNA enzyme topoisomerase I that relaxes DNA mainly during replication. Collision of a replication fork with a CPT-induced lesion generates a single DNA double strand break end, a DSBs substrate that is not efficiently repaired by NHEJ but specifically repaired by HR. Thus, in an asynchronous cell population, it is in majority cells in S phase that show γH2AX signal after CPT treatment, corresponding to DSBs predisposed to HR repair. As shown in figure 3.11A and B, CPT treatment seems to induced similar degree of DNA damage in both cell line HCT116 WT and p53-null as a similar percentage of S phase cells display γH2AX signal in both cell lines. On a side note, 4N cells in each FACS profile are due to cells clumping together. Trypsin treatment did not sufficiently separate the cells into a single cell suspension.

A



B

Cell line	no treatment	CPT
WT	2.2%	42.9%
p53 -/-	1.3%	50.4%

Figure 3.11: DNA damage induced by CPT in HCT116 cells.
(A) FACS analysis of DNA damages induced on HCT116 cell lines WT and p53 null treated with campthotecin (CPT) for 1h by staining for γH2AX using an anti-gH2ax antibody and an FITC-labelled secondary antibody. (B) Percentage of cell positive for γH2AX in cells in (A).

After 1h treatment with CPT, the drug was washed away and DSB were analysed by neutral comet assay at different timepoints to measure the recovery time (Figure 3.12A). One hour after CPT treatment, a similar extent of DNA DSBs is observed in both cell lines HCT116 WT and p53-null. After removal of drug, HCT116 WT progressively repaired DSBs induced by CPT treatment, returning to a basal level 2h after CPT removal. However, p53-null HCT116 repaired all CPT-induced DSBs in nearly 30 min (Figure 3.12B) revealing a more efficient repair of DSBs by HR in the absence of p53.

