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<th>Characterisation of three zinc finger proteins ZC3H8, ZC3H11A and ZC3H14, identified as new partners of ATM, the central regulator of biological response to DNA double strand breaks</th>
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<tr>
<td><strong>Author(s)</strong></td>
<td>Baldascini, Marta</td>
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<td><strong>Publication Date</strong></td>
<td>2019-12-16</td>
</tr>
<tr>
<td><strong>Publisher</strong></td>
<td>NUI Galway</td>
</tr>
<tr>
<td><strong>Item record</strong></td>
<td><a href="http://hdl.handle.net/10379/15652">http://hdl.handle.net/10379/15652</a></td>
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CHARACTERISATION OF THREE ZINC FINGER PROTEINS
ZC3H8, ZC3H11A AND ZC3H14, IDENTIFIED AS NEW
PARTNERS OF ATM, THE CENTRAL REGULATOR OF
BIOLOGICAL RESPONSE TO DNA DOUBLE STRAND BREAKS

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A thesis submitted to the National University of Ireland NUI Galway for the
Degree of Doctor of Philosophy

Head of Discipline: Dr. Kevin Sullivan
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December 2019
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Abbreviations

53BP1: TP53 Binding Protein 1
alt-NHEJ: Alternative NHEJ
ATM: Ataxia Telangiectasia Mutated
ATMi: ATM inhibitor

ATR: ATM and Rad3-related
ATRIP: ATR interacting protein
BARD1: BRCA1-associated RING Protein 1
BER: Base Excision Repair
BRCA1: Breast Cancer Susceptibility Gene
BRCT: BRCA1 C-terminus domain
BLM: Bloom’s Syndrome Gene
BrdU: 5-Bromo-2-Deoxyuridine
Cyclin-dependent Kinase

CDC25A: Cyclin-dependent Kinase 25A
CDC25B: Cyclin-dependent Kinase 25B
CDC25C: Cyclin-dependent Kinase 25C
CHK1: Checkpoint Kinase 1
CSK: Cytoskeleton Buffer

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
DSB: Double Strand Break
dsDNA: Double Stranded DNA
DUB: deubiquitinating enzyme
E6AP: E6 Associated Protein
FHA: ForkHead Domain

FITC: Fluorescein Isothiocyanate
FOG: co-factor of the Friend Of GATA
G1: Gap phase 1
G2: Gap phase 2
μg: Microgram
GC: Gene Conversion
GFP: Green Fluorescent Protein

Gy: Gray
γH2AX: phosphorylated H2AX (Ser139)
h: Hour
HECT: Homologous to E6AP Carboxy Terminus
HJ: Holliday Junction
HU: Hydroxyurea
IF: Immunofluorescence
IR: Ionising Radiation
IRIF: Ionising Radiation Induced Foci
LEC: Little Elongation Complex
μM: Micromolar
M: Molar
MDC1: Mediator of DNA damage Checkpoint 1
MG132: Proteasome inhibitor
min: Minute
MRN: MRE11-RAD50-NBS1
mRNA: Messenger RNA
MYM: Myeloproliferative and Mental Retardation
NaCl: Sodium Chloride
NBS1: Nijmegen Breakage Syndrome 1
NHEJ: Non Homologous End-Joining Recombination
PAB: Poly(A) Binding Protein
PALB2: Partner and Localiser of BRCA2
pATM: Phosphorylated ATM (S1981)
PBS: Phosphate Buffered Saline
PCNA: Proliferating Cell Nuclear Antigen
PCR: Polymerase Chain Reaction
PDIP38: Polymerase Delta Interacting Protein 38
PID: PALB2 Interacting Domain
PIKK: Phosphatidylinositol 3-Kinase
PFA: Paraformaldehyde
PIAS: Protein inhibitor of activated STAT
PML: Promyelocytic Leukemia Protein
RAP80: Receptor Associated Protein 80
RING: Really Interesting New Gene
RNF4: RING finger protein 4
RPA: Replication Protein A
RPA32 S4/S8: Phosphorylated Replication Protein A (S4/S8)
RNA: Ribonucleic Acid
rRNA: Ribosomal RNA
RNF168: Ring Finger Protein 168
RNF8: Ring Finger Protein 8
RS: Serine and Arginine Rich Region
S: Serine
SAE1: SUMO E1-activating enzyme 1
SAE2: SUMO E1-activating enzyme 2
ssDNA: Single Stranded DNA
SDS: Sodium Dodecyl Sulfate
SILAC: Stable Isotope Labelling with aminoacids
siRNA: Small Interfering RNA
SMA: Spinal Muscular Atrophy
snRNA: Small Nuclear RNA
snoRNA: Small Nucleolar Ribonucleoprotein particle
SSA: Single Strand Annealing
SSB: Single Strand Break
SSO: Short-Single Stranded Overhang
STAT: Signal transducer and activator of transcription
STUb: SUMO-targeted ubiquitin ligase
TDP2: Tyrosyl-DNA Phosphodiesterase 2
TEMED: N,N,N',N'-tetramethylethane-1,2-diamine
Thr: Threonine
TOPBP1: DNA Topoisomerase II Binding Protein 1
TRITC: Tetramethylrhodamine
TRITON: t-actylphenoxypolyethoxyethanol
Ub: Ubiquitin
Ubc9: Ubiquitin-conjugatin 9
UBC13: Ubiquitin-conjugating Enzyme 13
UDR: Ubiquitin-Dependent Recruitment Motif
UFD2: Homology Proteins (U-Box)
UV: Ultraviolet Light
V: Volt
XRCC1: X-Ray Repair Cross Complementing Protein 1
XRCC4: X-Ray Repair Cross Complementing Protein 4
Acknowledgements

I would like to thank my supervisor Prof. Noel F. Lowndes for giving me the opportunity to work in his lab; for trusting and believing in me.

I would like to thank Dr. Murilo T. D. Bueno for his mentoring during the KDM2A project which led to a co-authored publication.

I would like to thank all the members, present and past, in the lab. I thank them for the time, laughs, craziness and work we shared in these past four years. I also thank the entire Centre for Chromosome Biology and all my colleagues in the Department of Biochemistry and the Biomedical Sciences Building department and all the colleagues for their assistance and friendliness during my time in Galway.

I thank my friends Anna T., Laura M, Louise C. and Serena S. for the encouragement but, most important, for entertaining and distracting me when stressful moments came along.

Finally, a massive “THANK YOU” goes to my family. To my mum and dad and sister who have always been there for the good things and for the bad ones, even if far apart. They have always given me the strength and the freedom of taking my own decision and making my own mistakes; and they were always there to give me the right support. I am sure they will keep on doing what they can do best which is supporting me in everything.
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 F. Lowndes.

The experiment shown in Figure 2.11 was performed by Isabelle McDonald. The Flag-tagged ZC3H14 construct was generated by Dr. Murilo T.D. Bueno. The experiment shown in Figure 3.22 B was performed by Dr. Janna Luessing. The polyclonal DR-GFP HEK 293-T cell line and the polyclonal TK-GFP HEK 293-T cell line used in the experiment shown in figure 3.9 was generated by Dr. Murilo T.D. Bueno.
Abstract

The integrity of our genome is very important therefore, our cells have developed efficient DNA repair mechanisms to control this integrity and avoid mutations. Although the DNA double strand break response pathway is well known, there are still many aspects of this pathway that required further characterisation. The ATM protein is a major player in the DNA Damage Response (DDR). Through a proteomic screen of ATM three C3H1-type zinc finger proteins ZC3H8, ZC3H11A and ZC3H14 were identified as new ATM interacting partners. These proteins had been implicated in several aspects of RNA biogenesis but nothing has been reported about their implication in the DDR. We found these three zinc finger proteins being chromatin bound proteins and forming foci within the nucleus, with ZC3H14 co-localising with Nuclear Speckles (SC35). Depletion of ZC3H11A and ZC3H14 induces spontaneous damage, which results in block of the replication fork since accumulation of BrdU foci and pRPA2 (S4/S8) foci is visible upon ZC3H11A and ZC3H14 knock down. R-loop resolution is also affected when ZC3H11A and ZC3H14 are depleted suggesting that their role in RNA biogenesis might be important to avoid transcription/replication collision. Human cells are sensitive to IR, ICRF-193, but not to Olaparib, when ZC3H14 is depleted. Depletion of ZC3H14 also leads to persistent γH2AX foci and pATM S1981 foci confirming a role for this protein in DSBs repair. ZC3H14 is involved in the NHEJ, but not in the HR, repair pathway. The recruitment to site of DNA double strand breaks for 53BP1, BRCA1, FK2, RNF168 and RNF8, but not MDC1, is affected upon ZC3H14 depletion suggesting that ZC3H14 is required for DSBs repair pathway choice. ZC3H14 interacts with MDC1 and its depletion increases the total MDC1 protein levels, before and after damage, with the MDC1 lower band being more abundant than the upper band. Surprisingly, depletion of ZC3H14 does not affect the MDC1-RNF8 interaction.
CHAPTER 1

Introduction
1.1 The DNA damage response

Our cells face the daily threat of the DNA damage. This damage can bring a change in our genome sequence or in the DNA structure. In both cases, if it is not properly repair, this can lead the cells to tumorigenesis (Ciccia et al., 2010). Our DNA has limited chemical stability therefore, the spontaneous decay of our DNA is the major factor in mutagenesis, carcinogenesis and ageing (Lindahl, 1993). All biological macromolecules can decompose. For instance, nucleic acids can undergo spontaneous decomposition when in solution with the RNA being more vulnerable due to the presence of a 2’ hydroxyl group of ribose (Lindahl, 1993). Because of the presence of water in our cells the DNA hydrolysis represents a major threat. The DNA hydrolysis leads to a base loss, which produces instability in the DNA chain. Fortunately, the AP endonuclease initiates the DNA repair process by introducing a DNA strand break at the 5’ side of the free-base site (Lindahl, 1993). The gap is subsequently filled in by a DNA polymerase followed by a DNA ligase. The presence of oxygen in our cells can represents another major threat. The two major types of spontaneous premutagenic events in living cells are the hydrolytic deamination of a cytosine to uracil in the DNA, and oxidation of a guanine to 8 hydroxyguanine. The oxidative stress in our cells, generated by oxidation of the DNA, can be released by a specific DNA glycosylase (Lindahl, 1993). The uracil-DNA glycosylase removes the uracil and the lesion in the DNA is repaired by the AP endonuclease followed by the DNA polymerase and the DNA ligase. Another source of spontaneous damage is the presence of S-adenosylmethionine (Lindahl, 1993). A nonenzymatic methylation on guanines or adenines with formation of the 7-methylguanine and 3-methyladenine are the major DNA lesions. The 3-methyladenine is a cytotoxic lesion that leads to a block in replication. This type of lesion can be promptly repaired by a 3-methyladenine-DNA glycosylase, which excises the altered base. On the other hand, the 7-methylguanine is poorly repaired and so it rapidly accumulates in the DNA of mammalian cells (Lindahl, 1993). Damage on our DNA can come from endogenous sources or it can also
come from an external source. For instance, the exposure to ultraviolet light (UV) or any other type of radiation can generate a break on the DNA structure (Lorraine et al., 2011). In addition to DNA repair mechanisms cells are able to delay the cell cycle, when crossing a checkpoint, to allow the DNA repair before a major cell cycle transition (Ciccia et al., 2010).

1.1.1 Single strand break signalling

Although single strand break (SSB) occurs frequently in the cells the response to such damage and how it is repaired is not well understood. A critical and very early event in the SSBs repair pathway is the activation of the poly (ADP-ribose) polymerases (PARPs). These are enzymes that can modify themselves and other factors by attachment of mono and poly ADP-ribose molecules (Caldecott, 2014). Subsequently the X-ray repair cross complementing protein 1 (XRCC1) is recruited by direct binding to PARP through its phosphate-binding pocket, a region which is localised in the BRCT domain of XRCC1 (Breslin et al., 2015). The accumulation of XRCC1 at DNA damage induced by UV is also mediated by the phosphate-binding pocket (Breslin et al., 2015). The XRCC1 recruitment together with the PARylation of histones serves to transduce the signal to the downstream DDRs mediators and effectors required for efficient SSB repair.

When SSBs occur during genome replication and they are not repaired rapidly they can be converted to double strand breaks (DSBs). In this case the replication will stop in order to repair the damage. When damage occurs the very early step is PARylation, which occurs in the first few minutes after the actual break (Ciccia and Elledge, 2010); subsequently the recruitment of one of the three main phosphatidylinositol 3-kinases (PIKKs): Ataxia-telangiectasia Mutated (ATM), ATM and Rad3-related (ATR), and DNA-dependent protein kinase (DNA-PK) is crucial
to sense the damage and to induce the downstream signalling so that the cells can go through different biological responses. The double strand breaks (DSBs) activates both ATM and DNAPK, while ATR can be activated by many types of DNA damage that when processed result in single-stranded DNA (ssDNA) (Cimprich et al., 2009). ATR is mainly active during the S-phase progression in response to DNA damage and replication fork stalling (Shechter et al., 2004). The single strand of DNA is coated by the replication protein A (RPA) (Figure 1.1). RPA bound to ssDNA will then trigger the recruitment of the ATR interacting protein (ATRIP) and ATR itself. The recruitment of RPA is necessary for the localisation of the ATR-ATRIP complex to the single strand of DNA (Byun et al., 2005). While the ATR-ATRIP complex is localised to the single strand of DNA the Rad9-Rad1-Hus1 (9-1-1) complex is independently recruited to the junction between ssDNA and dsDNA. The 9-1-1 complex will then trigger the recruitment of TOPBP1, which activates ATR-ATRIP activator (Lee et al., 2007). In Xenopus the N-terminal region of TOPBP1, containing the BRCT repeats I and II, binds the 9-1-1 complex by specifically interacting with Rad9. This region is also necessary for the TOPBP1 interaction with the ATR-ATRIP complex. Once it is bound to the ATR-ATRIP complex, in vitro, TOPBP1 activates ATR via its ATR activation domain (AAD) (Kumagai et al., 2006). Although the mechanism through which TOPBP1 activates ATR is still unclear it seems that ATRIP interacts with TOPBP1 through a region that is necessary for the association of TOPBP1 and ATR and the subsequent ATR activation (Mordes et al., 2008). ATR activation is also mediated by the Ewing tumor-associated antigen 1 (ETAA1) factor (Lee et al., 2016). ETAA1 specifically interacts with RPA upon DNA replication stress and activates ATR via a conserved ATR-activating domain (AAD) located at its N-terminus.

Once activated ATR can phosphorylate and activate other factors to initiate the single strand DNA signalling cascade. It is known that the main ATR substrate is CHK1 (Liu et al., 2000).
CHK1 is phosphorylated on the Serine 345 upon damage. It has been shown that this phosphorylation is dependent on ATR and it is mediated by Claspin, which is found at the replication fork and brings ATR and CHK1 together (Kumagai et al., 2000). In both fission yeast and human cells CHK1 activation will result in a checkpoint arrest (Furnari et al., 1997; Sanchez et al., 1997). CHK1 binds and phosphorylates in vitro three CDC25 phosphatases CDC25A, CDC25B and CDC25C, which control the cell cycle transition by de-phosphorylating the cyclin-dependent kinases (CDK) necessary for cell cycle progression.
Figure 1.1: Single strand DNA signalling. RPA binds the single strand of DNA. The 9-1-1 complex is recruited to the junction between ssDNA and dsDNA. The recruitment of ETAA1 is then triggered by its interaction with RPA2. Subsequently the ATR-ATRIP complex is loaded on ssDNA due to the interaction between ATRP and RPA2. Simultaneously, TOPBP1 loaded on ssDNA and it interacts with the 9-1-1 complex. ETAA1 together with TOPBP1 will activate ATR, which will then phosphorylate CHK1 on the Serine 345. CHK1 will then transduce the signal by phosphorylating the CDC25 phosphatases which will cause a cell cycle arrest due to the inhibition of the cyclin-dependent kinases CDKs.
1.1.2 Double strand break signalling

DNA double strand breaks (DSB) are more dangerous lesions that can be repaired by two major pathways: homologous recombination (HR) and non homologous end-joining recombination (NHEJ). In addition to the HR and NHEJ repair pathway, alternative-NHEJ (alt-NHEJ) and single-strand annealing (SSA) are also responsible for DSBs repair. The pathway choice is determined by the extent of the DNA end processing (Ciccia and Elledge, 2010).

A crucial event necessary to sense the DSB is the phosphorylation of the histone variant H2AX on the Serine 139 (Rogakou et al., 1998). The H2AX phosphorylation on the Serine 139 spreads a few mega-bases from the break and it is dependent on ATM and MDC1 recruitment to the breaks (Figure 1.2). DSBs are sensed by the MRE11-RAD50-NBS1 complex (MRN complex) which localises to both sides of the break (Goldberg et al., 2003; Lukas et al., 2004). MDC1 interacts with NBS1 through its N-terminus enriched Ser-Asp-Thr (SDTD) repeats (Melander et al., 2008) (Figure 1.3). This region of MDC1 is constitutively phosphorylated by casein kinase 2 (CK2) and it is necessary for the interaction between MDC1 and NBS1 and also for the subsequent enrichment of NBS1 into foci (Spycher et al., 2008). MDC1 also presents an TQXF cluster domain at its N-terminus, which is phosphorylated by ATM. Consistent with this Ataxia telangiectasia (AT) cells show a reduction in MDC1 phosphorylation (Stewart et al., 2003). This ATM-dependent phosphorylation of MDC1 is crucial for the recruitment of the E3 ubiquitin ligase RNF8 (Kolas et al., 2007). RNF8 interacts with the phosphorylated N-terminus of MDC1 via its forkhead-associated (FHA) domain. RNF8 is found at site of damage together with the E2 ubiquitin-conjugating enzyme UBC13, which specifically generates K63-ubiquitin chains (Hofmann et al., 1999). RNF8 and UBC13, but not RNF168, ubiquitinate the histone linker type 1 (Thorslund et al., 2015) but can also ubiquitinate the histone variants H2A and H2AX (Mailand
et al., 2007). The E3-ligase RNF168 will subsequently be recruited to the site of damage by binding the RNF8-mediated K63 ubiquitin chains via its UDM1 module (Panier et al., 2012). However, the precise ubiquitination events are still to be determined. Initially, it was proposed that RNF8 firstly ubiquitinates H2A, while RNF168 will amplify this signal (Mailand et al., 2007). Later, it has been shown that RNF168 binds RNF8-dependent ubiquitylated histone H2AX, it mono-ubiquitinates H2AX on Lys13/15, as well as amplifying the K63 ubiquitination on H2A. This poly-ubiquitination of H2A on Lys63 is necessary for the retention of 53BP1 and BRCA1 at ionising radiation induced DSBs (Doil et al., 2009; Mattiroli et al., 2012). It seems that these two E3 ligases cross-talk in the DDR in order to transduce the signal to the subsequent effectors.
Figure 1.2: Double strand break signalling. i) Double strand break is induced by IR ii) The MRN complex is recruited at the break sites. iii) ATM binds the MRN complex and gets activated by its autophosphorylation on S1981. MDC1 binds ATM on the S1981 via its FHA domain. MDC1 is also recruited via its interaction with the MRN complex and its interaction with phosphorylated γH2AX via its BRCT domains. iv) MDC1 is phosphorylated by ATM and RNF8 is recruited to the break via its interaction with phosphorylated MDC1 through the FHA domain. v) RNF8, together with UBC13 will initiate the K63 ubiquitination of the histones H1. RNF168 will bind the RNF8 Lys63-mediated ubiquitination chains in order to amplify this ubiquitination event and trigger the recruitment of 53BP1 and BRCA1.
**Figure 1.3: Schematic of human MDC1.** MDC1 presents a FHA domain necessary for its interaction with phosphorylated ATM; SDTD and TQXF repeats phosphorylated by CK2 and ATM respectively, a PST rich domain and two BRCT domains necessary for its interaction with γH2AX.

### 1.1.3 DNA damage mediators

In the DNA damage response ATM, ATR and DNAPK are among the first DDR proteins recruited to the ssDNA and DSBs. They have roles in sensing breaks and transducing this information to downstream mediators and effectors, which will effect the different context specific biological responses to breaks in DNA (Marechal et al., 2017).

The MDC1 activation triggers the recruitment of the two E3 ubiquitin ligases, RNF8 and RNF168, which will ubiquitinate the histone H1 and H2AX via Lys63 dependent linkages; another necessary event for the recruitment of BRCA1 and 53BP1 (Kolas et al., 2007; Mailand et al., 2007; Doil et al., 2009). BRCA1 is an E3 ubiquitin ligase, which forms a heterodimer with the BRCA1-associated RING protein 1 (BARD1) (Wu et al., 1996). BRCA1 is an important tumor suppressor that binds the Abraxas protein, through its BRCT domains (Wang et al., 2007). Abraxas will then recruit the ubiquitin-interacting motif (UIM)-containing protein RAP80 to BRCA1 and together they will facilitate the recruitment of this last two on DNA damage sites through recognition of ubiquitinated proteins. Cells depleted for Abraxas or RAP80 show defect in HR repair although this defect is less severe than the defect in BRCA1-depleted cells, which
suggests that Abraxas or RAP80 mediates a subset of BRCA1 functions (Wang et al., 2007). Once BRCA1 is recruited to the break it binds and ubiquitinates CtIP, (Barber et al., 2006; Nakamura et al., 2010, Roy et al., 2012) to initiate resection. Resection allow subsequent recruitment of RPA2 and RAD51. BRCA1 is also required for the RAD51 recruitment the sites of DNA damage through its interactions with PALB2 and BRCA2 (Roy et al., 2012). Although it seems that resection is initiated by ubiquitinated CtIP (Barber et al., 2006; Nakamura et al., 2010, Roy et al., 2012) a recent study provide evidence for CtIP to act independently of BRCA1 (Polato et al., 2014). In mouse B cells deficient for CtIP resection is not affected by the loss of the BRCA1-CtIP complex but, it is the CDK-mediated phosphorylation of CtIP on T847 that is required to promote resection (Polato et al., 2014).

Effective recruitment of 53BP1 requires three elements of its primary structure: its oligomerization domain, its tudor domain, and a short carboxy-terminal extension of the tudor domain, termed the ubiquitination-dependent recruitment (UDR) motif. The Tudor domain of 53BP1 is necessary for its interaction with the Lysine20 of H4 (H4K20me2) whereas the UDR is required for interaction with H2A ubiquitinated on Lys 15 (Fradet-Turcotte et al., 2013; Zgheib et al., 2009). The oligomerisation domain is also required for the 53BP1 phosphorylation necessary for 53BP1 to bind RIF1 and blockage of BRCA1-dependent resection specifically in the G1 phase of the cell cycle (Escribano-Diaz et al., 2013). This event, together with the recruitment of the Shieldin complex will allow the cells to repair the damage via NHEJ.