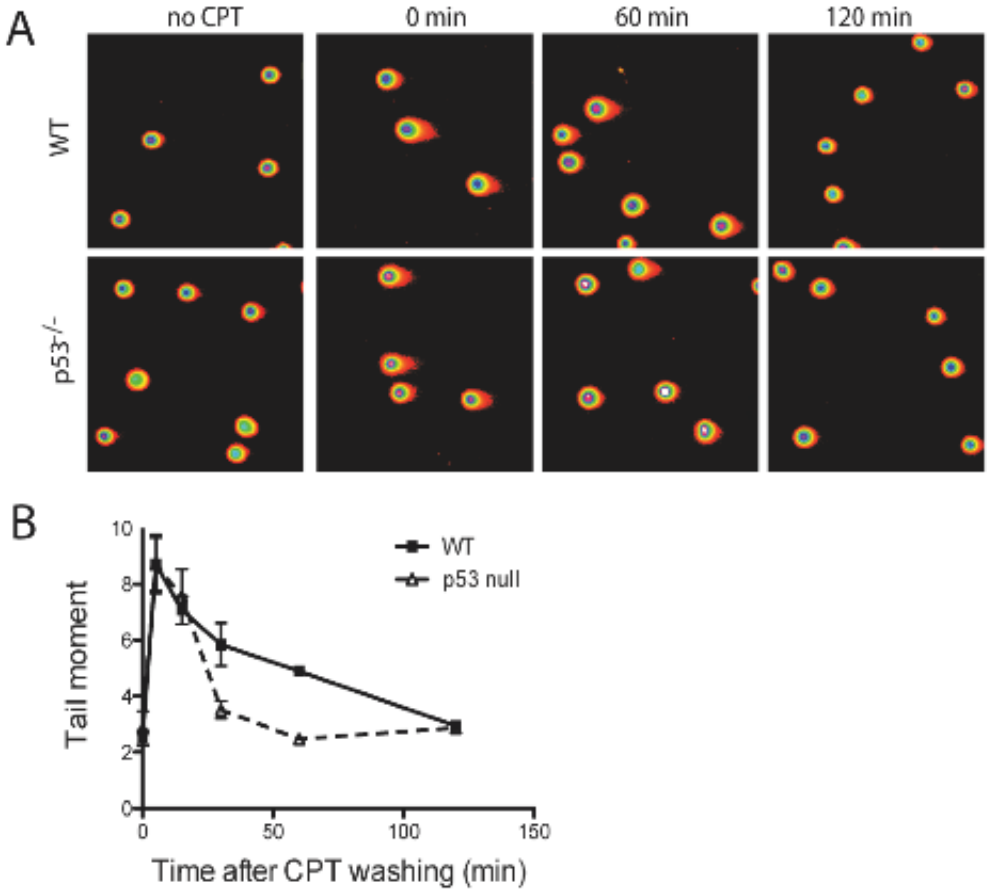


Figure 3.12: p53 inhibit homologous recombination.
(A) Representative images of neutral comet assay (spectrum view). Wild-type and p53-null HCT116 were treated for 1h with CPT. After PBS wash, cells were allowed to recover in drug-free media for the indicated time. (B) Quantification of DSBs analysed by comet assay in (A).

On the other hand, etoposide, inhibitor of topoisomerase II, is a drug that induces DNA DSBs during transcription. Etoposide induces two end DNA DSBs that are principally repaired by NHEJ. HCT116 WT and p53-null cells were exposed to etoposide for 1h.

Afterwards, the drug was washed away and samples were analysed by comet assay at different times of recovery. Repair of etoposide-induced DSBs in HCT116 WT cell was achieved rapidly, in only 15min. However, in HCT116 p53 null, etoposide-induced DSBs was accomplished in 1hour, revealing a more efficient repair of DSBs by NHEJ in presence of p53 (Figure 3.13B).