Recent studies have identified the Shieldin complex as a four-subunit putative single-stranded DNA-binding complex essential for REV7-dependent DNA end-protection and non-homologous end joining during class-switch recombination (Ghezraoui et al., 2018). The
Shieldin complex localises to site of DNA DSB in a 53BP1 and RIF1-dependent manner (Noordermeer et al., 2018).

53BP1 also plays an important role in DSB repair pathway choice between HR and NHEJ. Cells lacking BRCA1 and 53BP1 are able to repair the damage by using HR (Bunting et al., 2010; Zong et al., 2016). This is because a lack of 53BP1-dependent shieldin of DSB ends is permissive for extensive BRCA1-independent resection, which will be then repaired by gene conversion (GC) and/or single strand annealing (SSA). Due to its interaction with 53BP1 and requirement for shieldin recruitment depletion of RIF1 restores the resection and RAD51 loading in BRCA1-depleted cells (Escribano-Diaz et al., 2013).

1.2 DNA double strand break repair

While GC is RAD51-dependent, SSA is RAD52-dependent. SSA occurs when excessive resection exposes homologous repetitive sequences up to several kilobases away from the break. Alternative NHEJ (aNHEJ), also termed microhomology mediated end joining (MMEJ) occurs in absence of the classical NHEJ factors such as Ku or XRCC4 (Guirouilh-Barbat et al., 2007). MMEJ requires limited resection to reveal very short regions of homology, which are annealed with the resulting ssDNA tails being to produce small deletion (Yan C.T. et al., 2007). DNA polymerase θ (Polθ) promotes MMEJ of DSBs (Black et al., 2019). Importantly, the helicase is essential for Polθ MMEJ of long ssDNA overhangs which model resected DSBs (Black et al., 2019). SSA is mainly initiated by BRCA1, while the NHEJ and the alt-NHEJ are mainly initiated by 53BP1 and DNAPK. There is evidence that these main DNA damage repair factors crosstalk and inhibit each other in order to lead the cells through one pathway or the other. What exactly leads the cells to choose for one repair pathway or the other is not yet well understood (Zong et
al., 2016; Bunting et al., 2010). A main factor influencing the pathway choice seems to be the DNA end processing (Hartlerode and Scully, 2009). In HR the DNA ends are resected to yield 3’ single stranded DNA tail with a 3’ hydroxylated (3’OH) end which will then be repaired by annealing with the homologous single strand of DNA in case of SSA. In case of NHEJ DNA resection is not needed and simple ligation of the broken DNA occurs.

1.2.1 Homologous Recombination (HR)

In the case of homologous recombination once the double strand break has been sensed by MRN the localisation of BRCA1 and CtIP will catalyse resection thereby inhibiting NHEJ-dependent simple ligation. BRCA1 will activate CtIP by ubiquitination (Yu et al, 2006) and together they will initiate the 5’ to 3’ DNA resection, which is then extended by DNA2 and EXO1 (Hoa et al, 2015; Nakamura et al., 2010). The presence of sister chromatids is essential for repair by HR, this is why cells use this repair pathway when they are in S or G2 of the cell cycle (You et al., 2010). The formation of ssDNA with a 3’ terminus leads to the recruitment of RPA2, which protects the ssDNA from degradation (Figure 1.4). RAD51 subsequently is loaded onto the RPA-ssDNA in a BRCA2-dependent manner and replaces RPA (Wong et al., 1997). At this point a search for homology starts involving collision between the RAD51-coated ssDNA and DNA duplex as elsewhere in the nucleus. The close proximity of the sister chromatid immediately following replication greatly facilitates the homologous search. Once the homology is found the resected strand will invade the homologous strand of the DNA duplex by forming a D-loop structure (Figure 1.4 A-B). At this point the invaded strand of DNA is used as a template and the 3’ end revealed by resection will be extended by a DNA polymerase. It is still unclear which polymerase is required in HR but a study shows that DNA polymerase η can perform this function in vitro (McIlwraith et al., 2006). The HJ is then resolved by different enzymes; it can
be resolved by the Bloom’s syndrome protein (BLM) in complex with the topoisomerase IIIa to form a non-crossover product (Wu and Hickson 2003) or it can be resolved by the MUS81-EME1 complex (Chen et al., 2001), which will generate a crossover product (Figure 1.4 B). If the 3’ ssDNA goes through extensive resection then RAD52 will coat the single strand of DNA by binding the RPA-ssDNA complex (Ma et al., 2017). RAD52 forms a different complex with RPA compared to the RAD51 and plays a role in the annealing of complementary ssDNA (Shinohara et al., 1998). RAD52 promotes the RAD51-independent recombination through single strand annealing (SSA), where a sister chromatid is not required since the homologous sequence for the repair is found on the same DNA duplex (Figure 1.4 C).
**Figure 1.4: HR repair pathway.** The double strand break is sensed by the MRN complex which will activate the DDR cascade. BRCA1 will be recruited to the damage site, it will ubiquitinate CtIP and together they will initiate HR repair by inhibiting the 53BP1/RIF1 recruitment and triggering the resection. The 5’ DNA ends resection is performed by the exonucleases DNA2 and EXO1 which generate a 3’ resected ssDNA which is coated by RPA2 which protects this from degradation. RAD51 is subsequently brought to the single strand of DNA through its interaction with BRCA2. The resected strand will then invade the sister chromatid strand where there is a homologous sequence by forming a D-loop structure (A-B). A DNA polymerase will extend the resected strand by using the sister chromatid as a template. This way we can end up with a non-crossover product (A) or a crossover product (B). If there is a hyper-resection with a homology sequence nearby then the HR repair occurs in a RAD51-independent way also known as single strand annealing or SSA (C). This pathway is dependent on RAD52.
1.2.2 Non-homologous end joining recombination (NHEJ)

The NHEJ pathway is initiated by the recruitment of the Ku heterodimer complex Ku70/Ku80 to each end of broken DNA (Figure 1.5). The Ku complex forms a ring-like structure and the DNA helix, which is positioned through this ring (Walker et al., 2001). The topological principles of DSB recognition and alignment-based end joining are currently unknown. However, Ku has no sequence specificity when binding to DNA ends (Walker et al., 2001). Subsequently, the Ku heterodimer recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcS). The Ku-DNAPK interaction is essential for the activation of the serine/threonine kinase activity of DNA-PK (Gottlieb and Jackson, 1993). Two molecules of DNA-PK are brought to both broken DNA ends, one on each side, suggesting that DNA-PK bridges DNA ends together to facilitate their repair (De Fazio et al., 2002). The MRN complex also seems to play a role in the NHEJ repair pathway. A study suggests a role for the MRN complex in bridging DNA ends together with DNA-PK (Dinkelmann et al., 2009). This role for the MRN complex in bridging DNA ends seems to be conferred by RAD50 and its conserved motif (Cys-X-X-Cys), which forms a Zn (2+) ion dependent hook (Hopfner et al., 2002). This RAD50 motif is functional since if mutated it can lead to IR sensitivity in yeast and disruption of the MRN complex. This suggests that this RAD50 conserved motif is necessary for forming metal-mediated bridging complexes between two DNA ends.

Once DNA-PK is activated it can phosphorylate itself on different residues and it also phosphorylates different substrates required in the NHEJ repair cascade (Chan et al., 2002). Artemis interacts with DNA-PK, which phosphorylates Artemis (Ma et al., 2002). Free Artemis possesses a single strand 5’-3’ exonuclease activity but, once it forms a complex with DNA-PK, and gets phosphorylated by DNA-PK, it acquires a 5’-3’ endonucleolytic activity necessary for
the 5’ and 3’ overhang processing in NHEJ. The final step in the NHEJ repair pathway is the ligation of the DNA ends. This step is carried out by the XRCC4-LIG4 complex. This complex is phosphorylated by DNA-PK even though the phosphorylation of XRCC4 and XLF by DNA-PK is not essential for cell survival or DNA repair (Yu et al., 2003; Yu et al., 2008; Wang et al., 2004). DNA ligase IV is responsible for the ligation of blunt ends and its activity is stimulated by XRCC4 (Grawunder et al., 1998). DNA ligase IV can ligate one DNA strand independently from the other one, and ligate across gaps or incompatible DNA ends by generating short overhangs (Gu et al., 2007).
Figure 1.5: NHEJ repair pathway. When DSB occurs the Ku heterodimer (Ku70/80) is recruited to the site of damage. Subsequently two molecules of DNA-PK interact with Ku70/80 and it bridges the DNA blunt ends. By interacting with Ku70/80 and the DNA DNA-PK gets activated by autophosphorylation and it phosphorylates and recruits Artemis first and then the XRCC4-LIG4 (X4-L4) complex, composed of DNA Ligase IV, XRCC4 and XLF, which will ligate the two DNA blunt ends together.
1.2.3 The choice between HR and NHEJ

It is still not completely clear how the choice between the HR and the NHEJ is made at DSBs. There is evidence for BRCA1 to promote HR by antagonising 53BP1 but it remains unknown how BRCA1 function is limited to the S and G2 phases (Nakamura et al., 2019). BRCA1 recruitment requires recognition of histone H4 unmethylated at lysine 20 (H4K20me0), linking DSB repair pathway choice directly to sister chromatid availability (Nakamura et al., 2019).

Resection is a critical step for the HR initiation. CtIP, together with MRE11, initiates the short-range resection which is then extended by DNA2 and EXO1 (Hoa et al., 2015). Resected DNA ends are not a good substrate for the recruitment and binding of Ku, a main player in the NHEJ pathway. NHEJ occurs throughout the cell cycle and is the principle DSB repair pathway of cells in the G1 phase of the cell cycle, HR primarily occurs when the cells are in the S and G2 stages of the cell cycle when sister chromatids are available as a template for repair (Symington and Jean Gautier, 2011). HR in G1 would require a homologous chromosome which will be more difficult to find then the proximal sister chromatid upon replication. In budding yeast, for example, CDK1 is responsible for DSB-induced HR. CDK1 levels are low in G1, which could explain why the cells are more prone to repair the break via NHEJ. CDK1 regulates resection, and so the HR repair pathway by phosphorylating Sae2, the yeast equivalent of CtIP (Ira G. et al., 2004). Inhibition of HR by using an analogue-sensitive CDK1 protein results in an increased NHEJ. In Saccharomyces cerevisiae, DSB end resection and checkpoint activation seem to be dose-dependent (Zierhut and Diffley, 2008) and more efficient in G2/M then in G1; but the highest efficiency of DSB processing is reached at the S stage of the cell cycle at breaks encountered by DNA replication fork. Since resection is the important initiating step for HR initiation this has to be inhibited in order for the cells to repair via NHEJ repair.
HR and resection inhibition is due to the recruitment to site of damage of 53BP1, which recruits effector proteins with DNA end protection activity. 53BP1 interacts with REV7, one of the subunits of the Shieldin 1 complex. The Shieldin complex is necessary for the REV7-dependent DNA-end protection during the NHEJ class-switch recombination (Ghezraoui et al., 2018; Noordermeer et al., 2018). The Shieldin complex is recruited to DSBs in a 53BP1-RIF1-dependent manner, it binds the ssDNA via the FAM35A (SHLD2) subunit and protects the DNA from extensive resection (Noordermeer et al., 2018). In G1 cells RIF1 is recruited to site of damage by its direct interaction with 53BP1 and it inhibits the HR by recruiting shieldin which inhibits the recruitment of BRCA1 and CtIP to the breaks. The depletion of RIF1, on the other hand, restores the resection and the recruitment of RAD51 in BRCA1-depleted cells suggesting that the resection is a major feature for the initiation of the HR repair pathway and its inhibition will activate the NHEJ repair pathway (Escribano-Diaz et al., 2013).

Even if the two DSB repair pathways look like they antagonise each other all the time there is some evidence of crosstalk. Loss of 53BP1 reduces the sensitivity to PARP inhibition in BRCA1-mutated cells and it promotes DSB repair through HR (Bunting et al., 2010). In BRCA1-deficient cells, if 53BP1 is silenced or its localisation to DSB is inhibited then the DSB resection is converted to hyper-resection which leads the cells to repair the breaks through HR but more specifically through SSA which is an alternative error-prone RAD51-independent and RAD52-dependent HR pathway (Ochs et al., 2016).

1.3 Regulation of the DNA double strand break repair by Ubiquitination and Sumoylation

In presence of a double strand break the DNA around the break goes through distinct chromatin modifications. It is well known that one of the most important histone modifications during DNA
repair is the phosphorylation of the histone variant H2A on the Serine 139 (Savic et al., 2009; Iacovoni et al., 2010). All DSBs trigger this phosphorylation which, is bidirectional and spreads up to 1Mb from the site of damage (Iacovoni et al., 2010). The mechanism behind H2AX phosphorylation and its spreading is still not completely clear but this event is necessary for the recruitment of DDR factors such as MDC1 and the subsequent DSB repair. Histone methylation is another important epigenetic modification in the DNA double strand break repair pathway. For instance, 53BP1 is recruited to DSBs by direct binding between its Tudor domain, and H4K20me2, while its UDR binds specifically to H2A mono-ubiquitylated on Lys13 and Lys15 (Botuyan et al., 2006). The E3-ubiquitin ligase enzyme RNF8 is responsible for poly-ubiquitination of H1 and H2A yet to be discovered residues (Thorslund et al., 2015). This epigenetic modification is important for the recruitment of a second E3-ubiquitin ligase enzyme, RNF168, which binds the poly-ubiquitinated histone H2A and stimulates their further poly-ubiquitination (Doil et al., 2009). Additionally, RNF168 is responsible for the mono-ubiquitination of histone H2A on Lys13 and Lys15 required for efficient binding of 53BP1 to chromatin in the vicinity of DSB (Mattiroli et al., 2012).

Sumoylation is another important epigenetic modification in the genome stability (Jackson and Durocher 2013). Vertebrate cells possess two types of SUMO proteins: SUMO1 and the highly related pair SUMO2 and SUMO3, which appear to be redundant and only differ from each other by three residues at the N-terminus. Interestingly SUMO2/3 only share 50% similarity with SUMO1 (Wilkinson and Henley 2012). Many substrates can be modified by SUMO1 and SUMO2/3 even though it is not clear how the SUMOylation machinery distinguishes between these two and for most of these SUMO substrates the functional differences between SUMO1 and SUMO2/3 conjugation have yet to be defined. Many proteins are substrates for Ubiquitination as well as SUMOylation, often can be the same Lysine residue (Wilkinson and Henley 2012).
1.3.1 Ubiquitin conjugation machinery

Ubiquitins (Ub) are very small proteins that are conjugated to the amino group of lysines on target proteins and recognised in a way that leads to downstream events (Pickart and Eddins 2004). The ubiquitin can be attached to the target protein as a monomer or form polymers which are called polyubiquitin chains. This ubiquitin conjugation occurs on a Lysine residue of the target protein. The fate of the specific target depends on the type of ubiquitin modification. For example, polyubiquitin chains linked on Lys48 are the main signal for targeting substrates to the proteasome (Thrower et al., 2000), while Lys63 polyubiquitination is a signal for initiating the DNA repair pathway (Mattiroli et al., 2012). The ubiquitination of histone H2A on Lys11 is another important event in the DNA damage response. A recent study suggests that this Lys11 ubiquitination is responsible for silencing-transcription in the regions either side of DSBs (Atanu et al., 2017).

The conjugation of an ubiquitin (Ub) to the target protein requires three enzymes: Ub-activating enzymes (E1); Ub-conjugating enzymes or Ubc (E2) and Ub-protein ligases (E3). In general, each Ub has a specific E1 enzyme and more than one E2 and E3 enzyme (Dye and Schulman, 2007). The first step of the Ub conjugation is its own activation which is catalysed by the E1 enzyme. Firstly, an intermediate product is formed by E1-dependent adenylation of the Ub. This intermediate product will then bind the E1 on the cysteine residue to form an E1-Ub thiol-ester. This E1-Ub thiol ester product is then transferred from an E1 catalytic cysteine to an E2 catalytic cysteine. The E2-Ub thiol-ester product is released and it can be attached to the target protein by an E3 ligase enzymes. In the human genome, there are hundreds of E3 ligases genes, which could explain the specificity of ubiquitination. E3 ligases can be classified into three big families which share a common biochemical property of E2 binding: Homologous to E6AP Carboxy Terminus
(HECT), Really Interesting New Gene (RING), and UFD2 homology proteins (U-box) (Pickarta and Eddinsb, 2004).

The HECT E3 ligases differ slightly from the RING and the U-box. HECT E3 act like the E6 associated protein (E6-AP). This E3 ligase, involved in the human papillomavirus E6-induced ubiquitination of p53, forms an ubiquitin thiol-ester which is actually an intermediate. In this particular case the transfer of the ubiquitin molecule is from E1 to E2, from E2 to E6-AP and finally from this to the substrate. In this particular case the Ub conjugation cascade does not stop at the E2-Ub product but it goes further with one more intermediate, the E3-Ub (Scheffner et al., 1995). The first U-box E3 ligase was identified in yeast and it is known as Ufd2. This Ufd2 ligase is unusual, as it does not have a proper substrate but it polyubiquitinates others E3 ligase substrates (Koegl et al., 1999). U-box E3 ligases are often called E4 rather than E3 because they present a different kind of E3 ligase activity. On the other hand, E3 ligases that are part of the RING family show a RING domain, which consists of a short motif rich in Cysteines and Histidines, which includes two zinc atoms. BRCA1 contains a RING domain at its N-terminus. Mutations in the BRCA1 RING domain seem to predispose people to breast cancer suggesting that the BRCA1 E3 ligase activity is essential for its role as a tumor suppressor (Hashizume et al., 2001). A study shows that BRCA1 ubiquitinates CtIP and this event is necessary for the recruitment of CtIP at the site of damage (Yu et al., 2006) although, a recent study provides evidence for CtIP to act independently of BRCA1 (Polato et al., 2014).

1.3.2 SUMO conjugation machinery

SUMO proteins are synthesised as inactive precursors. Their activation is mediated by the SUMO-specific protease enzyme, which cleaves the C-terminus of the SUMO protein by
exposing a di-glycine motif necessary for the conjugation of the SUMO protein to lysine residues of the protein target (Wilkinson and Henley, 2012).

The SUMO conjugation machinery works similarly to the ubiquitin conjugation machinery. They are first activated in an ATP-dependent manner by the SUMO E1-activating enzyme SAE1 and SAE2 (Gong et al., 1999). In this step a thioester bond is formed between the active-site cysteine residue of SAE2 and the glycine residue of SUMO located at its C-terminus. Via a thioester linkage the activated SUMO is passed to the active-site cysteine of the conjugating enzyme Ubc9 (ubiquiting-conjugating 9) (Johnson et al., 1997). At this point a SUMO E3-ligase is required to attached the SUMO protein to the protein target. Similarly to RING E3-ligases, SUMO E3-ligases do not receive the SUMO protein through a thioester linkage, but act as scaffolds bringing SUMO-loaded Ubc9 into contact with the substrate protein (Wilkinson and Henley, 2012).

The first SUMO E3-ligases were identified in budding yeast and they are known as Siz proteins (Johnson et al., 2001). The mammalian homologous of the Siz proteins are the PIAS proteins (protein inhibitor of activated STAT (signal transducer and activator of transcription). This family is composed of five PIAS proteins all showing SUMO-dependent E3-ligase activity. They also possess a RING domain that is also found in the ubiquitin E3-ligases (Schmidt et al., 2002). A number of other RING domain-containing proteins have been reported to function as SUMO E3 ligases such as TOPORS (topoisomerase I-binding, arginine/serine-rich) and the mitochondrial E3 ubiquitin ligase 1 (Weger et al., 2005; Braschi et al., 2009).

Lower Eukaryotes posses only one SUMO protein while high Eukaryotes posses three SUMO isoforms known as SUMO1, SUMO2 and SUMO3 (Zhao, 2018). These three SUMO isoforms differ from each other for several reasons, for example, they can have different preferences for
E3 ligases or they can form poly or mono SUMO chains on different lysine residues of the target protein (Pitchler et al., 2017). SUMO1 is attached to the protein target as a mono-Sumoylation while SUMO2/3 are attached as a poly-Sumoylation (Tatham et al., 2001). SUMO1 does not form chains but it can be attached to lysine residues of SUMO2/3 chains leading to chain termination (Matic et al., 2008). SUMO1 is mostly found conjugated to proteins while, SUMO2/3 are found in a free form and become attached to substrates upon cellular stresses (Saitoh et al., 2000). Once attached to the substrate they can have different effects. They can change the substrate interaction with DNA, RNA or other proteins by simply changing the conformation of the protein; the effect of the sumoylation can be reversed when the SUMO is removed by SUMO-specific proteases or desumoylases (Zhao, 2018).

1.3.3 Ubiquitination and Sumoylation in the DNA damage response

Some important ubiquitination events in the DDR occur on the histones. For example, the histone H2A is ubiquitinated on Lys127 and Lys129 by the E3 ligase activity of the BRCA1/BARD1 complex. Even though the role of this ubiquitination is still poorly understood it seems to be necessary for repositioning 53BP1 and completion of resection through HR (Densham et al., 2016). The BRCA1 complex is recruited to breaks via RAP80 and its interaction with RNF8/RNF168 catalyses K63-linked ubiquitin chains (Sobhian et al., 2007). BRCA1 interacts with BRCA2 and subsequently binds PALB2 allowing the BRCA2-dependent recruitment of RAD51 to DSBs (Shirley et al., 2009). BRCA1 recruitment depends on the BRCA1-PALB2 interaction. The presence of PALB2 at the site of damage is dependent on MDC1 and RNF8 recruitment, suggesting that histone ubiquitination strongly regulates the PALB2 recruitment (Zang F. et al., 2012). BRCA1 recruitment to breaks is also dependent on its interaction with BARD1 (Yu et al., 2006). In response to DNA damage BARD1 interacts with Lys9-dimethylated
histone H3 (H3K9me2) in an ATM-dependent but RNF168-independent manner (Wu et al., 1996). The BRCT domain of BARD1 is crucial for its retention at DNA damaged sites via its interaction with HP1 (Wu et al., 1996).