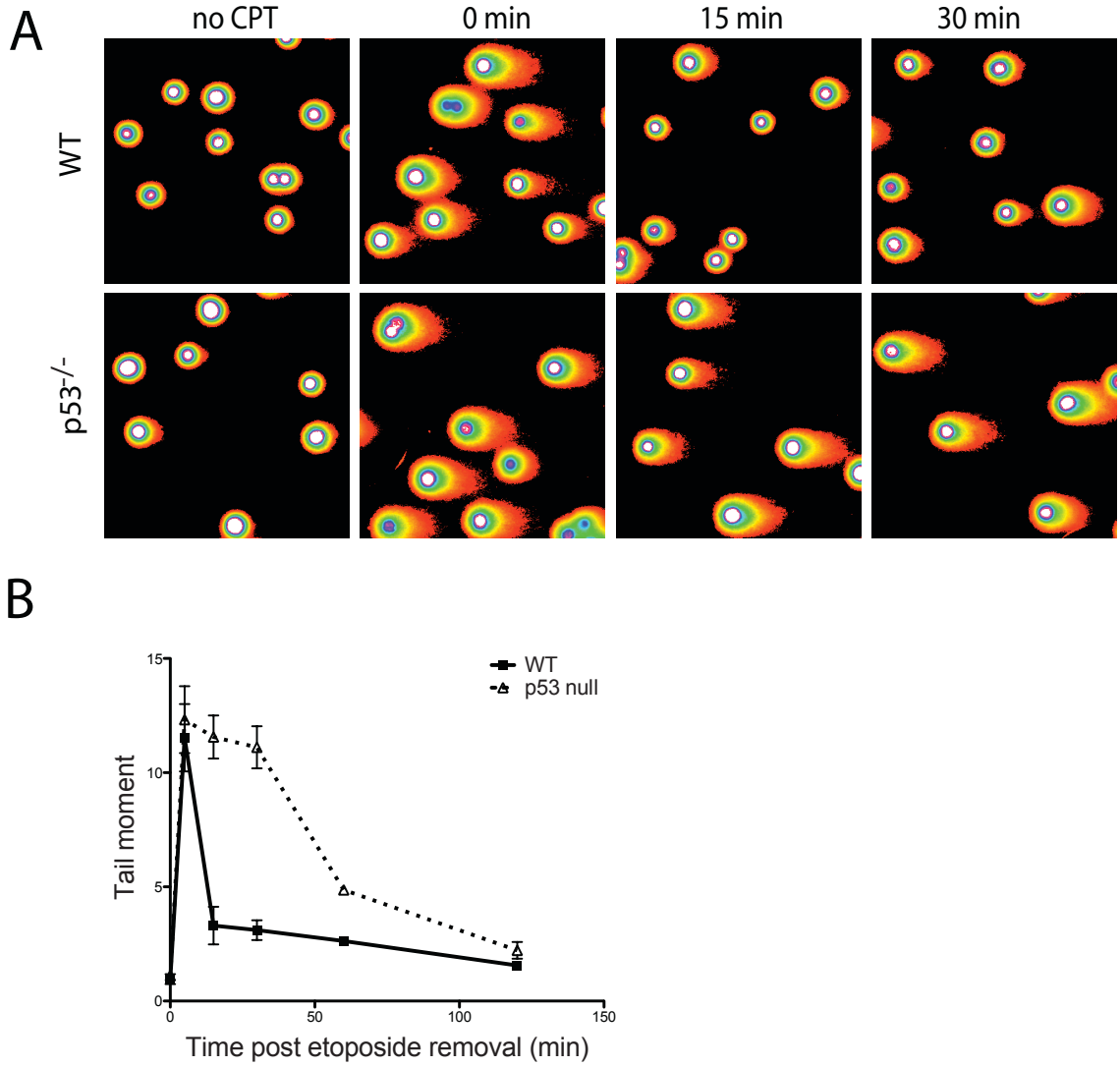


Figure 3.13: p53 enhance non-homologous end joining.
(A) Representative images of neutral comet assay (spectrum view). Wild-type and p53-null HCT116 were treated for 1h with Etoposide After PBS wash, cells were allowed to recover in drug-free media for the indicated time. (B) Quantification of DSBs analysed by comet assay in (A).

DISCUSSION

The mechanism behind 53BP1 recruitment to DSBs is still not fully characterized. It is known that 53BP1 oligomerisation domain and its Tudor domain are essential for foci formation (Zgheib et al., 2009). 53BP1 Tudor domain showed affinity for two histone marks H3K79me2 and H4K20me2 (Botuyan et al., 2006; Huyen et al., 2004). However, the relationship between the histone mark H3K79me2 and 53BP1 IRIF in vivo remains uncertain. Lack of H4K20me2 is consistently associated with a defect in 53BP1 localisation to DSBs, although the degree of this default varies from study to study. In HeLa cells, it was shown that lack of H4K20me2 abrogates 53BP1 foci formation for at least an hour following IR treatment (Botuyan et al., 2006; Yan et al., 2009; Yang et al., 2008). A different study conducted in MEF, also depleted for H4K20 dimethylation, showed that 53BP1 foci formation is merely delayed for the first 5min post-IR treatment (Schotta et al., 2008). The two cell lines used in those studies differ in their p53 status. The MEFs possess wild-type p53 while HeLa cells are defective for p53 function.

Earlier studies have established that 53BP1/p53 complex is associated with an upregulation of p53 transcriptional activity (Iwabuchi et al., 1998). Recently, a newly identified post-transcriptional modification of p53, p53K382me2, showed great affinity for the 53BP1 tandem Tudor domain (Kachirskaia et al., 2008; Roy et al., 2010). It was suggesting that the presence of 53BP1 at DSBs might help recruiting and stabilizing p53 at DNA damage site to regulate a p53 function other than its transactivation activity.

In this study, we observed that 53BP1 foci formation to DNA DSBs is abrogated in absence of p53 in human and mouse cells. This defect was detected at low to high doses of irradiation. Western blot analyses showed that 53BP1 protein levels were not affected by the absence of p53, suggesting that p53 regulate 53BP1 IRIF during the recruitment process and not at a transcriptional level. In the absence of p53, we observed a defect in 53BP1 accumulation in the first 15min post-IR in human cell and the first 5min post-IR in MEF. Intriguingly, the lack of H4K20me2 in MEF resulted only in a 5 min delay in 53BP1 IRIF formation (Schotta et al., 2008). Altogether, we can speculate that 53BP1 may be recruited at first by the interaction of its Tudor tandem domain with H4k20me2.