The histone variant H2B is also ubiquitinated (Weake and Workman, 2008). Mono-ubiquitinated H2B on Lys123 (H2BK123ub1) was identified for the first time in yeast Saccharomyces cerevisiae and mutation of the conserved ubiquitination site is shown to confer mitotic defects in mitotic cell (Robzyk et al., 2000). This H2BK123ub1 is required to promote error-free translation synthesis mediated by DNA polymerase eta (Polη) upon damage. In absence of H2BK123ub1, DNA polymerase zeta (Polζ) is responsible for mutagenic mechanisms (Northam et al., 2016). In mammalian cells, H2B ubiquitination is primarily mediated by an E3 ubiquitin ligase complex, which is composed of two RING finger proteins RNF20 and RNF40 (Shiloh et al., 2011). This H2B123ub1 mono-ubiquitination is also associated with gene transcription, probably by modulating the chromatin condensation at transcribed regions (Shiloh et al., 2011). Recent studies suggest that this modification can enhance or suppress the transcription of specific set of genes through a mechanism, which involves the hPAF1 complex and TFIIS protein. Because some of these genes are involved in genome stability RNF20 and RNF40 are classified as tumor suppressors. In mammalian cells these two RING finger proteins localise at sites of damage in an ATM-dependent manner and ubiquitinate histone H2B (Moyal et al., 2011). RNF8 together with UBC13 and the Lys63 ubiquitination of H2A is necessary for the recruitment of the BRCA1 complex (Wang et al., 2007). The BRCA1 complex contains the BRCA1/BARD1 heterodimer, RAP80, Abraxas and the Lys63-specific deubiquitinase BRCC36. RAP80 contains an Abraxas-interaction domain (AIR), but it also contains two ubiquitin-interacting motifs (UIMs), which bind the Lys63-ubiquitin chains (Sobhian et al., 2007). This suggests that the RNF8-UBC13 Lys63-dependent ubiquitination is necessary for the recruitment
of the BRCA1 complex and that RAP80 functions as a link between the K63-polyubiquitination chains and the recruitment of the entire complex.

The mono-ubiquitination of H2A on Lys13 and Lys15 by RNF168 is, on the other hand, necessary for the 53BP1 recruitment to the site of damage. In fact, 53BP1 is directly recruited to DSBs by its interaction with H2AK15/K13 via its ubiquitin-dependent recruitment (UDR) motif (Zgheib et al., 2008; Fradet-Turcotte et al., 2013). The recruitment of 53BP1 will inhibit the resection thus leading the cells towards NHEJ repair rather than HR repair. A recent study has associated RNF168 with HR repair (Luijsterburg et al., 2017) 53BP1 together with RIF1 suppresses HR in G1 cells. In S/G2 cells HR inhibition is relieved due to the presence of PALB2, which drives the cells towards HR repair. This is possible because PALB2 indirectly recognises histone ubiquitylation by associating with ubiquitin-bound RNF168 (Luijsterburg et al., 2017). This interaction occurs between the PALB2-interacting domain (PID) of RNF168 and the WD40 domain in PALB2. This strongly suggests that, while RNF8 is responsible for the initiation of the ubiquitination cascade, RNF168 is necessary for its amplification, which is necessary for the recruitment of downstream DDR factors (Luijsterburg et al., 2017).

In case of Sumoylation, mammalian SUMO E3-ligases PIAS1 and PIAS4 are required to promote the DNA double strand break response (Galanty et al., 2010). They are recruited to sites of damage via their SAP domain. PIAS4 is mainly required for the recruitment of 53BP1, RNF8 and RNF168, while PIAS1 is required for RAP80 and BRCA1 accumulation at sites of damage (Galanty et al., 2010). BRCA1, in fact, is found at DSBs together with SUMO1, SUMO2/3 and the SUMO-conjugating enzyme Ubc9 (Morris et al., 2009). The SUMO1 modification of BRCA1 by PIAS1 increases its ubiquitin-ligase activity (Morris et al., 2009). A very well characterised link between sumoylation and the DDR is the Rad52 sumoylation in budding yeast.
Rad52 acts in HR and its activity is promoted by SUMO E3-ligase Siz2-dependent sumoylation (Torres-Rosell et al., 2007). Emerging studies are suggesting a connection between ubiquitination and sumoylation. Many recent studies have been focused on SUMO-targeted ubiquitin ligases (STUbLs), which include human RNF4. These STUbLs contain SUMO binding motifs (SIMs) that bind SUMO proteins on target proteins and then ubiquitylate such proteins, often leading to their proteasomal degradation. RNF4 is found at DSBs and it is brought there via its interaction with MDC1, 53BP1 and RPA2 (Luo et al., 2012; Galanty et al., 2012; Jackson et al., 2013). The RNF4 recruitment to DSB is necessary for MDC1 sumoylation and subsequent ubiquitination, which is RNF4-dependent. MDC1 sumoylation and ubiquitination is important for its turnover and regulation in the DDR (Luo et al., 2012). RNF4 can also ubiquitinate RPA2 after it has been sumoylated, this event is necessary for the degradation of RPA2 and its replacement by RAD51 necessary in HR repair (Galanty et al., 2012).

1.4 PIKKs and the DNA damage response

PIKKs are responsible to activate a wide range of substrates. PIKKs possess a kinase activity that allows them to phosphorylate other proteins on Serine and Threonine residues. In the DNA damage response ATM (ataxia-telangiectasia mutated) is mainly involved in multiple responses to DSBs, while ATR (ATM- and Rad3-Related) is mainly involved in replication stress but also functions DSB repair. DNA-PKcs (DNA-dependent protein kinase) is involved in DSB repair via NHEJ. ATM and ATR seem to activate a wider range of substrates and so to have a wider range of DDR responses compare to DNAPK, which seems to have fewer targets consistent with a main role in the NHEJ repair pathway (Marechal and Zou, 2013).
1.4.1 The human PIKKs family: ATM, ATR and DNAPK

These three PIKKs were first identified based on sequence analysis (Keith et al., 1995). These kinases share common protein domains: the PIK-related kinase domain, the FAT-C terminal motif (FATC), a large region of α-helical HEAT repeats at the N-terminal some of which contribute to a second FAT domain localised upstream the kinase domain (Figure 1.6). The first PIKK to be identified was mTOR. It was initially identified in budding yeast to play a role in cell growth but not in the DDR (Kunz J. et al., 1993). On the other hand, ATM, ATR and DNA-PK all play a role in the DDR.

ATM forms a dimer, which is then dissociated when ATM is activated by autophosphorylation on the residue serine 1981 in the presence of DSBs (Bakkenist et al., 2003). ATR is found in a complex with ATRIP (Cortez et al., 2001). The structures for ATM and ATR has not been identified yet. However, the structure of DNA-PK is known (Sibanda et al., 2010). DNA-PK, which consists of the DNA-PK catalytic subunit and the Ku70/80 heterodimer, forms a hollow circular structure with the kinase domain located at the top of this ring. Although there is no evidence that these HEAT repeats bind DNA, it is known that, for ATM and ATR, HEAT repeats are required for protein-protein interactions. It has been shown in yeast that ATM (Tel1) binds the C-terminus of NBS1 through two separated pairs of HEAT repeats (You et al., 2005). This interaction is necessary for the ATM activation and its retention at the site of damage. ATR also binds ATRIP through the HEAT repeats located in its N-terminus region (Ball et al., 2005). The FAT and FATC domains in ATM and ATR play important roles in regulation and activation of the kinase activity of these two PIKKs. In absence of damage the FAT domain and the kinase domain of ATM interact with each other to inhibit ATM activity (Bakkenist et al., 2003). This interaction is disrupted by autophosphorylation on S1981, a residue that is located in the FAT
domain, and triggers ATM activation in presence of damage. Although the phosphorylation on S1981 is important to disrupt the interaction between the FAT domain and kinase domain of ATM and initiate the DNA double strand break repair, in ATM<sup>−/−</sup> mice, which show immune system deficiency and extreme radiosensitivity, the restoration of wild-type is only possible by complete restoration and expression of ATM (Di Siena et al., 2018). The FATC domain of ATM is required for binding of the acetyltransferase TIP60 which also contributes to ATM activation by its acetylation (Sun et al., 2005).

Phosphorylation of the threonine 1989 of ATR is recognised by TOPBP1 through its BRCT domain, it enables TOPBP1 to engage ATR-ATRIP and stimulates the ATR kinase activity. The FATC domain of ATR, on the other hand, still has no clear role but, is probably indispensable for its kinase activity (Marechal et al., 2013).

**Figure 1.6: Schematic of human ATM, ATR and mTOR.** The three human PIKKs share some conserved domains such as the FAT, the PIKK and the FATC domain.

### 1.4.2 ATM structure and function

The FAT and FATC domains, in ATM, are located at the C-terminus either side of its kinase domain (Bakkenist et al., 2003; Sun et al., 2005). The kinase domain is enzymatic, catalysing
the transfer of a phosphate from ATP to its main substrates. Phosphorylation follows binding of substrates HEAT repeats via various specific domains found on ATM substrate proteins (Marechal et al., 2017). In vitro, inactive ATM dimers were activated by the presence of free dsDNA and MRN complex (Lee et al., 2005). This recognition by the MRN complex, subsequently, triggers the recruitment of ATM to the site of damage and its activation (Shiotani B. et al., 2009).

1.4.3 ATM downstream signalling

Once activated, ATM can phosphorylate and activate many downstream factors. MDC1 is one of these ATM substrates. MDC1 has a SQ/T rich region at the N-terminal that is phosphorylated by ATM (Stewart et al., 2003). This phosphorylation is necessary for the subsequent transmission of the DDR signal to the downstream factors. The activation of the ATM effector checkpoint kinase 2 (CHK2) leads to the CHK2-dependent phosphorylation of the HP1-binding domain of KAP-1 on the S473, which results in mobilisation and dissociation of HP1-β from the heterochromatin. This event is necessary to allow DDR factors to access the heterochromatic DSBs for subsequent repair (Bolderson et al., 2012). The tumor suppressor protein p53 is an ATM effector and acts as a tumor suppressor by regulating apoptosis and checkpoint regulation in presence of damage. p53 can be phosphorylated by ATM and is also a CHK2 substrate (Nakagawa et al., 1999). Chk2−/− mouse cells are defective for p53 stabilisation and for p53-dependent transcription (Hirao et al., 2000). Human CHK2 is able to directly phosphorylate p53 which then increases its stability and accumulation in the cell. The ATM activation and subsequent H2AX phosphorylation also triggers the ubiquitination and the SUMOylation cascades. These two modifications can occur on histones and on different DDR factors and are important for the subsequent recruitment of 53BP1 and BRCA1.
1.5 Zinc finger proteins

Zinc finger (Znf) proteins are a large diverse family with different biological functions. Multiple zinc fingers domains are often found in zinc finger proteins with each zinc finger binding one zinc ion. The zinc finger domain was shown to be involved in different biological roles such as DNA and RNA binding, as well protein-protein interactions. In the zinc finger domain the zinc ion is usually coordinated to cysteine and histidine residues. The zinc ion is usually not involved in binding targets rather it is required to stabilise the zinc finger fold. There are different zinc finger domains classified in function of their structure and role (Laity et al., 2001).

1.5.1 Zinc finger proteins: classes, structures and functions

A zinc finger domain is characterised by the consensus sequence X₃-Cys-X₂₋₄-Cys-X₁₂-His-X₅-s-His-X₄. This consensus sequence forms a globular finger-like structure, which contains α-helices and β-sheets held together by the zinc ion (figure 1.7). The first zinc finger domain (Cys2His2 or C2H2) was identified in the transcription factor IIIA in *Xenopus laevis* (Miller et al., 1985). These zinc finger domains allow the IIIA transcription factor to bind the 5S of the rRNA gene.
Figure 1.7: Schematic of a C2H2-type zinc finger domain. The zinc finger domain typically contains a Zinc atom surrounded by β-sheets and α-helices.

C2H2 is the main class of the zinc finger proteins. Proteins falling into this class can bind DNA and RNA through the zinc finger domains (Laity et al., 2001). There is also some evidence for the presence of a consensus sequence, in these C2H2 type zinc finger proteins, which is necessary for the DNA binding (Laity et al., 2001). This consensus sequence (TGEKP) links two zinc finger domains and forms a cap at the C-terminus of the upstream domain (Laity et al., 2001). The hypothesis is that C2H2 DNA-binding zinc finger proteins can interact with a specific DNA sequence as this TGEKP linker sequence consensus is highly conserved through species (Laity et al., 2001). Although the majority of C2H2 zinc finger type proteins is involved in nucleic acid binding there is evidence, for some members of this family, to be involved in protein-protein interactions (Laity, Lee and Wright, 2001).

There are different zinc finger proteins, which are classified in function of how many cysteines and histidines they present in their domains and they are also classified by their function (Laity
et al., 2001). While C2H2 zinc finger proteins are mainly involved in DNA-RNA binding the C4H1 and C3H1 type zinc finger proteins are primarily involved in protein-protein interactions (Laity et al., 2001). GATA transcription factors are C4H1 zinc finger containing proteins implicated in adipogenesis in Drosophila and mammalian cells. GATA transcription factors recruit members of the Friend of GATA (FOG) family (Jack et al., 2010). The interaction between the GATA and the FOG co-factors has been shown to occur between the C4H1 domain at the N-terminus of GATA and three or four of the C3H1 domains of the FOG (Jack et al., 2010). The C2H2 domain in FOG doesn’t seem to play a role in this interaction. It has also been shown that a substitution of one histidine with a cysteine in the zinc finger domain does not affect the domain structure (Jack et al., 2010). Although this other class of zinc finger proteins are mainly involved in protein-protein interactions, there is also some evidence for DNA binding among some members of this family. The doublesex protein is a transcription factor which is involved in sexual differentiation in Drosophila. This protein presents two intertwined zinc finger domains CCHC and HCCC, which are able to bind DNA (Zhu et al., 2000).

1.5.2 Zinc finger proteins and the DNA damage response

Zinc finger domains are present in many human proteins. They have been characterised as proteins, typically involved in transcription, that interact with DNA, RNA or proteins. Proteomic, cellular and molecular studies of these proteins have highlighted a possible role for this large family in protecting our genome. Findings show a role for already existing and new zinc finger containing proteins in genome integrity, telomere maintenance and DNA repair (Lahity et al., 2001).
The ability of the C2H2 zinc finger proteins to bind other proteins can also be mediated by protein modification such as ubiquitination and sumoylation (Campbell et al., 2012). Some of these proteins, such as Really Interesting New Gene (RING) proteins and homeodomain proteins (PHD), have already been implicated in the DNA damage response. RNF8 and RNF168 are well known for transducing the DDR signal by histone ubiquitination. The RING domain is actually a C3HC4 type zinc finger domain. Although it is clear that the RING domain for RNF8 is responsible for its interaction with the E2 ubiquitin-conjugating enzyme UBC13, it is not clear what the RING domain for RNF168 is involved in (Campbell et al., 2012). RNF168 might bind another E2 ubiquitin-conjugating enzyme, since the two RING domains of the two E3 ligases are very similar in terms of structure (Campbell et al., 2012). The only difference is that while the RNF8 RING domain dimerises upon its interaction with UBC13, the RNF168 RING domain is a monomer.

A recent proteomic screen to identify proteins that localise to DNA breaks after damage showed that more than 120 proteins can localise to site of damage with most of them being DNA-binding proteins or transcription factors (Izhar et al., 2015). A random analysis of the transcription factors revealed that some of them have never been associated with the DDR, while some others were zinc finger proteins. Among these transcription factors which are zinc finger proteins, 70% were found to accumulate at DSBs.

The zinc finger ZNF451, also known as ZATT, is a C2H2-type zinc finger protein responsible for processing the protein-TOP2 DNA cleavage complex (TOP2cc) together with tyrosyl-DNA phosphodiesterase 2 (TDP2) (Schellenberg et al., 2017). TOP2 normally creates DSBs, which form intermediates (TOP2cc) where TOP2 is found to bind the DNA and that are used to resolve the stress coming from DNA supercoiling (Schellenberg et al., 2017). ZNF451 is a SUMO E3
ligase, which can sumoylate TOP2 and promote the TDP2 interaction with sumoylated TOP2 (Schellenberg et al., 2017). TDP2 with its hydrolase activity can so resolve the TOP2cc complex. It is still unclear whether the role of ZNF451 is actually mediated by its zinc finger domains. The ZNF506 is another C2H2-type zinc finger protein that is found at DSBs and facilitates MDC1 recruitment via de-phosphorylation of H2AX on Y142 (Nowsheen et al., 2018). ZNF506 is recruitment to DSB in an ATM-dependent manner. The ZNF506 activation triggers the EYA phosphatase recruitment to site of break, which is responsible for the H2AX de-phosphorylation at Y142 necessary for the MDC1 and other DDR factors recruitment to DSB.

The lethal(3)malignant brain tumour-like protein 2 (L3MBTL2) is an usual C2C2-type zinc finger protein (Lechtenberg et al., 2009). L3MBTL2 possess a transcription repression activity. Importantly, in the DDR it is found to interact with RNF8 and RNF168. L3MBTL2 seems to be the linker between RNF8 and RNF168 since, once L3MBTL2 is ubiquitinated by RNF8, then it recruits RNF168 to DSBs (Nowsheen et al., 2018).

Another class of zinc finger proteins, which is involved in the DDR, is the myeloproliferative and mental retardation MYM-type. Members of this family are characterised by a tandem zinc-binding MYM motif. The MYM motif gets is nomenclature from the fact that proteins with this motif have been identified in genes mutated in X-linked mental retardation and a myeloproliferative disorder (Van der Maarel et al., 1996). One of the members of this MYM-type zinc finger protein family, ZMYM3, has been recently associated with the DDR (Leung et al., 2017). ZMYM3 is found at sites of damage and recruited through its ability to bind histones and components of the nucleosome. ZMYM3 directly binds the BRCA1-A complex and mediates the function of this complex at DNA damage sites to facilitate DNA repair by HR (Leung et al., 2017). ZMYM3 acts to harness the HR-suppressive properties of the BRCA1-A
complex. ZMYM3 directly binds RAP80 and ABRA1 and facilitates BRCA1 accumulation at DSBs, which ensures its requisite loading for HR repair. (Leung et al., 2017)

Other zinc finger proteins, on the other hand, are responsible for transcription regulation at the DSBs. The zinc finger myeloid, Nervy, and DEAF-1 domain containing 8 (ZMYND8) protein has been recently identified as a new DDR factor required for the recruitment of the nucleosome remodelling and histone deacetylation (NuRD) complex to damaged chromatin (Gong et al., 2015). The NuRD complex is responsible for transcription regulation in the DDR. ZMYND8 bridges the NuRD complex with many other zinc finger proteins, which associate with DNA damage regions and regulate the ZMYND8-NuRD DDR axis (Spruijt et al., 2016). The histone demethylase KDM5A, for example, is responsible for the demethylation of the histone variant H3 on the lysine 4 (H3K4me3). This demethylation is essential for the subsequent recruitment of the ZMYND8-NuRD complex. KDM5A depletion results in impaired transcriptional silencing and DSB repair (Gong et al., 2017).

1.5.3 C3H1-type zinc finger proteins ZC3H8, ZC3H11A and ZC3H14

Very little is known about ZC3H8 and ZC3H11A. They are both involved in RNA biogenesis with ZC3H8 being a component of the little elongation complex (LEC) in human cells and playing a role in small nuclear RNA biogenesis (Hu et al., 2013). On the other hand, ZC3H11A is required for nuclear mRNA export together with PDIP3 (polymerase delta interacting protein 3) (Folco et al., 2012).

A little more is known about ZC3H14, which is considered a polyadenosine binding protein. The fate of a new transcript is primarily dependent on the proteins binding the transcript. These
transcript-binding proteins act as maturation factors involved in capping, splicing, polyadenylation or cleavage of the transcript. Other proteins can be, instead, involved in packing the transcript to stabilise it and exporting it out of the nucleus. One important class of proteins involved in post-transcriptional regulation of gene expression is the poly(A) binding protein class (Pab). These proteins can bind the poly(A) tail through a specific ribonucleoprotein consensus sequence (RNP) (Adam et al., 1986). Although this motif is specific for all Pabs there is another sub-class among these, where the binding to the poly(A) tail of a transcript is mediated through the C3H type zinc finger domain (Anderson et al., 1993).

Nab2 is one of the major Pabs associated with nuclear polyadenylated RNA in vivo (Anderson et al., 1993). Nab2 shows two distinct RNA-binding motifs: the RGG box and the C3H motif repeats, which are also found in the large subunit of RNA pol I, II and III. This C3H zinc motif of Nab2 in yeast has been found to be important for the protein to bind the poly(A) tail of transcripts (Kelly et al., 2007). Similarly to yeast, in human, the ZC3H14 protein contains C3H zinc finger homologous to the one found in Nab2 and it specifically binds the polyadenosine RNA through those repeats. A mutation in the human gene encoding for ZC3H14 causes an autosomal recessive intellectual disability (Pak et al., 2011). ZC3H14 mRNAs are found in the human central nervous system, in rodents ZC3H14 is found in hippocampal neurons and colocalises with poly(A) RNA in neuronal cell bodies, while in D. Melanogaster a mutation in the ZC3H14 gene reveals defects in development and in neurons that are involved in locomotion and flight (Pak et al., 2011). Nab2 is essential for controlling the poly(A) tail length of transcripts and its depletion results in hyperadenylation of the poly(A) (Soucek et al., 2013). Given that a mutation in the human homologue ZC3H14 gives an autosomal recessive intellectual disability; it is possible that the role of this C3H type zinc finger protein in controlling the poly(A) tail length might be related to the intellectual disability. The role of ZC3H14 is very conserved
through different species. Once firstly identified in yeast more studies have been conducted on Nab2/ZC3H14 especially to properly understand its role in human. Mutation in the dNab2 gene causes defects in locomotion and flight. Such defects can be rescued by introduction of the human dNab2, ZC3H14, in dNab2 null flies suggesting that the human gene might have a role in the development of certain regions of the human brain. Although ZC3H14 has been associated with an intellectual disability as yet there is no evidence for ZC3H14 functioning in the DDR.