Then, a second pathway involving p53 reinforce the accumulation of 53BP1 to DSBs. Such a model coincides with the recruitment process of the 53BP1 orthologue in fission yeast, Crb2. Initially, Crb2 is recruited to DSBs through the recognition and the binding of its Tudor domain to H4K20me2. Then, a second histone-independent pathway intervenes to support this accumulation (Du et al., 2006).

The characterization of 53BP1 accumulation to DSBs as a function of p53 status showed that in the absence of p53, 53BP1 IRIF significantly decrease in number and intensity in a cell cycle-dependent manner. The defect is prevalent in G1 and early S phase, then progressively diminishes in mid and late S phase to finally become null in G2 phase of the cell cycle. The G1 and S phases correspond to the predominant phase of the cell cycle for the NHEJ repair. It was shown that 53BP1 promotes this pathway by enhancing chromatin mobility of DNA end breaks (Dimitrova et al., 2008). In relation to DSBs repair, 53BP1 is also known to inhibit HR by down-regulating DNA end resection (Bunting et al., 2010; Dimitrova et al., 2008; Zimmermann et al., 2013). Indeed, this initial step in the HR pathway is dependent upon the balance between 53BP1 and BRCA1, which promote DNA end processing (Bunting et al., 2010). It has been shown that recruitment of BRCA1 to DSBs correlates with reduction of 53BP1 accumulation to these sites (Chapman et al., 2012). In this report, we observed that abrogation of 53BP1 IRIF in the absence of p53 is accompanied by more efficient BRCA1 foci formation. Transcription of BRCA1 is already known to be regulated by p53 as late response to DNA damage, in the 12 to 24 hours after IR exposure (Arizti et al., 2000) however we did not observe a difference in BRCA1 protein level at early timepoint (1hour to 2hours). Therefore, the enhanced BRCA1 foci formation in p53-null cells is not due to a higher BRCA1 protein level.

Further investigations of the homologous recombination pathway revealed that Rad51 foci formation was also enhanced in the absence of p53. In addition, cells lacking p53 were able to recover more efficiently from DNA damage through specific activation of homologous recombination repair pathways. On the contrary, p53 deficient cells recover less efficiently from DNA damage through specific activation of non homologous end joining repair pathway. Altogether, those results suggested a new function for p53 as a regulator of the balance between HR and NHEJ through the regulation of 53BP1 and BRCA1 recruitment to sites of DNA damage, as well as regulating BRCA1 stability. As our results showed a reduction of 53BP1 foci in G1 and

early S-phase of the cell cycle, we suspect that to counterbalance it, an accumulation of BRCA1 at DSBs may occur in those same phases of the cell cycle. Noticeably, an up-regulated homologous recombination repair, despite being an error free pathway, can be extremely threatening for genome integrity. If occurring in absence of the homologous sister chromaid, as in G0, G1 and early S-phase, the homologous recombination repair pathway may result in mutation or DNA rearrangement of genetic information. Also, using the HR repair pathway in a highly repetitive DNA region can lead to loss or addition of DNA sequences.

Discovered over 30 years ago, p53, also termed as the "*guardian of the genome*", is one of most studied proteins in biochemistry. It is mutated in the majority of human cancers and can be considered as an incredibly complex protein due to his numerous functions in the most diverse signalling pathways within the cell. New roles for p53 are continuously identified, all positioning p53 as an "effector" protein of the DNA damage response, mainly deciding the fate of damaged cells between life and death. Altogether, our results may highlight a new role of p53 intervening this time at an early stage of the DNA damage response, as a "mediator" protein, arbitrating the repair of DSBs between HR and NHEJ.

3.6 CHAPTER SPECIFIC REFERENCES

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CHAPTER 4

Conclusions and future perspectives.

Running title: Conclusions and perspectives

Keywords: 53BP1, BRCA1, IRIF, p53, H2AX, H3K79me, H4K20me, HR, NHEJ, DNA repair, DOT1, SUV420H1/2, CPT

In this study, we first investigated the implication of the histone mark H3K79me2 in the recruitment of 53BP1 to DNA DSBs in chicken system. As the methylation on this histone is catalysed only by the histone methyltransferase DOT1 (Feng et al., 2002; Frederiks et al., 2008; Ng et al., 2002), we decided to generate a DT40 cell line deficient in H3K79 methylation by knocking out the chicken *Dot1* gene. The newly generated cell line was defective for H3K79 methylation. We monitored the focal formation of chicken 53Bp1 upon DNA damage and did not observe any defect compared to the wild-type cell line. To confirm this result in human cells, we next decided to generate U2OS cell lines deficient for H3K79me2 by stable Dot1 shRNA transfection. The knock down appeared to be efficient, abrogating up to 97% of H3K79 methylation. We then analysed human 53BP1 accumulation in these new U2OS cell lines and two conflicting results emerged from 4 experiments of identical experimental design. In two cases, we did not observe a defect in 53BP1 IRIF in absence of H3K79me. However, in two other cases, we observed a partial but significant defect of 53BP1 accumulation at DSBs in cell depleted for DOT1 compared to wild-type cells. Regardless of these differences, the results together indicate that H3K79me2 is not essential for recruitment of 53BP1 after IR. However, we cannot exclude a role for H3K79me in regulating 53BP1 IRIF under specific conditions, such as, specific genome contexts or cell cycle phases. Indeed, a recent study proposed a role for H3K79 in the accumulation of 53BP1 to DNA damage sites in S and G2 phase of the cell cycle, when the methylation of H4K20 may potentially be reduced (Wakeman et al., 2012).

We next proposed to analyse the role of the histone mark H4K20me in 53BP1 IRIF using the chicken DT40 cell line. Unfortunately the lack of complete sequence for the chicken genome limited our investigation. However, parallel literature research revealed an interesting and overlooked element in the many 53BP1 relocation studies. All data using HeLa cells depleted for H4K20me2 showed a total defect in 53BP1 recruitment to DSBs even at late time points (Botuyan et al., 2006; Yan et al., 2009; Yang et al., 2008). On the other hand, data using MEF cells lacking H4K20me2 showed only a partial defect exclusively in the first minutes after irradiation (Schotta et al., 2008). The two cell lines used in those studies come from different organisms but also diverge for their p53 status which is positive for MEF (if no spontaneous mutations occur upon culturing) but non-functional in HeLa cells. Indeed, a newly identified post-translational modification on p53, p53K382me2, has been shown to be catalysed specifically upon

DNA damage and to be recognized by the Tandem Tudor domain of 53BP1 (Kachirskaia et al., 2008; Roy et al., 2010). To address a possible correlation between p53 status and the recruitment of 53BP1 at DNA damage sites, we decided to use two isogenic human HCT116 cell lines differing for their p53 status as a model system. We induced DNA damage by IR treatment (3Gy) and monitored 53BP1 and γH2AX focal formation in these cell lines. We detected significantly fewer and dimmer 53BP1 foci formed after IR in the p53-deficient HCT116 cells compared to those formed in wild type HCT116 cells. Whereas, the number of γH2AX foci formed after IR was similar in both cell lines. This result was additionally confirmed in MEF cells. Western blotting did not shown any difference in 53BP1 protein level as a function of p53 status in these cells. Altogether, we suggest here that this phenotype may be due to an ability of p53, possibly methylated at lysine 382 (p53k382me), to recruit and/or stabilize 53BP1 at DNA damage sites. However, we cannot exclude that it can also be an indirect result of a transactivation activity of p53. To clarify this point, we could use a mutant where the lysine 382 of p53 is mutated to an arginine. This mutant has been shown by Gozani and collaborators to keep the transactivation activity of p53 (Shi et al., 2007). Thus, the expression of p53K382R in the HCT116 p53-/- cell lines would allow restoration of the transactivation activity of p53 but not its ability to bind to 53BP1 and thus to recruit the latter at DNA DSBs.