1.6 Cellular compartments

1.6.1 Nuclear and sub-nuclear compartments

The primary function of the eukaryotic nucleus is to package the genetic material (Pollard et al., 2017). Inside the nucleus the DNA is found to bind different DNA binding proteins such as the nucleosomes, which are responsible for the DNA packaging. The nucleus is separated from the cytoplasm by a double nuclear membrane similar to the cellular membrane (Pollard et al., 2017). The nucleus is connected to the cellular cytoplasm through nuclear pores that are distributed all over the nuclear membrane. These nuclear pores are a complex network of proteins and important for export and import of proteins out of and into the nucleus. Inside the nucleus, the soluble fraction is termed the nucleoplasm (Pollard et al., 2017) while there are also several membrane-less organelles such as the nucleolus, paraspeckles, Cajal bodies, PML bodies and nuclear speckles (Handwerger et al., 2006). These membrane-less sub-nuclear organelles are dynamic structures typically involved in RNA metabolism. They can rapidly exchange components with the surrounding environment and also respond to external stress (Mitrea and Kriwacki, 2016). The paraspeckles are involved in gene expression (Handwerger et al., 2006). The Cajal bodies are the less well understood membrane-less organelles but seem to be linked
to the regulation of small nucleolar ribonucleoprotein particles (snoRNPs) (Handwerger et al., 2006). On the other hand, the nuclear speckles are linked to mRNA processing and proteins that are involved in splicing and poly(A) tail length accumulate in nuclear speckles (Handwerger et al., 2006).

1.6.2 Molecular organisation of nuclear speckles

Nuclear speckles are nuclear structures enriched in mRNA splicing factors and polyadenosine RNA-binding proteins. They are dynamic membrane-less organelles that vary in size and shape. Their dynamic structures allows continuous exchange of RNA and proteins between speckles and other nuclear structures (Lamond and Spector, 2003). By electron microscopy these nuclear structures are seen as interchromatin granule clusters. In the nucleoplasm they form regions where there is little or no DNA. Although there is some evidence for nuclear speckles localising at transcriptionally active sites more studies describe the nuclear speckles as storage, assembly and modification compartments (Xing et al., 1995).

An important signal for proteins to localise to nuclear speckles is the presence of serine and arginine rich regions (RS) (Lamond and Spector, 2003). Many splicing factors as well as kinases and phosphatases, which can phosphorylate and dephosphorylate components of the splicing machinery, have been localised to nuclear speckles (Mintz et al., 1999). Of particular interest in these nuclear structures is the presence of transcription factors and 3’ end RNA-processing factors. Although transcription does not occur within the nuclear speckles and there is no DNA localising to these regions, several sub-units of RNA polymerase II have been identified at the nuclear speckles together with a subset of transcription factors (Galganski et al., 2017). It is unclear what determines the localisation of this subset of transcription factors to the nuclear
speckles but their presence in these regions might relate to some regulatory steps affecting the modification or the accessibility of transcription factors. Additionally, poly(A) RNAs and non-coding RNAs have been identified at the nuclear speckles (Galganski et al., 2017) but it is still unknown, why such RNAs are located at these nuclear structures.

Ubiquitination and Sumoylation machinery is also found in nuclear speckles. Recent studies have identified many E3 ubiquitin ligase enzymes, together with de-SUMOylating enzymes at the nuclear speckles (Galganski et al., 2017). It seems that SUMO-1 is a typical signal to target proteins to these nuclear structures. This data might suggest that nuclear speckles are a centre for such processes but the role of ubiquitination and SUMOylation at the nuclear speckles remains to be determined. More studies, regarding nuclear speckles and their components, have to be carried out in order to address these observations and the relationship between these proteins and mRNA processing.

Because nuclear speckles are membrane-less organelles they are very dynamic structures and they can change in size and re-distribute during the cell cycle (Thiry, 1995). The biggest change for these organelles occurs when cells go through mitosis. During interphase, nuclear speckles are very stable but upon prophase initiation and nuclear envelope breakdown they disassemble. From metaphase to telophase nuclear speckles form mitotic interchromatin granules, which are probably required for modifications, assembly and delivery of pre-mRNA processing complexes to transcription sites in daughter nuclei. The mechanisms behind this nuclear speckle’s re-distribution during the cell cycle are still unknown (Lamond and Spector, 2003).

1.6.3 Nuclear Speckles and DNA damage

Nuclear speckles are dynamic nuclear structures whose roles in cells is still unclear. They have been associated with pre-mRNA processing simply because, from a proteomic screen, 54% of
proteins found at the nuclear speckles are pre-mRNA splicing components and 20% are RNA associated proteins (Saitoh et al., 2004). Although the association of nuclear speckles with proteins involved in mRNA processing, more studies have suggested the presence at the nuclear speckles of proteins that have nothing to do with RNA metabolism such as SUMOylated and ubiquitinated proteins and PIKKs such as DNA-PK suggesting that their role in human cells might extend beyond RNA biogenesis (Lamond and Spector, 2003).

Studies have linked nuclear speckles to the DNA damage; in particular to the UV-induced damage response (Campalans et al., 2007; Wong et al., 2013). UV radiation relocates the DNA glycosylase hOGG1 to the nuclear speckles (Campalans et al., 2007). This glycosylase is responsible for the initiation of the base excision repair (BER) of oxidised purines. One of the most dangerous lesions in the DNA is the presence of oxidised bases, such as the 8-oxoguanine, which, if not repaired, leads to the substitution of a T-A pair to a G-C pair. The hOGG1 is an important enzyme, which allows the repair of the DNA upon oxidative stress (Campalans et al., 2006). It has been shown that hOGG1 is usually distributed in the entire nucleus of human cells but, upon UV radiation forms foci that correspond to nuclear speckles. This hOGG1 relocation can be inhibited with anti-oxidant treatment, suggesting that reactive oxygen species are the signal for this protein to be relocated to nuclear speckles. Another important enzyme involved in the BER pathway is apurinic endonuclease APE1, which is responsible for the repair of the mismatch on the DNA caused by the excision of the oxidased base by the glycosylase. APE1, together with hOGG1, relocates to speckles upon UV-radiation. Transcription blockage does not induce the hOGG1 localisation to the nuclear speckles suggesting that such mechanism is entirely dependent on UV damage. The reason why this relocation occurs only upon UV damage has not been elucidated yet (Campalans et al., 2006). A protein involved in cell cycle progression, B-Myb, is found to be phosphorylated and relocated to nuclear speckles upon UV
treatment (Werwein et al., 2013). Little is known about the role of B-Myb at the nuclear speckles. Upon damage, the cells arrest or slow down their progression in the cell cycle in order to repair the damage so, for B-Myb, the hypothesis is that the protein is sequestered at these organelles till the cell proliferation restarts (Werwein et al., 2013).

Other membrane-less organelles have been associated with UV-induced damage. The polymerase delta interacting protein 38 (PDIP38) is also found at PML nuclear bodies upon UV radiation (Wong et al., 2013). The PDIP38 relocation to PML bodies is important for the accumulation of alternative splice variants of MDM2 upon damage. Some of the MDM2 spliced variants have been associated with different human cancers so this role for PIPD38 in the PML bodies might suggest that there is a correlation between nuclear structures and different types of genotoxic stress (Wong et al., 2013).

1.7 Summary

Human cells daily face the threat of endogenous and exogenous damage (Lindahl et al., 1993). This damage leads cells to mutations, which can subsequently result in tumorigenesis and cancer. This is the reason why human cells have developed different DNA damage repair pathways (Ciccia et al., 2010). Many proteins have been identified in playing a role in these repair pathways but the majority of them yet has to be discovered. ATM is the main player in the repair of DNA DSB repair. We carried out a proteomic screening for ATM (Pessina et al., 2014) and identified new players in the DDR. We identified three human zinc finger proteins ZC3H8, ZC3H11A and ZC3H14 as new ATM-interacting proteins. These proteins are part of the C3H1-type family of zinc fingers and they are known for playing different roles in the RNA biogenesis (Hu et al., 2012;
Folco et al., 2013; Soucek et al., 2013). Although they have a role in RNA processing, nothing has yet been reported concerning a role for ZC3H8, ZC3H11A and ZC3H14 in the DDR.

Here we provide new evidence for these zinc finger proteins, as ATM-interacting partners, in playing new quite distinct roles in genome stability. Based on the data accumulated around the role of ZC3H14 in the DDR, we hypothesise that ZC3H14 might be required for MDC1 phosphorylation on specific residues that are required for the MDC1 activation/turnover and subsequent transduction of the signal to the downstream DDR factors. These new findings provide evidences for an important new player in the DDR pathway, ZC3H14, which acts above the repair pathway choice. Considering the role of ZC3H14 as a polyadenosine binding protein, here we hypothesise that this C3H1-type of zinc finger protein might be required for efficient MDC1 splicing and transduction of the DNA double strand break signal to the downstream DDR factors.
CHAPTER 2

Characterisation of C3H-type zinc finger containing protein
ZC3H8, ZC3H11A and ZC3H14

Keywords: ZC3H8, ZC3H11A, ZC3H14, membrane-less organelles, RNA biogenesis Nuclear Speckles, DNA damage, single strand DNA (ssDNA), replication stress, replication fork, phosphoRPA2 (S4/S8), R-loops
2.1 Summary

Zinc finger proteins are very abundant in biology. A stable isotope labelling with amino acids in cell culture (SILAC) screen carried out in our laboratory identified several proteins, previously unreported to be interacting with ATM in presence and absence of DNA damage. Among them, we identified three zinc finger-containing proteins ZC3H8, ZC3H11A and ZC3H14. Apart from their C3H-type zinc finger domains very little is known about these three zinc finger proteins and their structures. The first few experiments showed in this chapter were performed to identify which zinger finger protein between ZC3H8, ZC3H11A and ZC3H14 were to pursue for further investigation. We performed a primary sequence analysis of ZC3H8, ZC3H11A and ZC3H14 and found that they are mainly hydrophilic and contain alpha-helix and beta-turns. We show that ZC3H8 forms foci primarily in the nucleus, while some focal structures are also visible in the cytoplasm. ZC3H14 forms foci exclusively in the nucleus. Interestingly ZC3H14, but neither ZC3H8 nor ZC3H11A, localise to Nuclear Speckles a subnuclear compartment primarily associated with mRNA processing. The fact, that they interact with ATM strongly suggests a role for these proteins in the DNA damage response. Among these three zing finger proteins ZC3H8 was the only one which showed a lower score, in terms of its interaction with ATM, compared to ZC3H11A and ZC3H14 (data from the SILAC). This is the reason why we decided to focused on the role that ZC3H11A and ZC3H14 might play in the DDR. We so observed spontaneous damage upon ZC3H11A or ZC3H14 knock down and, when examined further, we found that their depletion induces accumulation of single-stranded DNA (ssDNA) coated by pRPA2 (S4/S8). Furthermore, we provide preliminary evidence that DNA-RNA hybrids (R-loops) are not properly resolved when ZC3H11A or ZC3H14 are depleted.
2.2 Highlights

- ZC3H8, ZC3H11A and ZC3H14 are mainly hydrophilic proteins organised in alpha-helix and beta-turn structures
- ZC3H8 forms foci primarily in the nucleus and it is found as chromatin bound protein and also in the cytoplasm
- ZC3H14 forms foci exclusively in the nucleus and it is found only in the chromatin fraction
- ZC3H14, but neither ZC3H8 nor ZC3H11A, is found at the Nuclear Speckles
- Depletion of ZC3H11A or ZC3H14 induces spontaneous damage
- Accumulation of ssDNA and pRPA2 (S4/S8) upon ZC3H11A or ZC3H14 depletion
- R-loops formation/resolution is affected upon ZC3H11A or ZC3H14 knock down
2.3 Introduction

In our cells, macromolecules such as proteins, DNA and RNA are organised in membrane-bound compartments and non-membrane-bound compartments. In organelles containing a membrane these macromolecules are accumulated within lipid membranes where the hydrophilic region will contain hydrophilic molecules within the aqueous phase. On the other hand, membrane-less organelles do not show a bilayer structure and thus are more dynamic.

Nuclear Speckles, Cajal Bodies and PML Bodies are membrane-less organelles localised in the nucleus. The Nuclear speckles are enriched in mRNA splicing factors and found in the interchromatin region in mammalian cells. These are dynamic structures, which can change their pattern during the cell cycle due to phosphorylation and dephosphorylation of the nuclear speckles proteins. This regulation of phosphorylation results in release of these proteins, which will then migrate to the transcription sites (Lamond and Spector, 2003). Although Cajal bodies have been linked to the small nuclear RNA (snRNA) and to the small nuclear ribonucleoprotein particles (snoRNPs) their biological roles still remain unclear (Cioce and Lamond, 2005). The role of the PML bodies has yet to be fully elucidated. Their number is influenced by the cell cycle (Eskiw et al., 2003) and they are also localised at the nucleolar caps in senescent cells (Condemine et al., 2007) suggesting a role in RNA metabolism.

Nuclear Speckles have been also linked to the DNA damage. One example of this is polymerase delta interacting protein 38 PDIP38 which was first identified as a polymerase δ and PCNA interacting protein (Liu et al., 2003). PDIP38 is found to re-localise to spliceosome/nuclear speckles upon UV damage and it is required for alternative splicing of MDM2 upon UV-irradiation (Wong et al., 2013).
Here we focus on three C3H-type zinc finger proteins ZC3H8, ZC3H11A and ZC3H14, previously reported to have roles in RNA biogenesis. ZC3H8 is a component of the little elongation complex (LEC) in human cells and plays a role in small nuclear RNA biogenesis (Hu et al., 2013). ZC3H11A is required for nuclear mRNA export together with PDIP3 (polymerase delta interacting protein 3) (Folco et al., 2012), while ZC3H14 binds RNA for regulation of poly(A) tail length during mRNA processing and is found at the nuclear speckles in N2A mouse cells (Kelly et al., 2014). ZC3H8 localises to PML bodies in Drosophila (Hu et al., 2013), but we could not confirm this in human cells. On the other hand, we found ZC3H14 localising to nuclear speckles in human cells. We were able to link ZC3H11A and ZC3H14 to the DNA damage response. Furthermore, we observed spontaneous DNA damage upon ZC3H11A and ZC3H14 depletion. Specifically, this spontaneous damage may result from replication stress as we found BrdU foci colocalising with pRPA2 (S4/S8) foci upon ZC3H11A and ZC3H14 depletion. Interestingly, the R-loops resolution was also affected upon depletion of these two zinc finger proteins.
2.4 Materials and Methods

2.4.1 Cell culture

U2OS and U2OS GFP-ZC3H14 were cultured at 37°C in a humidified atmosphere of 5% CO₂ using DMEM (Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum (Gibco) and 1% penicillin-streptomycin (Sigma-Aldrich).

hTERT RPE-1 cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ using DMEM-F12 (Sigma-Aldrich) media supplemented with 10% Fetal Bovine Serum (Gibco) and 1% penicillin-streptomycin (Sigma-Aldrich).

2.4.2 siRNA knockdown, plasmid transfection and plasmid generation

1.5x10⁵ U2OS cells were plated in 35mm dishes and 24 hours later transfected with siZC3H11A Ambion (60nM) and siZC3H14 Dharmacon pool (80nM), (negative control siRNA (Ambion) was used as a control) by using oligofectamine. 1ml of fresh media was added one day after transfection. The cells were then left for further 24h at 37°C before further analysis. See appendix 1 table 4 for the siRNAs sequences.

For DNA transfection 1.5x10⁵ cells were plated in a 35mm dish and transfected 24 hours later with DNA by using lipofectamine 2000 (1:3 DNA:lipofectamine ratio). 5 hours after transfection, the media was switched to media containing fetal bovine serum only and the cells were left for 48 hours at 37°C before further analysis. The plasmid pEGFP-C1-ZC3H14 was generated by cloning the ZC3H14 cDNA resistant to the siRNA (isoform 1) into the pEGFP-C1 plasmid. XhoI and HindIII restriction enzyme sites were used to clone the ZC3H14 cDNA into the pEGFP-C1 vector (see appendix 1 table 1 for primers sequences). The ZC3H14 cDNA resistant to the siRNA was generated by site-directed mutagenesis of the ZC3H14 cDNA (see
appendix 1 table 1 for primers sequences). 4 point mutations, G2040A, T2049C, A2184G and T2196A were inserted in the region of ZC3H14 siRNA 1 and 2 of the Dharmacon pool binding.

2.4.3 Immunofluorescence

For analysis of the different nuclear structures and γH2AX foci 1.5x10^5 U2OS cells or RPE1 cells were plated in a 35mm dish 24 hours prior fixation. Cells were fixed in 4% PFA diluted in 1X PBS for 10 minutes at RT. They were washed three times in 1ml of 1X PBS and then permeabilised for 2 minutes at RT in 0.125% Triton X-100 diluted in 1X PBS. Three more washes in 1X PBS were performed prior blocking in 1% BSA for 1h at 37°C. The cells were washed again three times with 1ml of 1X PBS and incubated for 1 hour at 37°C in 1% BSA containing the primary antibody. The same incubation was performed with secondary antibody. Vectashield mounting-DAPI was used to stain the nuclei.

For analysis of ssDNA and phosphorylation of RPA2 on Ser4/8 upon ZC3H11A and ZC3H14 knockdown, 1.5x10^5 U2OS cells were plated in a 35mm dish. 24 hours later siRNA transfection was performed as previously described (scramble RNA was used as a control). One day later BrdU was added at the final concentration of 500uM and the cells were left further 24 hours at 37°C. The BrdU was then washed off and, where specified, the cells were treated with 2mM Hydroxyurea for 2 hours. At this point the coverslips were collected and pre-extracted with CSK (100mM NaCl; 300mM Sucrose; 10mM Pipes ph 6.8; 3mM MgCl₂; 0.3% Triton X-100) for 10 min at RT, while remaining cells were lysed for 1 hr on ice by adding 100ul of 1X lysis buffer containing phosphatase/protease inhibitors and Benzonase (1/1000), followed by centrifugation at 14000 rpm for 10 minutes at 4°C. The supernatant was collected and proteins were quantified by Bradford. The lysates were then analysed by western blot. Antibodies used for the western
blot analysis were diluted in primary antibody buffer containing 2% BSA, 0.1% Sodium Azide, 25mM Tris-HCl pH 7.4, 150mM NaCl. Secondary antibodies were diluted in 5% milk TBST 1X. The coverslips were fixed for 10 minutes at RT in 4% PFA diluted in 1X PBS and permeabilised with 0.125% Triton X-100 for 2 minutes at RT. The cells were then blocked at 37°C for 1 hour in 1% BSA and subsequently incubated 1 hour at 37°C with 1% BSA containing anti-BrdU and anti pRPA2 S4/S8. Same incubation was performed for the secondary antibody. In between each of the above steps, cells were washed three times in 1ml of 1X PBS. Vectashield mounting-DAPI was used to stain the nuclei.

For the R-loops study 1x10^5 U2OS were plated in a 35mm dish and 24 hours later transfected with siRNAs as previously described. 24 hours after transfection, cells were transfected with pEGFP plasmid expressing RNAseH1 (empty vector was used as a control) as previously described. The coverslips were then recovered and processed and remaining cells were lysed and lysates were analysed as previously described. The coverslips were pre-extracted for 10 minutes at RT with CSK and fixed in 4% PFA diluted in 1X PBS for 10 minutes at RT followed by 2 minutes permeabilisation at RT in 0.3% Triton X-100 diluted in 1X PBS. The cells were then blocked in 5% BSA for 1 hr at RT, followed by 1 hr incubation at RT with primary antibody diluted in blocking buffer and 1 hr incubation at RT with secondary antibody diluted in blocking buffer. Each of the steps above was followed by three washes with 1 ml of 1X PBS. DAPI (Vectashield mounting medium) was used to stain the nuclei.

2.4.4 Chromatin Binding Assay

In 10cm dish 90% confluent U2OS cells were treated with 50uM Etoposide, where specified, for 1hr. The cells were then harvested and washed twice with 1 ml of ice-cold 1X PBS. Half of the
cells were resuspended in 400ul of 2X laemmli buffer (Input), while the other half was lysed in 200ul of CSK buffer I containing protease inhibitors (10mM Pipes pH 6.8; 100mM NaCl; 1mM EDTA; 300mM Sucrose; 1mM MgCl$_2$; 1mM DTT; 0.5% Triton X-100) for 15 minutes on ice. The lysates were then centrifuged at 1000g for 6 minutes at 4°C. The supernatant (S1 fraction) containing the triton-soluble proteins (not chromatin bound proteins) was further clarified by centrifugation at 12000g for 5 minutes and transferred to a fresh tube. The pellet was washed once in 200ul of CSK buffer I and then resuspended in 200ul of CSK buffer II (10mM Pipes pH 6.8; 50mM NaCl; 300mM Sucrose; 6mM MgCl$_2$; 1mM DTT) containing Benzonase (1/1000) and protease inhibitor followed by 40 minutes incubation on ice. 250mM Ammonium Sulfate was then added to the pellet and incubated for further 15 minutes at 37°C. The samples were then centrifuged at 12000g for 10 minutes at 4°C and the supernatant was recovered (S2 fraction) containing released chromatin-associated proteins. The protein concentration was measured by Bradford and the input with the S1 and S2 fractions were analysed by western blot. Antibodies used for the western blot analysis were diluted in primary antibody buffer containing 2% BSA, 0.1% Sodium Azide, 25mM Tris-HCl pH 7.4, 150mM NaCl. Secondary antibodies were diluted in 5% milk TBST 1X.
2.5 Results

2.5.1 Primary analysis of the human ZC3H8, ZC3H11A and ZC3H14 protein sequences

Little is known about human C3H type Zinc finger ZC3H8, ZC3H11A and ZC3H14 proteins. We know, from the Uniprot database, that ZC3H8 (Q8N5P1) has three C3H Zinc finger domains localised in the C-terminus of the protein. ZC3H11A (O75152) has three C3H Zinc finger domain localised in the N-terminus, while ZC3H14 (Q6PJT7) has five C3H Zinc finger domain, two in its C-terminus and three centrally located. Additionally, ZC3H14 has a Piwi-like domain spanning from residue 172 to residue 199 (Figure 2.1). All three zinc finger proteins present several SQ/TQ sites spanned throughout the entire protein sequence, with those of ZC3H11A being mainly found in a C-terminal cluster, while for ZC3H8 and ZC3H14 the SQ/TQ sites are primarily N-terminally located.