Lack of H4K20me in MEF resulted in a modest 53BP1 IRIF defect restricted to the first 5 minutes post-irradiation (2Gy) (Schotta et al., 2008). In our data, using similar IR doses (3Gy), lack of p53 in MEF resulted in a more striking 53BP1 IRIF defect extending from 5min until 2hours post-irradiation. Finally, lack of γH2AX in MEF treated with 3Gy IR resulted in the initial recruitment of 53BP1 during the first 30min after irradiation, which was rapidly lost afterwards (Celeste et al., 2003). Altogether, we suggest that the general recruitment of 53BP1 to DSBs results from a three-step process. First, 53BP1 is recruited via a histone-dependant pathway involving its Tandem Tudor domain binding to the histone mark H4K20me. Secondly, an additional phase of 53BP1 recruitment occurs again via its Tandem Tudor domain but this time involving its interaction with p53, possibly p53K382me2. Finally, as shown in the current literature, 53BP1 is retained at DSBs by a complex histone γH2AX dependent pathway (Figure 4.1).

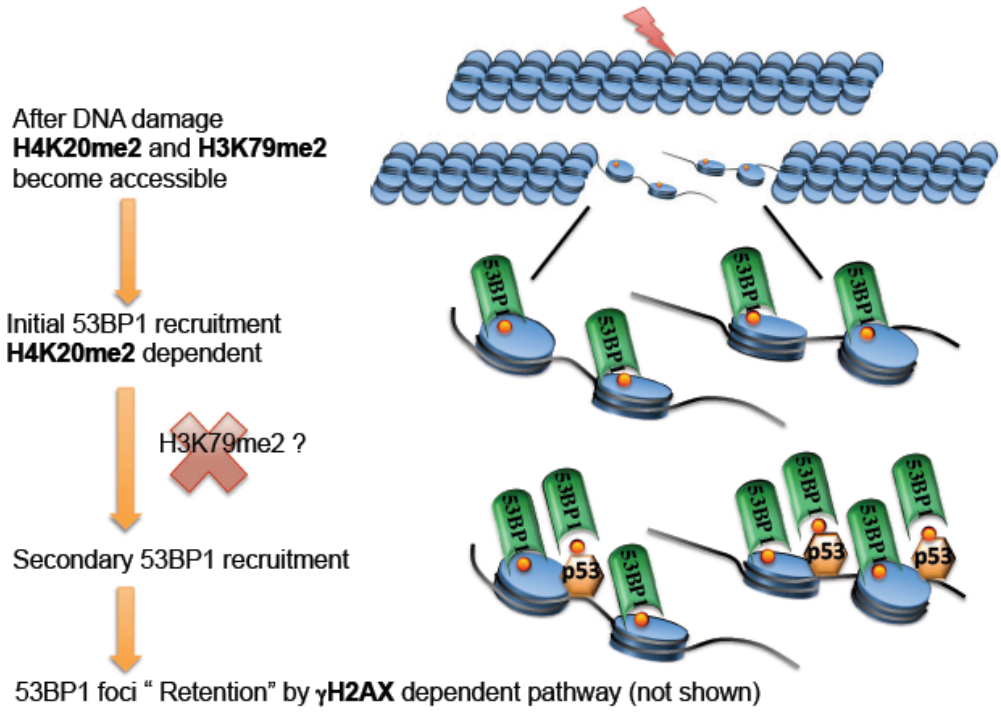

Figure 4.1: Model for the general recruitment of 53BP1 to DNA damage sites.
Upon DNA DSBs, chromatin conformation is altered and previously hidden histone marks become exposed. Via its Tandem Tudor domain, 53BP1 is initially recruited by recognition with the histone H4 dimethylated on its lysine 20. Then a second pathway involving the binding of p53k382me2 with the tandem tudor domain of 53BP1 allows the reinforcement of 53BP1 at DSBs. Finally, 53BP1 proteins are stabilized and retained at DSBs through a γH2AX dependant pathway until completion of the repair.

The perspective of a new histone independent pathway in the recruitment of 53BP1 at DSBs is particularly interesting as such a pathway is already identified in fission yeast. In this organism, the 53BP1 ortholog, Crb2, is mainly recruited to IR damage sites via the binding of its Tudor domain with the histone mark H4K20me2. However, in presence of persistent DSBs generated by IR, Crb2 is recruited through a second histone-independent pathway involving the protein Cut5 (Du et al., 2006). In higher cells, the orthologs of Cut5 is TopBP1(Garcia et al., 2005). TopBP1 is a mediator of the DNA damage response that colocalizes with 53BP1 at late times after IR and specifically in G1 phase cells (Cescutti et al., 2010). As defective 53BP1 IRIF formation in absence of p53 is more significant in G1-early S phase of the cell cycle, it would be interesting to assess a possible role for TopBP1 in the recruitment of 53BP1 through its p53 dependant pathway. Moreover, TopBP1 has been shown to be able to interact via its BRCT domain with the DNA binding domain of p53 (Liu et al., 2009). Therefore, TopBP1 would be a good candidate for the recruitment of p53 and further on 53BP1 at DSBs.

We next investigated the biological relevance of this new p53 dependent pathway for 53BP1 IRIF. Using a 3D-Structural Illimination Microscope, Chapman and collaborators demonstrated that within individual foci, BRCA1 and 53BP1 are positioned in adjacent but yet different sub-focal volume. They also demonstrated that enrichment of either BRCA1 or 53BP1 within a foci correlates with a reduction of the other one (Chapman et al., 2012). As p53 enhances 53BP1 IRIF, we therefore asked whether p53 could also influence the recruitment of BRCA1. Indeed, the percentage of cells positive for BRCA1 foci as well as number of BRCA1 foci per cell was greater in p53 deficient cells than that in WT cells.

BRCA1 and 53BP1 focal reciprocity visualised by Chapman and collaborators correlates with their reciprocal functions in homologous recombination repair. Both BRCA1 and 53BP1 regulate DNA end resection, an early step of HR, either by promoting or inhibiting it respectively (Bunting et al., 2010; Lowndes, 2010; Schlegel et al., 2006; Zimmermann et al., 2013). Therefore, we evaluated the role of p53 in regulating homologous recombination repair. First, we observed that Rad51 IRIF were formed more efficiently in p53 deficient cells compared to WT cells. Then, using neutral comet assay, we quantified double strand breaks in cell treated with camptothecin (CPT). CPT is a topoisomerase I inhibitor that generates single end double strand break in S-phase cells specifically repaired by HR. Our data demonstrated progressive repair of DSBs over 2hours in wild type cells whereas in p53 defective cells, HR required for DSBs to return to basal levels was only 30 min. A similar experiment was performed using etoposide that generated two end DNA DSBs mainly repaired by NHEJ. Upon this treatment, fast DSBs repair was achieved in only 15min in HCT116 WT cell lines whereas a much slower repair was observed in HCT116 p53-null cell, between 1 to 2 hours.

In conclusion, these results together suggest a new role for p53 in the DNA damage response. Currently, p53 is considered to be an “effector’ of the DNA damage response deciding the fate of cells as a function of the degree of damage encountered. When DNA damage occurs, p53 activates cell cycle checkpoints in order to facilitate repair of the damage. However, when the damage is too severe, p53 drives cells towards apoptosis or senescence. In this study, we propose a new function for p53 in the early selection of the most appropriate repair pathway to use in the presence of DSBs. In this new role, p53 acts like as a “mediator” protein in the DNA damage response regulating

the recruitment of 53BP1 and BRCA1 which in turn regulate the balance between HR and NHEJ.

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Appendices