Figure 2.1: Human ZC3H8, ZC3H11A and ZC3H14 protein structures. The ZC3H8, ZC3H11A and ZC3H14 protein sequences were obtained from the Uniprot database. The coloured boxes in the sequence highlight the C3H1 Zinc finger domains and the Piwi-like. The different colours for the Znf domains represents how conserved these domains are with in the three proteins. The yellow stars indicate the SQ/TQ sites.
The protein sequence of each C3H-type zinc finger protein obtained from the Uniport website was used in the ExPASy bioinformatic website to obtain more information about the secondary structure of these proteins. By using the ProtScale tool, we were able to identify predicted alpha-helices and beta-turns. The ProtScale tool uses the Deleage-Roux apha-helicity and beta-turincity plot. In this method each amino acid has a score. The score for the amino acid changes between the alpha-helix and the beta-turn plot. In the plot the amino acid position is on the X-axis while the amino acid score is on the Y-axis. A cut-off score is taken at 0.99. Using the ZC3H8 Uniprot ID, we obtained the apha-helicity and beta-turincity plot (Figure 2.2). ZC3H8 mainly contains an alpha-helical structure with beta-turns in aa145 to 155.
**Figure 2.2: ZC3H8 alpha-helix and beta-turn structures.** The Deleage and Roux plot for the alpha-helix (A) and the beta-turn (B) uses a cut-off of 0.99. The amino acid position is reported on the X-axis while the score is on the Y-axis. The zinc finger domains for ZC3H8 are localised from position 191 to position 271 of the protein sequence.

ZC3H11A consists of an equal distribution of alpha-helices and beta-turns throughout the entire protein sequence (Figure 2.3). We identified some alpha-helix spikes corresponding to the
central region of the protein (~350th amino acid) and the C-terminus, and a couple of spikes for the beta-turns around position 220 and 300.

**Figure 2.3: ZC3H11A alpha-helix and beta-turn structures.** The Deleage and Roux plot for the alpha-helix (A) and the beta-turn (B) uses a cut-off of 0.99. The amino acid position is reported on the X-axis while the score is on the Y-axis. The zinc finger domains for ZC3H11A are localised from position 2 to position 86 of the protein sequence.
Something similar is observed in the case of ZC3H14 (Figure 2.3). ZC3H14 contains an equal distribution of alpha-helix and beta-turn structures, throughout the entire protein.

**Figure 2.4: ZC3H14 alpha-helix and beta-turn structures.** The Deleage and Roux plot for the alpha-helix (A) and the beta-turn (B) uses a cut-off of 0.99. The amino acid position is reported on the X-axis while the score is on the Y-axis. The zinc finger domains for ZC3H14 are localised from position 595 to position 719 of the protein sequence.
The ExPASy bioinformatic website allows to investigate the total hydrophobicity/hydrophilicity of a protein. We used the Eisenberg et al plot, which assigns a score to each amino acid. A positive score is given to the hydrophobic amino acid, while a negative score is given to the hydrophilic ones. The output is a plot (Hphob/Eisenberg plot) showing the position of the amino acids on the X-axis and the score on the Y-axis. The Hphob/Eisenberg et al. plots for ZC3H8, ZC3H11A and ZC3H14 shows many spikes falling in the negative region of the plot suggesting that these proteins are mainly hydrophilic (Figure 2.5 A-B-C). Apart from their zinc finger domains, ZC3H8, ZC3H11A and ZC3H14 are largely unstructured proteins since no other domains have been identified. This is the reason why they are primarily hydrophilic.

![Hphob/Eisenberg plots for ZC3H8, ZC3H11A and ZC3H14.](image)

**Figure 2.5**: Hphob/Eisenberg plots for ZC3H8, ZC3H11A and ZC3H14. The Eisenberg method to determine whether a protein is hydrophilic or hydrophobic is based on giving a positive score to hydrophobic residues and a negative score to the hydrophilic residues. The position of the amino acid is then plotted on the X-axis, while the score is on the Y-axis. A) ZC3H8. B) ZC3H11A. C) ZC3H14.
2.5.2 ZC3H8 and ZC3H14 are nuclear proteins found primarily in the chromatin fraction

We asked whether ZC3H8 and ZC3H14 are nuclear or cytoplasmic. Note that a previous PhD student in our laboratory showed that ZC3H11A forms foci exclusively in the nucleus and is a chromatin bound protein (Danielle Hamilton, PhD thesis 2013).

We carried out IF for ZC3H8 and ZC3H14 in U2OS cells and observed similar patterns for both proteins. Specifically, ZC3H14 is primarily in the nucleus (Figure 2.6 B), while ZC3H8 is primarily in the nucleus with some small foci apparent in the cytoplasm (Figure 2.6 A). Also, the two zinc finger proteins show a slightly different nuclear pattern. ZC3H14 foci are bigger in size compared to ZC3H8 foci.

![Image of IF for ZC3H8 and ZC3H14 in U2OS cells](image)

**Figure 2.6: Nuclear localisation of ZC3H8 and ZC3H14.** U2OS cells were grown on a coverslip and subsequently stained for ZC3H8 (Ab) and ZC3H14 (Ab) respectively. DAPI was used to stain the nuclei. The experiment was repeated twice. Images were acquired with an Olympus IX73 inverted microscope (DV Core, Imsol system). A) ZC3H8. B) ZC3H14.
We next decided to investigate if ZC3H8 and ZC3H14 were chromatin binding proteins as previously described for ZC3H11A (Danielle Hamilton, PhD thesis 2013). To do so we carried out a chromatin binding assay (Figure 2.7) and observed a strong band for both proteins appearing in the chromatin bound fraction (S2). In case of ZC3H8, a fainter band was visible in the non-chromatin bound proteins fraction (S1). The faint band could correspond to the ZC3H8 foci around the nucleus observed in our immunofluorescence experiments. In addition, ZC3H8 and ZC3H14 localisation did not change in presence of Etoposide, which induces DSB.
Lyse the cells in CSK buffer I for 15min on ice

Spin down at 1000 rpm for 6min at 4°C

The supernatant (S1 fraction) is recovered and loaded on to an SDS-page gel. The pellet is resuspended in CSK buffer II and incubated for 40min on ice

Add 250mM of Ammonium Sulfate and incubate further 15min at 37°C

Spin down at 12000rpm for 10min at 4°C. Recover the supernatant (S2 fraction) and load it on an SDS-page gel

B

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Figure 2.7: ZC3H8 and ZC3H14 are chromatin bound proteins. A) Schematic of experimental protocol. B) U2OS cells were treated with 50μM of Etoposide for 1hr. The input corresponds to the untreated cells, with S1 being the cytoplasmic fraction and the S2 the nuclear. Anti-Tubulin and anti-H3 antibodies were used as controls. The middle band detected with anti-ZC3H14 antibody is a non-specific band (ns). See Figure 2.12 B.

The ZC3H8 and ZC3H14 antibodies, also, showed a different pattern. ZC3H8, which size is 38 KDa, came up as a single band at around 40 KDa. In case of ZC3H14 we could see three bands, very close to each other, coming up between 64 and 98 KDa. These bands could correspond to different ZC3H14 spliced variants as previously described by Leung SW et al., 2009 as they
migrate close to the size expected for their specific sequence 82 kDa for isoform 1 and 64 kDa for isoforms 2 and 3.

We were also confident of the results obtained since the band for the Tubulin protein was visible only in the cytoplasmic fraction (S1) while the band for the Histone H3 was visible only in the nuclear fraction (S2) suggesting that the fractionation of chromatin from non-chromatin bound proteins was very efficient.

2.5.3 ZC3H14, but neither ZC3H8 nor ZC3H11A, co-localises with the Nuclear Speckles

It is known that ZC3H8 is found in the Cajal bodies in Drosophila (Shilatifard et al., 2013), while ZC3H14 is found in the Nuclear Speckles in N2A mouse cells (Corbett et al., 2014). ZC3H11A co-localisation remains to be determined. To determine whether ZC3H8 and ZC3H14 could be found in the Cajal bodies or PML or nuclear speckles in human cells we performed IF analysis. We used Coilin or PML or SC35 as markers of Cajal bodies, PML bodies and nuclear speckles respectively. ZC3H8, ZC3H11A and ZC3H14 were not found in the Cajal bodies using two different human cell lines, U2OS and RPE1 (Figure 2.8 A-B). Furthermore, these three zinc finger proteins did not localise to PML bodies in either cell lines. Furthermore neither ZC3H8 nor ZC3H11A or ZC3H14 co-localised with the PML bodies in human U2OS and RPE1 cell lines (Figure 2.9 A-B).
Figure 2.8: ZC3H8, ZC3H11A and ZC3H14 do not co-localise with Cajal Bodies. U2OS (A) and RPE1 (B) were stained with ZC3H8, ZC3H11A, ZC3H14 and Coilin respectively. DAPI was used to stain the nuclei. Images were acquired with an Olympus IX73 inverted microscope (DV Core, Imsol system).
Figure 2.9: ZC3H8, ZC3H11A and ZC3H14 do not co-localise with PML Bodies. U2OS (A) and RPE1 (B) were stained with ZC3H8, ZC3H11A, ZC3H14 and PML, respectively. DAPI was used to stain the nuclei. Images were acquired with an Olympus IX73 inverted microscope (DV Core, Imsol system).

Similarly, to mouse N2A cells (Kelly et al., 2014) we observed co-localisation of ZC3H14 with the nuclear speckles marker, SC35, in both U2OS and RPE1 human cells(Figure 2.10 A-B). We could not see any co-localisation between the nuclear speckles marker SC35 and ZC3H8 or
ZC3H11A in both U2OS and RPE1 cells indicating that neither of these proteins localises to nuclear speckles, PML bodies, nor Cajal bodies (Figure 2.10 A-B). The localisation of ZC3H14 in Nuclear speckles was also confirmed by examining the GFP tagged ZC3H14 localisation in U2OS cells stabling expressing the ZC3H14 siRNA resistant construct (Figure 2.11). Importantly, knockdown of ZC3H14 abolished ZC3H14 focal localisation, indicating the specificity of the antibody used.
Figure 2.10: ZC3H14, but neither ZC3H8 nor ZC3H11A, co-localises with the nuclear speckles. U2OS (A) and RPE1 (B) were stained with ZC3H8, ZC3H11A, ZC3H14 and SC35 respectively. DAPI was used to stain the nuclei. Images were acquired with an Olympus IX73 inverted microscope (DV Core, Imsol system).
Figure 2.11: GFP tagged ZC3H14 co-localises with nuclear speckles. U2OS and U2OS-GFP-ZC3H14 were transfected with siRNA against ZC3H14, where the GFP-ZC3H14 construct is resistant to the siRNA. They were stained for ZC3H14 and SC35. Images were acquired with an Olympus IX73 inverted microscope (Imvisage, Imsol system).

2.5.4 Depletion of ZC3H11A or ZC3H14 induces spontaneous damage

The ATM proteomic screen carried out in our laboratory allowed us to identify ZC3H11A as an ATM interactor (Pessina et al., 2014). In this proteomic screen ZC3H14 had a lower interaction score than ZC3H11A but was stronger than ZC3H8. Based on these scores we decided to focus on ZC3H11A and ZC3H14. ZC3H11A depletion induces spontaneous damage in U2OS cells (Danielle Hamilton, PhD thesis 2013). Based on this, we asked whether ZC3H14 depletion had a similar phenotype. Upon DNA damage induced by ionising radiation we observed γH2AX foci after IR throughout all the samples but some of these foci were also visible upon ZC3H11A or ZC3H14 knock down in undamaged cells, suggesting that depleting these two proteins might have resulted in spontaneous damage (Figure 2.12 A). In about 7% of ZC3H11A depleted cells γH2AX foci were observed in the absence of damage, while 12% of ZC3H14 depleted cells contained γH2AX foci in the absence of damage compared to the control.
Figure 2.12: Spontaneous damage upon ZC3H11A or ZC3H14 knockdown. A) U2OS cells were transfected with siRNA against ZC3H11A and ZC3H14 respectively. 48 hours later they were irradiated at 3Gy and let recover for 1 hour at 37°C. They were subsequently stained for γH2AX S139. DAPI was used to stain the nuclei. The percentage showed for the siZC3H11A (-IR) sample and for the siZC3H14 (-IR) sample represents the percentage of cells with more than 5 γH2AX foci. Images were acquired with an Olympus IX73 inverted microscope (DV Core, Imsol system). B) Corresponding knock down of ZC3H11A and ZC3H14.
To make sure that such phenotype was due to the ZC3H11A or ZC3H14 knock downs the lysates were analysed by western blot (Figure 2.12 B). The ZC3H11A signal appeared as the expected single band of 98 kDa, while of the three bands we previously observed from an anti-ZC3H14 blot (Figure 2.7) we could assess that the middle band was likely to be an unspecific band. The top and the bottom band probably corresponded to the ZC3H14 spliced variants 1 and 2 as previously reported by Leung S. W. et al. (Leung et al., 2009).

2.5.5 Accumulation of single-stranded DNA upon ZC3H11A or ZC3H14 knockdown

Spontaneous damage upon ZC3H11A or ZC3H14 knock down could be an indication that depletion of these two C3H-type zinc finger proteins causes replication stress. Replication stress can be a result of stalled replication fork progression when such forks encounter difficult to replicate regions of the genome. Upon replication fork stalling, ssDNA accumulates and triggers recruitment of RPA2 and the consequent phosphorylation of the RPA2 subunit on residue S4 and S8.

The hydroxyurea (HU) blocks the DNA replication thereby increasing ssDNA. As expected, in the positive control 90% of the cells showed BrdU foci overlapping with pRPA2 (S4/S8) foci (Figure 2.13 A). Cells had an accumulation of ssDNA upon depletion of ZC3H11A and ZC3H14 compared to control cells. In the siZC3H11A treated cells about 5% of the population showed BrdU foci colocalising with the pRPA2 (S4/S8) foci, while in the siZC3H14 treated cells about 12% showed accumulation of ssDNA. In ZC3H14 depleted cells we could observe a more severe phenotype in terms of BrdU foci compared to ZC3H11A depleted cells.
**Figure 2.13: Accumulation of ssDNA upon ZC3H11A and ZC3H14 knock down.**

**A**

U2OS cells were knocked down for ZC3H11A and ZC3H14 respectively (siGFP was used as a control). Cells were grown for 24 hours in presence of BrdU, which was then removed and HU was added for 2 hours at final concentration of 2mM. Cells were then stained for BrdU and pRPA2 S4/S8. DAPI was used to stain the nuclei. The percentages on the merge channel represent the percentage of cells showing BrdU and pRPA2 (S4/S8) foci. Images were acquired with an Olympus IX73 inverted microscope (DV Core, Imsol system).

**B**

Western blot analysis of pRPA2 S4/S8. U2OS cells were knocked down for ZC3H11A and ZC3H14 respectively (siGFP was used as a control). 2 mM Hydroxyurea was added, as indicated, for 2 hours.
Surprisingly, when we analysed the lysates by western blot we could not detect any pRPA2 S4/S8 signal in cells depleted for ZC3H11A or ZC3H14 compared to HU treated cells (Figure 2.13 B). The western blot analysis is less sensitive than the immunofluorescence, the 5% and 12% of cells showing pRPA2 S4/S8 foci when ZC3H11A or ZC3H14 are depleted might be too little to be detected by western blot. On the other hand, we can’t exclude the possibility that 5% and 12% we observed in term of cells showing pRPA (S4/S8) foci when ZC3H11A or ZC3H14 are depleted might have been apoptotic cells, although this is still a high percentage if compared to siCTL.

2.5.6 R-loops formation is affected upon ZC3H11A or ZC3H14 depletion

It is known that ZC3H11A is involved in mRNA export (Folco et al., 2012), while ZC3H14 plays a role in mRNA processing particularly in controlling the poly(A) tail length (Kelly et al., 2014. Kelly et al., 2017). Replication and transcription occur simultaneously during S-phase. At the same time, newly RNA molecules can be bound by mRNA processing factors. It is known that if the DNA replication and transcription proceed in the same direction the DNA-RNA hybrids, if not resolved quickly, can be an obstacle for the replication which will subsequently stop (García-Muse and Aguilera, 2016).

Since ZC3H11A and ZC3H14 are both mRNA binding proteins involved in processing new mRNA (Folco et al., 2012; Kelly et al., 2014) it is possible that their loss could lead to an accumulation of DNA-RNA hybrids, which in turn, could impede progression of the replication fork. We depleted ZC3H11A or ZC3H14 and stained cells with the anti-S9.6 an antibody that recognises DNA-RNA hybrids (Figure 2.14). In control cells, we could observe signal consistent with R-loop accumulation in the nucleoli. The R-Loop signal was removed upon expression of RNaseH1 enzyme, which disrupts RNA-DNA hybrids, suggesting that the signal we observed
indeed corresponds to R-loops. Loss of ZC3H11A resulted in reduction of the S9.6 signal suggesting that ZC3H11A might be required for efficient formation of R-loops. On the other hand, when ZC3H14 was depleted the S9.6 signal in the nucleoli was more intense with larger nucleoli relative to the control. Thus, there might be a defect in R-loop resolution when ZC3H14 is absent.

**Figure 2.14: R-loop formation is affected upon ZC3H11A and ZC3H14 depletion.** U2OS were transfected with ZC3H11A or ZC3H14, respectively. 24 hours later the RNAseH1 enzyme was expressed as indicated. The cells were subsequently stained for S9.6 and DAPI was used to stain the nuclei. Images were acquired with an Olympus IX73 inverted microscope (DV Core, Imsol system). The percentages indicate the amount of cells showing the phenotype.
2.6 Discussion

There is newly evidence for a role for C3H-type zinc finger proteins in mRNA processing but little is known about any potential function in the DNA damage response pathways. Our identification of them as ATM-interacting proteins suggests a function for ZC3H8, ZC3H11A and ZC3H14 in the DDR.

We previously identified ZC3H11A as chromatin bound protein forming foci exclusively in the nucleus (Danielle Hamilton, PhD thesis 2013). Here, we could confirm that ZC3H8 and ZC3H14 are chromatin bound proteins with ZC3H14 being exclusively in the chromatin bound proteins fraction and ZC3H8 being partially in the non-chromatin bound proteins fraction. ZC3H14 is found in Nuclear Speckles, which are not chromatin structure. Although, it could be possible that nuclear speckles are disrupted during chromatin fractionation or not all ZC3H14 is found at nuclear speckles but a small amount of the ZC3H14 protein might be at chromatin. ZC3H8 forms foci primarily in the nucleus and some foci are visible in the cytoplasm while ZC3H14 forms foci exclusively in the nucleus. The cytoplasmic ZC3H8 foci could result from additional structures in the cytoplasm or could merely result from non-specific background. Previously it has been shown that ZC3H14 is found at the nuclear speckles in neuronal N2A cells (Leung et al., 2009). Here we could confirm the localisation to nuclear speckles for ZC3H14, and demonstrate that neither ZC3H11A nor ZC3H8 localise to nuclear speckles in human cells. In Drosophila ZC3H8 has been reported to localise to the Cajal bodies (Shilatifard et al., 2013) but we could not see ZC3H8 colocalising with this nuclear structure in human cells. This could be due to a different role that the human ZC3H8 might play compared to the ZC3H8 Drosophila orthologues.

We focused on the possible role of ZC3H11A and ZC3H14 in the DNA damage response suggested by their identification as ATM-interacting partners but, excluded ZC3H8 from further
analysis given its relatively lower interaction score in our proteomic studies. In previous unpublished work we obtained evidence that ZC3H11A functions in the DDR; in particular U2OS cells are sensitive to ionising radiation (IR) upon ZC3H11A depletion and persistent γH2AX foci are visible upon ZC3H11A depletion after IR (Danielle Hamilton, PhD thesis 2013). Before damage, the average number of γH2AX foci per cell in the ZC3H11A depleted population looks higher than the control cells. We confirmed this data for ZC3H11A and, while we could see that 7% of the ZC3H11A knocked down population was showing spontaneous damage compare to the control cells, about 12% of the ZC3H14 knocked down population showed γH2AX foci compare to the control cells. This suggests that ZC3H11A and ZC3H14 are required for genome integrity. Although the number of U2OS cells showing spontaneous damage when ZC3H11A or ZC3H14 are depleted is quite high we cannot be sure about this phenotype. This spontaneous damage could be an off-target effect of the actual siRNAs. A rescue experiment for ZC3H11A and ZC3H14 would confirm the data above and exclude the off-target effect hypothesis.

Depletion of ZC3H11A or ZC3H14 also leads to an accumulation of BrdU and pRPA2 (S4/S8) foci. This might suggest the presence of replication stress upon ZC3H11A or ZC3H14 resulting depletion. This could explain why we see such an accumulation of spontaneous damage when we deplete ZC3H11A or ZC3H14. The percentages of cells showing BrdU and pRPA2 (S4/S8) foci was higher in the ZC3H14 depleted population compared to the ZC3H11A depleted one suggesting that, ZC3H14 might play a major role in the DDR compared to the ZC3H11A role. Phosphorylation of RPA2 on S4/S8 was detectable by western blot in U2OS treated with HU but not in control cells or cells knocked down for ZC3H11A or ZC3H14. This might suggest that even though we could see 5% and 12% of cells showing pRPA2 S4/S8 foci when ZC3H11A or ZC3H14 are knocked down by immunofluorescence, these percentages might have been very low to detect the pRPA S4/S8 signal by western blot. Another hypothesis is that the 5% and 12%
we observed in terms of pRPA2 S4/S8 foci when we deplete ZC3H11A or ZC3H14 are apoptotic cells, although these percentages are still high compare to the 0% observed in the siCTL sample. Further analysis is required to investigate whether ZC3H11A and ZC3H14 have a role in replication and resection.

Next question was to try to understand why we were observe replication stress upon ZC3H11A and ZC3H14 depletion. The DNA replication and transcription can occur simultaneously leading to the DNA-RNA hybrids formation (R-loops), which if not properly resolved can cause a block in the DNA replication (García-Muse and Aguilera, 2016). ZC3H11A and ZC3H14 are found to be involved in mRNA processing (Folco et al., 2012; Kelly et al., 2014) so we wondered whether we could observe a defect in terms of R-loops resolution upon ZC3H11A or ZC3H14 depletion. Our data suggests opposite phenotypes upon ZC3H11A or ZC3H14 depletion. The depletion of ZC3H14 resulted in bigger and brighter R-loops foci in the nucleoli while, in case of ZC3H11A depletion, resulted in reduction of the R-loop signal. We do not really understand what the role for ZC3H11A and ZC3H14 might be in terms of replication but this data suggests that these two zinc finger proteins might play more separate roles in cells.
CHAPTER 3

ZC3H14 is required for genome integrity functioning between MDC1 and RNF8 to regulate the recruitment of repair and signalling factors to DSBs

Keywords: Genome integrity, DNA damage response, DSBs, Double strand break repair, HR, NHEJ, ubiquitination, 53BP1, BRCA1, FK2, RNF8, RNF168, MDC1.
3.1 Summary

The relatively poorly described ZC3H14 protein has been assigned functions as an RNA binding protein. In neuronal cells from mice ZC3H14 co-localises with poly(A) RNA in neuronal cell bodies indicating brain-specific function (Pak et al., 2011). The budding yeast ortholog of ZC3H14, known as Nab2 was shown to be involved in mRNA maturation (Soucek et al., 2016). Analysis of ZC3H14 function in a neuronal cell line (N2A cells) as well as in vivo complementation studies in the Drosophila model system identify a role for ZC3H14 in proper control of poly(A) tail length in neuronal cells (Kelly et al., 2014). Consistent with the presence of ZC3H14 mRNA transcripts in the human central nervous system and its role in neuronal mRNA maturation, a mutation in the ZC3H14 gene causes a human brain disorder which leads to intellectual disability (Pak et al., 2011).

Here we show a novel role for ZC3H14 in the DNA damage response in cultured human cells. First identified as an ATM-interacting protein (Pessina and Lowndes, 2013), we confirmed the interaction between ZC3H14 and ATM. We also observed that U2OS cells were sensitive to ionising irradiation upon ZC3H14 depletion. Persistent damage was detectable upon ZC3H14 knock down as we could observe persistent γH2AX foci, as well as pATM S1981 foci upon depletion of this zinc finger protein relative to control cells. Preliminary data suggests defect in the NHEJ repair pathway but not in the HR repair pathway when ZC3H14 is depleted compare to control cells. Although, immunofluorescence data strongly suggests that ZC3H14 acts above DNA repair pathway choice since ZC3H14 depletion affects both 53BP1 IRIF and BRCA1 IRIF. Depletion of ZC3H14 also caused defective FK2 IRIFs, RNF168 IRIFs and RNF8 IRIFs but not MDC1 IRIFs. When we immunoprecipitated MDC1 we could detect an interaction between MDC1 and tagged ZC3H14. ZC3H14 depletion also leads to increased MDC1 protein levels.
with the MDC1 lower band being more abundant than the upper MDC1 band in cells depleted for ZC3H14 compared to control cells. Surprisingly, ZC3H14 does not appear to regulate the MDC1-RNF8 interaction. These results suggest that ZC3H14 is acting upstream of pathway choice.

3.2 Highlights

- ZC3H14 interacts with ATM
- Persistent γH2AX foci and pATM (S1981) foci upon ZC3H14 depletion
- ZC3H14 cannot be detected at sites of DNA DSB
- Defective 53BP1 and RIF IRIFs upon ZC3H14 depletion
- Defective BRCA1 in non S-phase cells upon ZC3H14 depletion
- Defective damage-dependent ubiquitination upon ZC3H14 depletion
- Defective RNF8 and RNF168 focal recruitment upon ZC3H14 knock down
- ZC3H14 depletion does not impact upon the levelling of several DDR factors
- Normal MDC1 recruitment to DSBs upon ZC3H14 depletion
- ZC3H14 depletion leads to increased total MDC1 protein levels
- ZC3H14 interacts with MDC1 but it is not required for the MDC1-RNF8 interaction
- U2OS are sensitive to IR and ICRF193, but not to Olaparib, upon ZC3H14 depletion
- ZC3H14 is involved in the NHEJ repair pathway but not in the HR repair pathway
3.3 Introduction

Our genome integrity is threatened daily. These threats can bring a change into our genome sequence. During DNA replication a nucleotide insertion or deletion might occur and this will bring a change in the genome sequence. DSB can arise by exposure to an external agent such as ionising radiation (IR) or it can arise when DNA replication fork encounters an unrepaired DNA-lesion (Pfeiffer et al., 2000). Such changes are detected in our cells by the DNA damage response proteins, which will activate a cascade of events necessary to initiate the repair (Marechal A. et al., 2013). The two major DNA DSBs repair pathways are homologous recombination (HR) and the error prone nonhomologous end-joining recombination (NHEJ). In the NHEJ 5’ DNA ends are blocked by the Ku70-Ku80 heterodimer and resection is inhibited. NHEJ promotes directly ligation of blunt DNA ends in an error prone manner, often resulting in small deletions, insertions or substitutions at the break site (Chapman et al., 2012). In contrast, HR is largely error free and is triggered when the resection is initiated. The resection generates 3’ ssDNA ends which are bind by the RAD51 and the RPA2 protein. This structure invades homologous duplex DNA which is used as a template for the repair (Chapman et al., 2012).

Following DSB the histone variant H2AX is phosphorylated on S139 in the proximity of the break (Rogakou et al, 1998). This phosphorylation event spreads a few megabases from each side of the break and functions as platform for the recruitment of the subsequent DDR factors necessary for the repair. The MRN complex is retained at sites of damage by its direct interaction with MDC1 via NBS1 (Chapman and Jackson, 2008) (Figure 1,2). MDC1 is constitutively phosphorylate by Casein Kinase 2 (CK2). This kinase phosphorylates the SDTD consensus sequence of MDC1 promoting so the direct binding of MDC1 with NBS1 via its FHA domain (Chapman and Jackson, 2008). MDC1 is also phosphorylated by ATM (Goldberg et al., 2003;
Kolas et al., 2007). ATM preferentially phosphorylates MDC1 on the TQXF consensus sequence. This phosphorylation event is necessary for the recruitment of the ubiquitin E3 ligase RNF8, which binds the pT719 residue of MDC1 via its FHA domain (Kolas et al., 2007). RNF8, together with UBC-13, an E2 ubiquitin-conjugating enzyme, will initiate the formation of ubiquitin chains on Lysine-63 of the linker-histone H1 (Thorslund et al., 2015). This ubiquitination recruits RNF168 to the site of damage, which then initiates the ubiquitination on Lysine-63, Lysine 13 and 15 of the histone variant H2A (Doil et al., 2009; Mattiroli et al., 2012).

The H2AX ubiquitination is an important event necessary for the recruitment of 53BP1 and BRCA1 and the subsequent DNA DSB repair. 53BP1 is recruited to sites of damage by its interaction with the H4K20me2 and H2AK13/K15 via the Tudor domain and the UDR, respectively (Zgheib et al., 2008; Fradet-Turcotte et al., 2013). 53BP1 is subsequently phosphorylated by ATM and binds RIF1 (Chapman et al., 2013). RIF1 and 53BP1 cooperate to block the DNA DSB resection and initiate the NHEJ in G1 cells. The block of the resection is mainly provided by the 53BP1-RIF1 dependent recruitment of the shieldin complex (Noordermeer et al., 2018). This complex encounters four subunits, C20orf196 (SHLD1), FAM35A (SHLD2), CTC-534A2.2 (SHLD3) and REV7, where the SHLD2 binds the ssDNA and protects the DNA ends to mediate 53BP1-dependent DNA repair. Loss of the Shieldin complex results in impaired non homologous end-joining. In BRCA1-deficient cells shieldin promotes DNA DSB end-joining by restricting DSB resection and to inhibit homologous recombination by antagonizing BRCA2/RAD51 loading (Dev et al., 2018). 53BP1 regulates the NHEJ class-switch recombination and NHEJ long range V(D)J recombination. REV7 interacts with 53BP1 to promote non-homologous end joining during class-switch recombination, but REV7 is not required for 53BP1-dependent V(D)J recombination (Ghezraoui et al., 2018). The NHEJ repair pathway is antagonised by BRCA1 in S-phase. The BRCA1-BARD1 complex is
brought to site of damage by RAP80 (Sobhian et al., 2007). BRCA1 interacts with CtIP which is responsible for the initiation of 3’ DNA ends resection and subsequent recruitment of RPA2 on ssDNA promoting DSB repair via HR (Sartori et al., 2007; Hoa et al., 2015).

Here we report new evidences for a new player in the DDR, ZC3H14. ZC3H14 is found to interact with ATM. Depletion of ZC3H14 results in increased IR sensitivity in U2OS and RPE1 cells suggesting an important role for this zinc finger protein in the DNA damage response pathway. Here we investigated on the role that ZC3H14 might play in the DDR and where, in this pathway, this new zinc finger protein might act.
3.4 Materials and Methods

3.4.1 Cell culture

U2OS, U2OS-DSB reporter, U2OS ZC3H14 KO and HEK293-T cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ using DMEM (Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum (Lonza) and 1% penicillin-streptomycin (Sigma-Aldrich).

RPE-1 hTERT cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ using DMEM F12 (Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum (Lonza) and 1% penicillin-streptomycin (Sigma-Aldrich).

3.4.2 Plasmids and siRNA

Flag-tagged ZC3H14 was generated by cloning the ZC3H14 cDNA into the pCMV2C plasmid by Dr. Murilo T.D. Bueno. The plasmid pEGFP-C1-ZC3H14-WTr was generated by cloning the ZC3H14 cDNA resistant to the siRNA (isoform 1), by using Xhol and HindIII restriction sites, into the pEGFP-C1 plasmid. The ZC3H14 cDNA resistant to the siRNA was generated by site-directed mutagenesis of the ZC3H14 cDNA. 4 point mutations, G2040A, T2049C, A2184G and T2196A were inserted in the region of ZC3H14 siRNA 1 and 2 of the Dharmacon pool binding.

The ZC3H14 mutant lacking of the Piwi-like domain (pEGFP-C1-ZC3H14ΔPiwi) was generated by Fusion PCR of the ZC3H14r cDNA (see appendix 1 table 1 for primers sequence). The ZC3H14 cDNA lacking of the Piwi-like domain (aa172-199) was then cloned, using XhoI and HindII restriction sites, into the pEGFP-C1 plasmid. The ZC3H14 mutant lacking of the c-terminus of the protein where the five Zinc finger domains are located (pEGFP-C1-ZC3H14ΔZF) was generated by PCR of the ZC3H14r cDNA (see appendix 1 table 1 for primers sequence).
sequence). The PCR product lacking of the region coding for the c-terminus (aa595-736) was then cloned, using XhoI and HindIII restriction sites, into the pEGFP-C1 plasmid. Plasmid transfection was performed using Lipofectamine 2000 (1:3 DNA:lipofectamine ratio). 5 hours after transfection, the media was changed to media containing fetal bovine serum only and the cells were left for 48 hours at 37°C before further analysis. Cells were transfected with siRNAs by using oligofectamine 24 hours after plating. Negative control siRNA (Ambion AM4611) and siGFP were used as controls. A pool of four siRNAs for ZC3H14 was used at final concentration of 80nM. The DNA-PK siRNA was used at the final concentration of 50nM, while the siRNAs for BRCA1 and DNA ligase 4 were used at the final concentration of 40nM. See appendix 1 table 4 for the details about the siRNAs sequences.

3.4.3 Immunoprecipitation

For the WT GFP-ZC3H14/ATM CoIP, HEK293-T cells were plated in a 10cm dish. 24 hours later, the cells were transfected with WT ZC3H14 (pEGFP-C1 was used as a control). 48 hours later the cells were irradiated (3Gy) and let recover for 1h at 37°C. They were then harvested and lysed in 1ml of 1X lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 0.5% NP40, 10% Glycerol) containing phosphatase/protease inhibitors and Benzonase (1/1000), followed by centrifugation at 14000 rpm for 10 minutes at 4°C. 5mg of crude cell extract was incubated with 5ug of mouse anti-GFP antibody (Roche 11814460001) for 2h under rotation at 4°C. 60ul of Sepharose G-beads 4 Fast-flow (Sigma), which were washed twice in 200ul of 1X lysis buffer w/o benzonase, were added to the crude cell extract/antibody mix and incubated for further 2 hours under rotation at 4°C. The flow through was recovered and the beads were washed three times with 200ul of 1X lysis buffer (tubes were inverted upside down for 3 times in each wash).
The beads were resuspended in 10ul of 2X laemmli buffer, boiled at 95°C and span down at max speed. The samples were then analysed by SDS-page gel. The proteins were transferred to a nitrocellulose membrane at 100mA ON at 4°C. The membrane was blocked in 5% milk TBST 1X for 1 hour at RT and then incubated ON at 4°C with primary antibodies. The day after the membrane was washed for 30 minutes in TBST 1X at RT, incubated with the secondary antibodies for 1 hour at RT, washed again for 30 minutes with TBST 1X prior to X-Ray films analysis. Primary antibodies were diluted in primary antibody buffer containing 2% BSA, 0,1% Sodium Azide, 25mM Tris-HCl pH 7.4, 150mM NaCl. Secondary antibodies were diluted in 5% milk TBST 1X.

For the Flag-tagged ZC3H14 immunoprecipitation, HEK293-T cells were plated in a 10cm dish 24 hours later the cells were transfected with pCMV2C-FLAG-ZC3H14 plasmid. The cells were left further 48 hours at 37°C when they were irradiated (3Gy) and let recover for 1h at 37°C. In case of ZC3H14 knock down, the cells were transfected with the siZC3H14 (80 nM) 24 hours after plating. 24 hours later a second round of siRNA transfection was performed together with DNA transfection and left in the incubator for further 24 hours. They were then harvested and lysed in 1ml of 1X lysis buffer containing phosphatase/protease inhibitors and Benzonase (1/1000), followed by centrifugation at 14000 rpm for 10 minutes at 4°C. 10mg of crude cell extract was incubated with 30ul of Flag magnetic beads for 2 hours at 4°C under rotation. The beads were washed three times with 500ul of lysis buffer 1X without benzonase (tubes were inverted upside down for 3 times in each wash), resuspended in 10ul of 2X laemmli buffer, boiled at 95°C and span down at max speed. The samples were then analysed by SDS-page gel. The proteins were transferred to a nitrocellulose ON at 100mA at 4°C. The membrane was blocked in 5% milk TBST 1X for 1 hour at RT and then incubated ON at 4°C with primary antibodies. The day after the membrane was washed for 30 minutes in TBST 1X at RT, incubated
with the secondary antibodies for 1 hour at RT, washed again for 30 minutes with TBST 1X prior to X-Ray films analysis. Primary antibodies were diluted in primary antibody buffer containing 2% BSA, 0.1% Sodium Azide, 25mM Tris-HCl pH 7.4, 150mM NaCl. Secondary antibodies were diluted in 5% milk TBST 1X.

For the MDC1/GFP-ZC3H14 CoIP, HEK293-T cells were plated in a 10cm dish. 24 hours later the cells were transfected with WT ZC3H14 (pEGFP-C1 was used as a control). 48 hours later the cells were irradiated (3Gy) and let recover for 1h at 37°C. They were then harvested and lysed in 1ml of 1X lysis buffer containing phosphatase/protease inhibitors and Benzonase (1/1000), followed by centrifugation at 14000 rpm for 10 minutes at 4°C. 5mg of crude cell extract was incubated with 5ug of rabbit anti-MDC1 antibody (Bethyl A300-053A) for 2h under rotation at 4°C. 60ul of Sepharose G-beads 4 Fast-flow (Sigma), which were washed twice in 200ul of 1X lysis buffer w/o benzonase, were added to the crude cell extract/antibody mix and incubated for further 2 hours under rotation at 4°C. The flow through was recovered and the beads were washed three times with 200ul of 1X lysis buffer (tubes were inverted upside down for 3 times in each wash). The beads were resuspended in 10ul of 2X laemmlili buffer, boiled at 95°C and span down at max speed. The samples were then analysed by SDS-page gel. The proteins were transferred to a nitrocellulose membrane by a constant voltage of 85V for 80 minutes at 4°C. The membrane was blocked in 5% milk TBST 1X for 1 hour at RT and then incubated ON at 4°C with primary antibodies. The day after the membrane was washed for 30 minutes in TBST 1X at RT, incubated with the secondary antibodies for 1 hour at RT, washed again for 30 minutes with TBST 1X prior to X-Ray films analysis. Primary antibodies were diluted in primary antibody buffer containing 2% BSA, 0.1% Sodium Azide, 25mM Tris-HCl pH 7.4, 150mM NaCl. Secondary antibodies were diluted in 5% milk TBST 1X.
For the MDC1/RNF8 CoIP 4x10 cm dishes of U2OS WT and U2OS ZC3H14 KO were plated, per condition. Once they were 90% confluent they were radiated at 5Gy and harvested 15 minutes after. Cells were lysed for 1 hour on ice with 1ml of lysis buffer 1X containing phosphatase/protease inhibitors and Benzonase (1/1000). The supernatant was recovered after 10 min centrifugation at max speed at 4°C. 3mg of lysate was incubated for 2,5 hours at 4°C, under rotation, with 3ug of rabbit anti-MDC1 antibody (Bethyl A300-053A) (anti-rabbit IgG was used as control). 60ul of sepharo se G-beads 4 Fast flow (Sigma) were washed twice with 200ul of 1X lysis buffer w/o Benzonase and incubated for 2,5 hours at 4°C, under rotation, with the lysate/antibody mix. They were then washed 3 times (tubes were inverted upside down for 3 times in each wash) with 1X lysis buffer w/o Benzonase. The first and third wash were performed with 200ul of lysis buffer while, the second wash was performed with 100ul of lysis buffer. Beads were resuspended in 10ul of 2X laemml buffer, boiled at 95°C and span down at max speed. The samples were then analysed by SDS-page gel. The proteins were transferred to a nitrocellulose membrane at 100mA ON at 4°C. The membrane was blocked in 5% milk TBST 1X for 1 hour at RT and then incubated ON at 4°C with primary antibodies. In case of the RNF8 blot the membrane was incubated 48 hours with primary antibody. The day after the membrane was washed for 30 minutes in TBST 1X at RT, incubated with the secondary antibodies for 1 hour at RT, washed again for 30 minutes with TBST 1X prior to X-Ray films analysis. Primary antibodies were diluted in primary antibody buffer containing 2% BSA, 0.1% Sodium Azide, 25mM Tris-HCl pH 7.4, 150mM NaCl. Secondary antibodies were diluted in 5% milk TBST 1X.
3.4.4 Clonogenic survival assay

1.5x10^5 U2OS cells were plated in a 35mm dish. 24 hours later they were transfected with siRNA (negative control siRNA was used as a control) and left for further 48 hours at 37°C. Cells were trypsinised and 500 cells were plated in a 60mm dish. Cells were treated with indicated doses of ionising radiation, Olaparib (Santa Cruz sc-302017) and ICRF-193 (Sigma Aldrich I4659). For the ATM inhibitor treatment, this was added at final concentration of 10uM 1 hour before irradiation. The cells were left at 37°C till colonies appear (10/14 days). Colonies were stained with 1,9-dimethylmethylene blue-70% methanol solution, washed with milli-Q water and then counted. Colony numbers were normalised to the untreated control for each siRNA condition.

3.4.5 GFP Reporter Assay

Polyclonal HEK293-T cells with an integrated DR-GFP and TK-GFP plasmid (Janna Luessing, unpublished) were plated (1.5x10^5) in a 35mm dish and transfected, the day after, with siRNAs (negative control siRNA was used as a control). 24 hours after siRNA transfection the cells were transfected with ISceI plasmid and left for 48 hours at 37°C. The cells were then trypsinised, washed and resuspended in ice-cold 1X PBS prior analysis by FACS. The total level of GFP was normalised to the scramble.

Some cells were lysed in 1X lysis buffer as previously described for western blot analysis.

3.4.6 Immunofluorescence

1.5x10^5 U2OS were plated in a 35mm dish in presence of a coverslip. 24 hours later they were transfected with siRNAs (negative control siRNA or sieGFP were used as controls) and left for
further 48 hours at 37°C. The cells were irradiated (3Gy) and let recover for 1 hour at 37°C. The coverslips were processed for the immunofluorescence analysis while, the cells left in the dish were lysed for 40 minutes on ice with 100ul of 1X lysis buffer containing protease/phosphatase inhibitors and Benzonase (1/1000). The supernatant was recovered after centrifugation at 14000rpm for 10 min at 4°C. 30ug of lysates was used for western blot analysis. The coverslips were washed three times in 1X PBS and subsequently fixed in 4% PFA in 1X PBS for 10 minutes at RT and the permeabilised for 10 minutes at RT with 0.125% Triton X-100. They were then blocked for 1 hour at 37°C in 1% BSA followed by 1 hour incubation at 37°C with primary antibody firstly, diluted in 1% BSA, and secondary antibody secondly. Three washes with 1 ml of 1X PBS were performed in between each of the above steps. DAPI (vectashield mounting medium) was used to stain the nuclei.

In case of RNF8 and RNF168 staining the cells were fixed in 4% PFA diluted in 1X PBST (1X PBS containing 0.1% Triton X-100) for 10 minutes at RT. The cells were washed three times with 1 ml of 1X PBS and permeabilised for 3 minutes at RT with 0.5% Triton X-100 diluted in 1X PBS. The cells were washed three times in 1X PBS and then blocked ON at 4°C with 10% FBS in 1X PBST. They were subsequently incubated for 2 hours at RT with primary antibody diluted in blocking buffer. Cells were washed three times with 1X PBS and incubated for 40 minutes at RT with the secondary antibody diluted in blocking buffer. Vectashield mounting-DAPI was used to stain the nuclei.

3.4.7 Analysis of ZC3H14 at site of damage in the U2OS-DSB reporter cell line

U2OS-DSB reporter cells were plated in a 35mm dish with a coverslip. The day after they were exposed to 1μM hydroxytamoxifen (4OHT) together with 5μM Shield-1 for 5 hours. The cells
were then harvested and subsequently fixed in 4% PFA in 1X PBS for 10 minutes at RT and the permeabilised for 10 minutes at RT with 0.125% Triton X-100. They were then blocked for 1 hour at 37°C in 1% BSA followed by 1 hour incubation at 37°C with primary antibody firstly, diluted in 1% BSA, and secondary antibody secondly. Three washes with 1 ml of 1X PBS were performed in between each of the above steps. DAPI (vectashield mounting medium) was used to stain the nuclei.

3.4.8 Microscopy and images analysis

Images were acquired with an Olympus IX73 inverted microscope (Imvisage, Imsol system). Each image was taken as Z-stacks (0.5µm thickness) which were then deconvolved with the Huygens software (Scientific Volume Imaging). Quantification of the different DDR factors foci was performed with the ImageJ software. The parameters for the foci quantification were set on the positive control (siCTL+IR) and kept constant throughout the entire analysis. Images of 16-bit were converted to 8-bit and split into three channels creating three individual images. The DAPI channel was used to adjust the threshold in order to highlight the nuclei. The FITC or TRITC channel, corresponding to the foci, was used to highlight these as single points (black dots on a white background). Equal noise tolerance was then kept constant throughout the images analysed. The number of foci per cell was then calculated by dividing the raw integrated density by the maximum grey value for each cell.

3.4.9 Analysis of DDR factors protein levels

1.5x10⁵ U2OS cells were plated in a 35mm dish and transfected with siZC3H14 (sieGFP was used as a control) the day after. The cells were left for 48 hours at 37°C. They were irradiated
(3Gy) and let recover for 1 hour at 37°C. The cells were lysed for 40 minutes on ice with 100ul of 1X lysis buffer containing phosphatase/protease inhibitors and Benzonase (1/1000) followed by centrifugation at 14000rpm for 10 minutes at 4°C. The supernatant was then collected and 30ug of lysates were loaded on a SDS-page gel. Proteins were subsequently transferred to a nitrocellulose membrane for 80 minutes at 85V at 4°C. The membrane was then blocked for 1 hour in 5% milk in TBST 1X at RT, washed for 30 minutes in 1X TBST and incubate ON at 4°C with the different antibodies diluted in primary antibody buffer (2% BSA, 0.1% Sodium azide, 25mM Tris-HCl pH7.4, 150mM NaCl) followed by 1 hour incubation at RT with HRP-conjugated secondary antibody diluted in 5% milk. The membrane was washed for 30 minutes in TBST 1X and exposed to X-ray films.

3.4.10 MDC1 analysis by western blot

1.5x10³ U2OS cells were plated in a 35mm dish and transfected with siRNA against ZC3H14 (negative control siRNA was used as control) the day after. 48 hours after transfection the cells were radiated at 5Gy and harvested at different timepoints. They were lysed with with 80ul of lysis buffer 1X, containing benzonase (1:1000), for 1 hour on ice. The lysates were recovered after 10 minutes centrifugation at max speed (4°C). Between 30ug and 50ug of total cells extract was loaded on a 6.5% SDS-page gel 80:1 cross-linking ratio (see appendix 1 table 6 for the recipe) and run at 120V for 90 minutes. The proteins were then transferred to a nitrocellulose membrane at 0.16 A for 1 hour in transfer buffer (4°C). The membrane was blocked in 5% milk in TBST 1X, washed and incubated ON at 4°C with anti-MDC1 antibody. The day after it was washed for 30 minutes at RT in TBST 1X, incubated for one hour at RT with HRP-conjugated secondary antibody, washed again for 30 minutes and analysed by X-ray films.
The ratio between the MDC1 top and bottom band on the western blot was determined by the Ly-Cor system using the Image studio digits Ver5.2 software.
3.5 Results

3.5.1 ZC3H14 interacts with ATM

In our laboratory we performed two SILAC analysis in DT40 chicken cells to find potential ATM interactors (Pessina et al., 2014 Fabio Pessina, PhD thesis, 2014). ZC3H14 was identified as a novel ATM partner in both analysis. Interestingly this interaction was only detectable after damage.

We confirmed this result by co-immunoprecipitating GFP-tagged ZC3H14 with ATM in presence and absence of damage (Figure 3.1). Using an ant-GFP antibody to precipitate overexpressed GFP-ZC3H14 we could detect an interaction between the GFP tagged ZC3H14 and ATM. Surprisingly this interaction was visible before and after damage, while the SILAC data only showed interaction between ATM and ZC3H14 after damage. This result might be due to the fact that when we looked at the ZC3H14 interaction with ATM by SILAC screening we did not overexpress tagged ZC3H14 but we were looking at the interaction between endogenous ZC3H14 and ATM.
Figure 3.1: ZC3H14 interacts with ATM. HEK293-T cells were transfected with GFP WT ZC3H14 (GFP empty vector was used as a control). 48 hours after transfection they were radiated at 3 Gy, allowed to recover for 1 hour at 37°C and crude cell extracts were prepared. 5 mg of crude cell extract was used to immunoprecipitated GFP tagged ZC3H14 using anti-GFP antibody. The beads were resuspended in 10 μl of 2X laemmlie buffer and loaded on an SDS-page gel together with 30 μg of lysates coming from the input and analysed by western blot.
3.5.2 Persistent γH2AX foci and pATM (S1981) foci upon ZC3H14 depletion

The ZC3H14 interaction with ATM suggested a role for this C3H1-type zinc finger protein in the DNA damage response. We next wanted to look at the kinetics of DNA repair. Using γH2AX foci as an indicator of DSBs, we knocked down ZC3H14 and analysed γH2AX (S139) foci at different timepoints after IR (Figure 3.2 A-B). In control cells we could see γH2AX foci at 1 hour and fewer foci at 4 hours, while they were lost at 8 and 24 hrs after IR. Depletion of ZC3H14 resulted in persistence of γH2AX foci into the 8 and 24hrs samples. Quantification of this data (Figure 3.2 B) confirmed the reproducible persistence of γH2AX foci relative to control cells upon depletion of ZC3H14. This data suggests that ZC3H14 is required for efficient DSB repair. Because ZC3H14 interacts with ATM and this is recruited and activated by autophosphorylation on the SER1981 upon DSB we wondered if the ZC3H14-dependent persistent γH2AX phenotype observed for γH2AX foci could also be detected using an antibody specific to phosphorylation of ATM at Serine 1981 (ATM-S1981p). Similarly to γH2AX, ATM-S1981p foci upon ionising radiation were clearly visible at 1 hour and 4 hours post-irradiation, with clearly fewer ATM-S1981p foci at 4 hours post IR. Only minimal ATM-S1981p foci were detected at 8 and 24 hours after ionising radiation (Figure 3.3 A-B). Just as we observed for γH2AX foci, ATM-S1981p foci were reproducibly persistent after IR upon ZC3H14 depletion. Thus, γH2AX and ATM-S1981p, suggests that ZC3H14 has a role in the efficient repair of DDR.
Figure 3.2: Persistent γH2AX foci upon ZC3H14 depletion. A) U2OS cells were transfected with siRNA against ZC3H14 (scramble RNA was used as a control). 48 hours after transfection they were radiated at 3Gy and harvested at different timepoints. The cells were stained for γH2AX. Images were acquired with an Olympus IX73 inverted microscope (DV Core, Imsol system). B) Quantification of the IF images showed in A (n=3). The statistical analysis was done by paired t-test (p<0.0001).
Figure 3.3: Persistent pATM(S1981) foci upon ZC3H14 depletion. A) U2OS cells were transfected with siRNA against ZC3H14 (scramble RNA was used as a control). 48 hours after transfection they were radiated at 3Gy and harvested at different timepoints. The cells were stained for pATM(S1981). Images were acquired with an Olympus IX73 inverted microscope (DV Core, Imsol system). B) Quantification of the IF images showed in A (n=3). The statistical analysis was done by paired t-test (p<0.0001).
3.5.3 ZC3H14 cannot be detected at sites of DNA double strand breaks

ATM is the main player in the DDR and is found at sites of damage. ZC3H14 likely interacts with ATM and is required for efficient DNA double strand break repair we next investigated whether ZC3H14 is also found at DNA DSB sites by immunofluorescence. We induced damage by IR and 1 hour later we harvested U2OS cells and stained them with anti-γH2AX and anti-ZC3H14 antibody (Figure 3.4). Interestingly, while ZC3H14 localisation into nuclear speckles was unaffected by IR, we could not detect any ZC3H14 foci co-localising with γH2AX after IR. If only a small portion of ZC3H14 localises to sites of DSBs this would not be detectable against the large fraction in nuclear speckles.

Even though, ZC3H14 interacts with ATM and its depletion affects the DSBs repair suggesting a role for ZC3H14 in the DNA damage response pathway we could not detect ZC3H14 IRIFs (Figure 3.6). Therefore, we then decided to examine the localisation of ZC3H14 to sites of damage in a more sensitive system, the U2OS-DSB reporter cell line (Shanbhag et al., 2010; Tang et al., 2013). In this cell line it is possible to examine DSBs that occur on a specific locus by using the LacO system from bacteria (Shanbhag et al., 2010; Tang et al., 2013). 256 copies of the LacO construct are integrated in the genome of these U2OS cells (Figure 3.5 A). This LacO construct contains a sequence that is recognised by the FokI endonuclease. The U2OS-DSB reporter cell line also expresses a LacI fusion protein. Specifically, the LacI construct used expresses a fusion of LacI to the estrogen receptor (ER), the mCherry fluorescent protein, the FokI endonuclease and a destabilising domain (DD). This LacI-ER-mCherry-FokI-DD fusion protein is expressed, stabilised and transported into the nucleus by the presence of the hydroxytamoxifen (4OHT) and Shield-1, which blocks the DD-dependent proteolysis. Once in the nucleus, this LacI fusion protein binds LacO and DSBs are generated within the 256 copies
of the LacO array via the FokI component of the LacI fusion protein. The DSB is so detectable as a single red spot within the nucleus.

The U2OS-DSB reporter cell line was grown for 24 hours and DSBs induced by exposure of the cells to 4OHT and Shieldin-1 for 5 hours. The cells were then harvested and stained with anti-ZC3H14, anti-53BP1 and anti-γH2AX antibody (Figure 3.5 B). 53BP1 was clearly localising with the FokI and γH2AX. Unfortunately, we could not detect ZC3H14 at the FokI site of damage.

**Figure 3.4: ZC3H14 cannot be detected in IRIFs.** U2OS were radiated with a 3Gy IR dose and let to recover for 1h at 37°C. They were fixed, permeabilised and stained for ZC3H14 and γH2AX. DAPI was used to stain the nuclei. Images were acquired with an Olympus IX73 inverted microscope (DV Core, Imsol system).
Figure 3.5: ZC3H14 cannot be detected at the FokI DSB-induced sites  

A) Schematic of the U2OS-DSB reporter cell line system.  

B) U2OS-DSB reporter cells were plated and seeded for 24 hours after. DSB was induced by 5 hours treatment with 4OHT and Shieldin-1. Subsequently, they were fixed, permeabilised and stained with anti-53BP1 and anti-γH2AX (i) as control, and anti-γH2AX and anti-ZC3H14 antibody (ii). Images were acquired with an Olympus IX73 inverted microscope (DV Core, Imsol system).
3.5.4 Defective 53BP1 and RIF1 IR induced foci (IRIF) upon ZC3H14 depletion

Since we saw that ZC3H14 is required for efficient DNA DSB repair we started investigating whether ZC3H14 is involved in the NHEJ repair pathway or in the HR repair pathway. We started by looking at 53BP1 which is a DDR factor involved in the NHEJ repair pathway. We knocked down ZC3H14 and looked at the recruitment of 53BP1 by immunofluorescence (Figure 3.6 A).

In absence of damage 53BP1 presents a pan-nuclear pattern with a few big bright foci, known as G1 nuclear bodies, visible only in G1 cells. After damage 53BP1 localises to the breaks resulting in loss of the pan-nuclear pattern and the appearance of clear foci co-localising with γH2AX. Interestingly, when ZC3H14 is depleted 53BP1 IRIF cannot be detected even though DSBs are been induced as shown by the γH2AX IRIF signal (Figure 3.6 A-Bi). What we can see in these cells is the pan-nuclear staining with fewer foci compare to our control cells. No difference is recorded, in terms of 53BP1 G1 nuclear bodies, before damage between control cells and cells knocked down for ZC3H14. This data might suggest a potential role for ZC3H14 in the NHEJ repair pathway. Total cell extracts from control cells and cells knocked down for ZC3H14 were analysed by western blot (Figure 3.6 Bii) to confirm that the defective 53BP1 IRIF observed in cells depleted for ZC3H14 was actually due to the absence of the ZC3H14 protein.

Importantly, expression of the GFP-tagged ZC3H14 resistant to the siRNA was able to rescue the 53BP1 IRIFs indicating that loss of 53BP1 IRIF upon ZC3H14 depletion is not a result of an off-target effect of the siRNA used (Fig 3.7 A-B). The 53BP1 IRIFs rescue in the sample expressing the GFP-tagged ZC3H14 rescues 53BP1 IRIF to nearly 80% of that observed in control cells.
Figure 3.6: 53BP1 IRIF is affected upon ZC3H14 depletion. A) U2OS cells were transfected with siRNA against ZC3H14. 48 hours after transfection they were radiated at 3Gy and harvested 1 hour later. The cells were stained for 53BP1 and γH2AX. DAPI was used to stain the nuclei. Bi) Quantification of the IF images showed in A (n=3). The statistical analysis was done by paired t-test (p<0.0001). Bii) Western blot analysis of total cell extracts from the immunofluorescence experiment in A.
Figure 3.7: GFP-tagged ZC3H14 rescues the 53BP1 IRIFs. A) U2OS cells were transfected with siRNA against ZC3H14. 24 hours later they were transfected with the GFP plasmid expressing ZC3H14 resistant to the siRNA. 24 hours after DNA transfection the cells were irradiated with 3Gy dose and let to recover for an hour at 37°C when they were fixed, permeabilised and stained for γH2AX and 53BP1. DAPI was used to stain the nuclei. B) Quantification of the IF images showed in A (n=3). The statistical analysis was done by paired t-test (p<0.0001). C) Western blot analysis showing the level of expression of the GFP tagged ZC3H14 over the endogenous.
Next we examined the recruitment of RIF1 which, together with 53BP1, is recruited to the breaks in order to inhibit the resection and promote the repair by NHEJ. RIF1 focal recruitment is dependent on the phosphorylation of 53BP1 on its N-terminal phospho-SQ/TQ domain (Chapman et al., 2013). Since we could see a defect on 53BP1 IRIF upon ZC3H14 depletion, as a consequence, we were expecting to see the same phenotype for RIF1. We repeated the IF experiment showed in Fig 3.10 A, this time staining the cells for 53BP1 and RIF1 (Figure 3.8 A-B). Similar to 53BP1, RIF1 shows a pan-nuclear pattern upon damage and forms IRIFs when damage occurs. As for 53BP1, we observed defective RIF1 IRIFs upon ZC3H14 depletion, consisting with ZC3H14 functioning upstream of 53BP1 focal recruitment.
Figure 3.8: Defective RIF1 IRIF upon ZC3H14 depletion. A) U2OS cells were transfected with siRNA against ZC3H14. 48 hours after transfection they were radiated at 3Gy and harvested 1 hour later. The cells were stained for RIF1 and 53BP1. DAPI was used to stain the nuclei. Images were acquired with an Olympus IX73 inverted microscope (DV Core, Imsol system). B) Quantification of the IF images showed in A (n=3). The statistical analysis was done by paired t-test (p<0.0001).
3.5.5 Defective BRCA1 IRIF in non-replicating cells upon ZC3H14 depletion

Since ZC3H14 depletion results in defective 53BP1 and RIF1 recruitment after IR we were wondering if similar phenotype was detectable for another important DDR factor, BRCA1. It is known that BRCA1 forms foci in S-phase cells in absence of damage (Scully et al., 1997). Arrest of DNA synthesis leads to loss of these S-phase specific focal structures, suggesting that BRCA1 is recruited to DNA replication structures. To discriminate cells in S-phase from cells in other stages of the cell cycle we used an anti PCNA antibody (Figure 3.9 A). Prior to damage, and as expected, BRCA1 foci were visible in PCNA positive S-phase cells, while upon damage BRCA1 IRIFs are visible in every single cell as indicated by the white arrows in Figure 3.13A. When ZC3H14 is depleted, in absence of damage, BRCA1 foci are visible in S-phase cells but not in the PCNA negative non-replicating cells. After damage, depletion of ZC3H14 leads to a loss of BRCA1 foci in non-replicating cells (Figure 3.9 A-B). A second marker, such as RAD52, could be used to confirm whether ZC3H14 acts above the BRCA1 focal recruitment or not.

This data, together with the defective 53BP1 IRIFs upon ZC3H14 knock down, suggests that this C3H1-type zinc finger protein acts in the DDR upstream the recruitment of both 53BP1 and BRCA1.
Figure 3.9: Defective BRCA1 IRIF in no S-phase cells upon ZC3H14 depletion. A) U2OS cells were transfected with siRNA against ZC3H14. 48 hours after they were radiated and harvested 1 hour later. The cells were fixed, permeabilised and subsequently stained for BRCA1 and PCNA. DAPI mounting media was used to stain the nuclei. White arrows are indicating non S-phase cells. Images were acquired with an Olympus IX73 inverted microscope (DV Core, Imsol system). B) Quantification of BRCA1 foci in PCNA negative cells shown in the images in A (n=3). The statistical analysis was done by paired t-test (p<0.0001).
3.5.6 Ubiquitination at sites of DNA damage requires ZC3H14

When ZC3H14 is depleted we observed defective recruitment of 53BP1, RIF1 and BRCA1 into IRIFs. This data strongly suggests a role for ZC3H14 upstream of 53BP1 and BRCA1 focal recruitment.

In order, for 53BP1 and BRCA1, to be recruited at sites of damage, the histone H1 has to be mono and poly-ubiquitinated on Lys63, together with mono-ubiquitination of Lys13 and Lys15 of histone H2A. These ubiquitination events are a result of the recruitment of two important E3 ligases, RNF8 and RNF168, which are required for the subsequent recruitment of the downstream factors 53BP1 and BRCA1.

To test whether ZC3H14 might regulate these ubiquitination events, once again, we knocked down ZC3H14 and looked at the formation of polyubiquitin chains by staining the cells for FK2, an antibody specific to ubiquitin (Figure 3.10 A). Before damage we detected only a few FK2 foci in both control cells and cells knocked down for ZC3H14, while 53BP1 showed the usual pan nuclear staining with large, bright foci corresponding to the G1 nuclear bodies in the G1 cells (Figure 3.10 A-B). Upon damage, FK2 IRIFs could not form when ZC3H14 was depleted. As observed for 53BP1 IRIFs, the FK2 IRIFs were also rescued by expression of the GFP-tagged ZC3H14 resistant to the siRNA (Figure 3.11 A-B). Defective enrichment of ubiquitin in IRIF upon ZC3H14 knock down suggests that ZC3H14 acts early in the DNA damage response, perhaps, being required for RNF8 and RNF168 focal recruitment.
Figure 3.10: Ubiquitination is affected upon ZC3H14 depletion. A) U2OS cells were transfected with siRNA against ZC3H14. 48 hours after transfection they were radiated at 3Gy and harvested 1 hour later when they were fixed, permeabilised and stained for FK2 and 53BP1. DAPI was used to stain the nuclei. B) Quantification of the images shown in A (n=3). The statistical analysis was done by paired t-test (p<0.0001).
Figure 3.11: GFP-tagged ZC3H14 rescues the FK2 IRIFs. A) U2OS cells were transfected with siRNA against ZC3H14. 24 hours later they were transfected with the GFP plasmid expressing ZC3H14 resistant to the siRNA. 24 hours after DNA transfection the cells were irradiated with 3Gy dose and let to recover for an hour at 37°C when they were fixed, permeabilised and stained for FK2. DAPI was used to stain the nuclei. B) Quantification of the IF images showed in A (n=3). The statistical analysis was done by paired t-test (p<0.0001).
3.5.7 RNF8 and RNF168 focal recruitment is affected upon ZC3H14 depletion

Histones ubiquitination is an early event that occurs when DNA double strand breaks accumulate in the cells. One important ubiquitination event is the one that occurs on the Lys63 (K63) of the histone-linker H1 (Thorslund et al., 2015). This H1 ubiquitination event is mediated by RNF8, which also ubiquitinates the histone H2A. Subsequently, RNF8 binds the ubiquitinated H2A and amplifies local concentration of lysine 63-linked ubiquitin conjugates, which are necessary for the recruitment of 53BP1 and BRCA1 (Doil et al., 2009).

Since we saw a defect in ubiquitination upon ZC3H14 depletion we decided to test if such defect was due to a defective focal recruitment of RNF8 and RNF168 upon ZC3H14 knock down. Once again, we depleted ZC3H14 and we looked at RNF8 and RNF168 IRIFs by immunofluorescence (Figure 3.12 A-B, Figure 3.13 A-B). When ZC3H14 is knocked down both RNF8 and RNF168 IRIFs are barely visible compared to control cells. Importantly, once more, γH2AX IRIF are unaffected by ZC3H14 depletion.

Data suggests that ZC3H14 acts above both of these E3 ubiquitin ligases. The RNF8 recruitment at sites of damage is dependent on the phosphorylation of MDC1 (Kolas et al, 2007). MDC1 is phosphorylated by ATM and interacts with RNF8, which is then recruited at DSBs (Kolas et al., 2007).
Figure 3.12: Defective recruitment of RNF8 into IRIF, but normal formation of γH2AX, upon depletion of ZC3H14. A) U2OS cells were transfected with siRNA against ZC3H14. 48 hours after transfection they were radiated at 3Gy and harvested 1 hour later. Cells were fixed and permeabilised and then stained for RNF8 and γH2AX respectively. DAPI was used to stain the nuclei. B) Quantification of the images in Ai (n=3). The statistical analysis was done by paired t-test (p<0.0001).
Figure 3.13: Defective recruitment of RNF168 into IRIF, but normal formation of γH2AX, upon depletion of ZC3H14. A) U2OS cells were transfected with siRNA against ZC3H14. 48 hours after transfection they were radiated at 3Gy and harvested 1 hour later. Cells were fixed and permeabilised and then stained for RNF168 and γH2AX respectively. DAPI was used to stain the nuclei. B) Quantification of the images in Ai (n=3). The statistical analysis was done by paired t-test (p<0.0001).
3.5.8 Expression levels of DDR factors are not affected upon ZC3H14 depletion

To ensure that the defect we observed for RIF1, 53BP1, BRCA1, RNF168 and RNF8 upon ZC3H14 depletion was not due to a defect on their level of expression we analysed their expression in western analyses (Figure 3.14). We could not detect any difference in terms of total levels of expression for RIF1, 53BP1, BRCA1, RNF168 and RNF, nor for ATM, between control cells and cells knocked down for ZC3H14 before or after damage. This suggested that the defective recruitment of these factors functioning downstream of ATM upon ZC3H14 depletion is not due to a reduction in their levels of protein expression. There is also no evidence for subcellular re-organisation of DDR factors before and after damage. Thus, ZC3H14 appear to be a novel factor required for the focal recruitment of their DDR factors at sites of DNA damage.
Figure 3.14: DDR factors level of expression. U2OS cells were transfected with siRNA against ZC3H14 (sieGFP was used as a control). 48 hours after transfection the cells were radiate at 3Gy and harvested 1 hour later. They were lysed on ice for 40 minutes with 1X lysis buffer. 30ug of lysate was loaded on a SDS-page gel and analysed by western blot.
MDC1 is an important player in the DNA damage response. Its recruitment to sites of damage is necessary for the subsequent recruitment of RNF8 into IRIF. This RNF8 recruitment to DSBs occurs directly through RNF8 interaction, via its FHA domain, with TQXF phosphorylated sites localised at the N-terminus of MDC1 (Kolas et al., 2007). Since the depletion of ZC3H14 led to defective RNF8 focal recruitment we next looked at the MDC1 IRIFs upon ZC3H14 knock down (Figure 3.15 A). In absence of damage MDC1 is diffused within the nucleus while, upon gamma radiation, accumulates into foci, which co-localise with the γH2AX IRIFs. When we knocked down ZC3H14 we could not detect any defect for MDC1 IRIFs (Figure 3.15 A-B) indicating that this zinc finger protein must act in the DNA damage response pathway between the focal recruitment of MDC1 and RNF8.
Figure 3.15: MDC1 focal recruitment is not affected upon ZC3H14 depletion. A) U2OS were transfected with siRNA against ZC3H14 24h after seeding. Two days later they were radiated, let recover for 1h at 37°C and subsequently fixed and stained for MDC1 and γH2AX. DAPI was used to stain the nuclei. B) MDC1 IRIFs quantification of the images shown in Ai (n=3).
3.5.10 Perturbation of MDC1 isoforms upon ZC3H14 depletion

RNF8 interaction with MDC1 is mediated through the fork head domain of RNF8 and the phosphorylation of MDC1 on TQXF sites (Kolas et al., 2007). As observed defective RNF8 IRIFs, but not MDC1 IRIFs upon ZC3H14 depletion suggests that ZC3H14 might regulate the MDC1-RNF8 interaction.

MDC1 presents many TQXF sites which can be phosphorylated by ATM, ATR and DNA-PK upon damage (Stewart et al., 2003). MDC1 is also sumoylated and ubiquitinated upon damage (Luo et al., 2012). MDC1 Lys48-linkage ubiquitination, mediated by a SUMO-targeted E3 ubiquitin ligase StUbl termed RNF4, leads to degradation of MDC1 (Luo et al., 2012). Surprisingly, this MDC1 degradation does not affect its recruitment to DSBs (Luo et al., 2012).

We examined MDC1 protein levels upon ZC3H14 depletion and knock out (Figure 3.16 B-C). This ZC3H14 knock out U2OS cell line was generated in the laboratory by CRISPR-Cas9 approach (Isabelle McDonald, Master thesis, 2018). The type of mutations generated in this ZC3H14 knock out U2OS cells by the CRISPR-Cas9 approach are reported in the table 5 of the appendix. In control cells, MDC1 shows up as two strong bands, with similar intensity, which migrate between the 250 and 148 kDa marker proteins. A middle band can also be detected between the two main MDC1 bands upon long exposure. Upon DNA damage, at 15 minutes after IR, the two main MDC1 bands migrate more slowly due to phosphorylation (Stewart et al., 2003). Lou and colleagues have also reported sumoylation and ubiquitination of MDC1 after IR that leads to its degradation (Lou et al., 2012). In control siRNA treated cells 15 minutes after damage there is no evidence for degradation, in fact the faster migrating lower band becomes more abundant, while at later time points the abundance of the upper and lower MDC1 bands
clearly diminishes, being barely detectable in the 3 hrs timepoint. For some reason, both the upper and lower forms of MDC1 are slightly, but reproducibly increased in abundance at 1 hour relative to the 30’ sample (Figure 3.16 B compare lane 4 with lane 3). Upon ZC3H14 depletion (siZC3H14), in undamaged cells, the level of both MDC1 isoforms is significantly increased relative to control cells with the lower, more quickly migrating form, being relatively more abundant than the upper form (Figure 3.16 B compare lane 6 with lane 1). Upon damage the level of both MDC1 isoforms is significantly increased relative to control cells (Figure 3.16 B compare lane 7 with lane 2). Furthermore, the proportion of signal in the lower form appears significantly increased relative to the upper form. In ZC3H14 depleted cells after DNA damage degradation is again detected at all timepoints examined, with the more abundant lower isoform persisting for longer than the less abundant upper isoform. To ensure that this MDC1 phenotype was not due to off-target effects of the ZC3H14 siRNA we looked at the MDC1 protein pattern in the U2OS ZC3H14 KO cells (Figure 3.16 C and Figure 3.17). In these ZC3H14 KO cells we could detect a more abundant MDC1 lower band compared to the upper MDC1 band, before and after damage, compared to control cells. MDC1 degradation upon damage was also detected in U2OS ZC3H14 KO cells.
Figure 3.16: MDC1 is degraded upon damage. A) Schematic of RNF8 and MDC1. The region of each protein required for their interaction is indicated by the red arrow. B) U2OS cells were transfected with siRNAs against ZC3H14 24 hours after seeding. 48 hours after transfection they were radiated at 5Gy and harvested at different timepoints. They were lysed and 30ug of total cell extracts were loaded on a 6% SDS-page gel (37:1 cross-linking ratio) they were then analysed by western blot analysis. The blots shown are representative of 4 repeated experiments. C) U2OS cells WT and ZC3H14 KO were radiated at 5Gy and harvested at different timepoints. They were lysed and the lysates were run on a 6,5% 80:1 cross-linking ratio gel and analysed by western blot. The blots shown are representative of 4 repeated experiments.
**Figure 3.17: MDC1 total protein levels is increased upon ZC3H14 depletion.** U2OS cells were transfected with siRNAs against ZC3H14 24 hours after seeding. 48 hours after transfection they were radiated at 5Gy and harvested at different timepoints. They were lysed and 30μg of total cell extracts were loaded on a 6% SDS-page gel (37:1 cross-linking ratio) they were then analysed by western blot using the Ly-Cor system. The blots shown are representative of 4 repeated experiments.

We also looked at the MDC1 protein levels in h-TERT RPE1 cells (Figure 3.18). Once again, we could detect two strong MDC1 bands in control cells, while in the ZC3H14 depleted cells the lower band is relatively more abundant than the upper one. MDC1 degradation upon damage was also detectable in the h-TERT RPE1 cells, suggesting that ZC3H14 is not required for post-IR degradation of MDC1, albeit the kinetic of MDC1 degradation were faster in RPE1 cells than in U2OS cells. Nevertheless, and despite some differences in the kinetic of degradation, MDC1 is clearly degraded after IR in both cell types indicating that ZC3H14 is not required for post-IR dependent degradation of MDC1. Moreover, depletion of ZC3H14 results in relatively greater levels of the fastest migrating isoform of MDC1 indicating that ZC3H14 is required for the normal abundance of the fastest migrating form.

Little is known about the nature of the isoforms of MDC1 detected by SDS-page. Both forms are phosphorylated after DNA damage (Stewart et al., 2003; Kolas et al., 2007) (Figure 3.16 B-C and Figure 3.17 A). However, the major effect of ZC3H14 depletion is the relative abundance
of the upper to lower which are roughly equally abundant in control cells but upon loss of ZC3H14 the lower, faster migrating, form is more abundant than the upper form.

MDC1 has also been reported to be both SUMOylated and ubiquitinated and subjected to degradation after DNA damage (Lou et al., 2012) and post-IR dependent degradation does not require ZC3H14. However, the SUMOylated/ubiquitinated forms are not detected by SDS-page, presumably because of their rapid degradation. Interestingly, MDC1 recruitment into IRIF remains stable until repair is complete (Lou et al., 2012 and see Figure 3.15 A) suggesting that it is the nucleoplasmic pool of MDC1, rather than the MDC1 recruited to chromatin flanking DSBs, that is subjected to degradation.

Little is known about the MDC1 pattern and what those bands are. They are likely to be MDC1 splicing isoforms. The possibility that the upper band could be a modified form of MDC1 is less likely given the apparent size difference detected in SDS-page.

![Figure 3.18: MDC1 pattern in h-TERT RPE1 cells.](image)

Cells were transfected with siRNAs against ZC3H14 (scramble RNA was used as a control) 24 hours after seeding. 48 hours later they were radiated at 5Gy and harvested at different timepoints. The cells were lysed and the lysates were run on a 6,5% 80:1 cross-linking gel. They were then analysed by western blot. The blots shown are representative of 3 repeated experiments.
3.5.11 ZC3H14 interacts with MDC1 but it is not required for the MDC1-RNF8 interaction

Since we observed a defect on RNF8, but not on MDC1 IRIFs upon ZC3H14 depletion we wondered if ZC3H14 might affect the interaction between MDC1 and RNF8. We first investigated whether ZC3H14 might interact with MDC1. We pulled down MDC1, in presence and absence of damage, and looked for the interaction with GFP-ZC3H14 (Figure 3.19). We could detect a constitutive interaction between MDC1 and GFP-ZC3H14 before and after damage. It is important to notice that when we use a 3Gy dose of γ radiations and harvest the cells one hour later, the MDC1 phospho-shift is not as great as when we use a 5Gy dose (see Figure 3.16 B) and there is also less MDC1 degradation. In fact, by testing different IR doses (Figure 3.20) we could asses that the MDC1 phospho-shift and degradation is dependent on the IR dose. The higher the IR dose the greater is the MDC1 phospho-shift and the faster is the degradation.
Figure 3.19: MDC1 interacts with ZC3H14. HEK 293T cells were plated and transfected with GFP-ZC3H14 construct (GFP empty vector was used as a control) 24 hours after. 48 hours after transfection they were radiated at 3Gy and harvested 1hr later. The cells were lysed and 5mg of total cell extract was used to pull down MDC1. Total cell extracts and IP samples were loaded on an SDS-page gel and analysed by western blot.

| IP: MDC1 |
|-----------------|-----------------|-----------------|
| IR (3Gy 1h)     | -               | -               |
| GFP-ZC3H14      | -               | +               |
| 250 kDa         | -               | -               |
| 148 kDa         | -               | +               |
| 98 kDa          | -               | +               |

Figures: MDC1 and GFP-ZC3H14 bands in different conditions.

Figure 3.20: MDC1 phospho-shift and degradation are dose dependent. U2OS cells were seeded for 24 hours and then radiated with different IR doses and harvested at different timepoints. Cells were lysed and 30μg of crude cell extract were loaded on a 6% SDS-page gel and analysed by western blot.

| INPUTS |
|-----------------|-----------------|-----------------|
| MDC1            | MDC1            |
| GFP-ZC3H14      | GFP-ZC3H14      |
| 250 kDa         | 250 kDa         |
| 148 kDa         | 148 kDa         |
| 98 kDa          | 98 kDa          |

Figure: MDC1 and GFP-ZC3H14 bands in different conditions for different IR doses and timepoints.
The fact we could detect an interaction between ZC3H14 and MDC1 might suggest that ZC3H14 regulates the MDC1-RNF8 interaction previously reported (Mailand et al., 2007). To test such hypothesis, we pulled down MDC1 in U2OS WT and ZC3H14 KO cells and looked for the interaction with RNF8 (Figure 3.21). Although, it has previously been shown a constitutive interaction between MDC1 and RNF8 (Mailand et al., 2007), here we detected RNF8 to interact with MDC1 only upon damage (Figure 3.21 compare lane 3 with lane 2). This interaction was also visible in ZC3H14 KO cells upon damage but not in absence of damage (Figure 3.21 compare lane 5 with lane 4). Although the MDC1 protein level is affected upon ZC3H14 depletion compare to control cells, with the lower MDC1 band being more abundant than the upper MDC1 band compare to control cells (Figure 3.17), this does not impact on the MDC1-RNF8 interaction. This data suggests that the upper band of MDC1 is not required for its interaction with RNF8 whether this is a MDC1 isoform or a modified form of MDC1.
Figure 3.21: MDC1-RNF8 interaction is not affected in U2OS ZC3H14 KO cells. U2OS WT and ZC3H14 KO cells were plated in 10 cm dishes. When 90% confluent they were radiated at 5Gy and harvested 15 min after. The cells were lysed and 3mg of total cell extract was used to pull down MDC1. The cell extracts and the IP samples were loaded on an SDS-page gel and analysed by western blot.
3.5.12 U2OS are sensitive to IR and ICRF193, but not to Olaparib, upon ZC3H14 depletion

Our data supports a role for ZC3H14 in efficient DNA DSB repair. To support this hypothesis we examined the viability of U2OS and RPE1 upon ZC3H14 depletion after treatment with different DNA damage agents. We first looked at cell viability using different IR doses. We ran clonogenic survival assays in U2OS and RPE1 cells depleted for ZC3H14 and also in cells treated with ATM inhibitor (positive control). In both the transformed U2OS and immortalised h-TERT RPE1 cells ATMi resulted in fewer cell colonies. Also, in both cell types depletion of ZC3H14 resulted in identical clonogenic survival phenotypes to ATMi (Figure 3.22 A-B). This data, together with the persistence of γH2AX foci upon ZC3H14 depletion, strongly suggests a role for this zinc finger protein in the repair of DNA double strand breaks. Interestingly, ZC3H14 depletion results in highly similar survival defects to inhibition of ATM, the central regulator of the cellular responses to DSBs, indicating an important role for ZC3H14 in survival upon ionising radiation.
Figure 3.22: Cells are sensitive to IR upon ZC3H14 depletion. A) U2OS cells were transfected with siRNA against ZC3H14. 48 hours after transfection 100 cells were plated in duplicate per each IR dose and, only for the ATMi treated, they were treated with 10uM of ATM kinase inhibitor (KU-55933) for 1 hour before radiation. The cells were left at 37°C for 10 days when colonies were stained and counted. The number of colonies was normalised to the control (non-radiated cells) within the same population. Error bars indicate the standard deviation (n=3). B) RPE1 cells were transfected with siRNA against ZC3H14 (scramble RNA was used as a control). 48 hours after transfection 100 cells were plated in duplicate per each IR dose and, only for the ATMi treated, they were treated with 10uM of ATM kinase inhibitor (KU-55933) for 1 hour before radiation. The cells were left at 37°C for 10 days when colonies were stained and counted. The number of colonies was normalised to the control (non-radiated cells) within the same population. Error bars indicate the standard deviation (n=3).
We then tested the cellular viability upon treatment with two further DNA damage agents, Olaparib and ICRF193. Olaparib is an inhibitor of the poly (ADP-ribose) polymerase. PARP is recruited to ssDNA (Caldecott, 2014) and PARP inhibition leads to an accumulation of ssDNA breaks, which if not repaired efficiently during replication, are converted to single-ended DNA double strand breaks that are efficiently repaired by a HR-dependent mechanism (Helleday, 2011). The ICRF193, on the other hand, has been linked to the NHEJ repair pathway. ICRF193 is a drug that inhibits the topoisomerase II. Studies show that MEF Lig4<sup>−/−</sup> and Ku70<sup>−/−</sup> cells are sensitive to ICRF-193 while MEF Rad54<sup>−/−</sup> cells show no sensitivity to ICRF-193 treatment (Adachi et al., 2003). Since Lig4 and Ku70, but not Rad54, are involved in the NHEJ repair pathway ICRF-193 has been linked to this repair pathway.

Consistent with the role for BRCA1 in HR (Hartlerode & Scully, 2009) its depletion results in sensitivity to Olaparib relative to control cells (Figure 3.23 A). However, when ZC3H14 is depleted these behave similarly to control cells, suggesting that ZC3H14 is not important in the HR repair pathway.

DNA-PK is important for NHEJ (Hartlerode & Scully, 2009) and is sensitive to ICRF193 relative to control cells (Figure 3.23 B). Upon depletion of DNA-PK U2OS cells are sensitive to ICRF193 compare to control cells. ZC3H14 depletion, on the other hand, displayed decreased viability when cells were treated with ICRF193 compare to control cells suggesting a role in NHEJ. Interestingly, the sensitivity of ZC3H14 depleted cells to ICRF193, relative to DNA-PK depleted cells, was similar. This suggests an important role for ZC3H14 in NHEJ.
Figure 3.23: Cells are sensitive to ICRF193, but not to Olaparib, upon ZC3H14 depletion. A) U2OS cells were transfected with siRNA against ZC3H14 and BRCA1 (positive control). 48 hours after transfection 100 cells were plated in duplicate per each Olaparib dose. The cells were left at 37°C for 10 days when colonies were stained and counted. The number of colonies was normalised to the control (untreated) within the same population. Error bars indicate the standard deviation (n=3). B) U2OS cells were transfected with siRNA against ZC3H14 and DNA-PK (positive control). 48 hours after transfection 100 cells were plated in duplicate per each ICRF193 dose. The cells were left at 37°C for 10 days when colonies were stained and counted. The number of colonies was normalised to the control (untreated) within the same population. Error bars indicate the standard deviation (n=3).
3.5.13 ZC3H14 in involved in the NHEJ but not in the HR repair pathway

When DNA double strand breaks occur the cells have two main options for their repair: homologous recombination (HR) or non-homologous end joining recombination (NHEJ). In case of HR resection is critically required for the formation of a free 3’ single-stranded DNA end and subsequent DNA strand invasion and repair. In case of NHEJ, DNA resection is not needed since no template is required in order to repair the break. In fact, resection inhibits NHEJ. Thus, inhibition of resection is required for NHEJ and is achieved largely by 53BP1-dependent recruitment of the SHEILDIN complex (Ghezraoui et al., 2018; Noordermeer et al., 2018; Harveer Dev et al., 2018). The two non-resected ends are substrates for NHEJ-dependent ligation by DNA ligase 4 (Grawunder U. et al., 1998).

Since we observed a defect for both 53BP1 and RIF1 IRIF upon ZC3H14 depletion (Figure 3.6 and figure 3.8) we hypothesised a possible role for this protein in the NHEJ repair pathway. To test such hypothesis we generated two polyclonal HEK 293-T cell lines stably transfected with plasmids suitable for assaying HR or NHEJ (Dr. Murilo T.D. Bueno). One polyclonal cell line was generated by stably transfecting cells with the DR-GFP plasmid (Vriend et al., 2014). The other polyclonal cell line was generated by stably transfecting cells with the TK-GFP plasmid (Dr. Janna Luessing) in order to look at the NHEJ repair pathway. In both cell lines DSBs are induced by the I-SceI restriction enzyme and the repair is measured as GFP expression (Figure 3.24 Ai-Bi). In case of the TK-GFP construct two I-SceI cut sites are beside the Tyrosine Kinase (TK) gene. The eGFP gene is downstream the TK domain. If both sites are simultaneously cut and the interfering TK gene fragment is lost the CMV promoter can be ligated close to the eGFP resulting in green cells. Any resection at either I-SceI site would prevent ligation of CMV to GFP and would not allow eGFP expression. For the DR-GFP construct there is a Sce-GFP gene containing mutations that inactivate the GFP. This mutated GFP is also engineered to contain an
ISceI cut site compared to the iGFP (wild type GFP gene) downstream, which contains a BglI site. Upon an I-SceI-induced DSB the iGFP gene downstream can function as a template for gene conversion, converting the Sce-GFP mutation and allow expression of full-length WT GFP. Both polyclonal stable cell lines were depleted for ZC3H14 and GFP positive cells counted by FACS analysis upon transfection of these cells with a plasmid expressing the I-SceI restriction enzyme. We observed that ZC3H14 is involved in the NHEJ but not in the HR repair pathway (Figure 3.24 Aii-Bii). In terms of NHEJ efficiency when ZC3H14 is knocked down we get a 20% GFP expression compared to control cells. A similar percentage is detected in cells depleted of DNA ligase 4, which is a protein involved in the NHEJ pathway. In terms of HR efficiency when ZC3H14 is knocked down we get a 100% efficiency similar to control cells, while in case of BRCA1 knock down the HR efficiency drops to 50% since BRCA1 has important function in HR.

Interestingly, we have observed that ZC3H14 depletion does not affect HR-dependent DSB repair even though a slightly sensitivity to Olaparib is visible at higher dose when ZC3H14 is depleted (Figure 3.23 A), yet both 53BP1 (Figure 3.6) and BRCA1 (Figure 3.9) recruitment to DSBs is defective. When ZC3H14 is depleted we observe defective BRCA1 IRIFs in PCNA negative cells only while BRCA1 foci are not affected in PCNA positive cells (Figure 3.9). This might explain the reason why we cannot detect any defect in the HR repair pathway (Figure 3.24 Bii). Cells in S-phase might still be able to efficiently repair the DSBs by HR when ZC3H14 is depleted. Also, upon depletion of BRCA1, BRCA1-dependent HR is rescued by co-depletion of 53BP1 (Bunting et al., 2010; Ochs et al., 2016). In absence of BRCA1 and 53BP1, DSBs is repaired by SSA (Bunting et al., 2010). Based on what is known in the literature, our data could suggest a role for ZC3H14 in SSA. ZC3H14 depletion might cause hyper-resection which is then repaired by SSA repair pathway. Further analysis might be required to assess the levels of resection upon ZC3H14 depletion and its role in SSA.
Figure 3.24: ZC3H14 is involved in the NHEJ, but not in the HR, repair pathway. 

Ai) The TK-GFP construct contains a CMV promoter followed by the TK domain, the eGFP domain, the IRES and a Puro cassette for the selection. Two IScel cut sites are localised at the sides of the TK domain. Aii) HEK 293-T TK-GFP polyclonal cells were transfected with siRNAs against DNA ligase 4, BRCA1 and ZC3H14. 24 hours later they were transfected with a plasmid expressing the IScel restriction enzyme in order to induce the cut and they were left for 48 hours at 37°C when they were trypsinised and analysed by FACS. The level of GFP expression was normalised to the control cells (siCTL).

Bi) The DR-GFP presents a mutated GFP (SceGFP) which contains an IScel cut site and a wild type GFP (iGFP) which contains a BglI cut site. The two GFP domains are separated by a puro cassette for the selection. Bii) HEK 293-T DR-GFP polyclonal cells were transfected with siRNAs against DNA ligase 4, BRCA1 and ZC3H14. 24 hours later they were transfected with a plasmid expressing the IScel restriction enzyme in order to induce the cut and they were left for 48 hours at 37°C when they were trypsinised and analysed by FACS. The level of GFP expression was normalised to the control cells (siCTL).
3.6 Discussion

Human cells have developed different mechanisms to act against different threats. DNA damage is one of the most dangerous threats our cells can go through because, if it is not properly repaired, it can lead to cancer. This is the reason why our cells have a complex pathway through which they recognise the presence of DNA damage and coordinate a complex series of cellular responses to maintain genome stability. One of the most important player in this pathway is ATM, a member of the PIKKs family, which once is recruited at sites of damage, phosphorylates many downstream proteins required for cell signalling, repair and apoptosis (Matsuoka et al., 2007). Because of its central role our laboratory performed a quantitative proteomic screen for ATM partners. We identified many potential ATM-interacting proteins including the C3H-type zinc finger protein, ZC3H14, as a novel ATM interactor (Pessina et al., 2014).

Zinc finger motifs are abundant proteins with the C2H2 type being the biggest family. With its five zinc finger domains, which contain three cysteines and one histidine, ZC3H14 belongs to the C3H1 family. A part from this difference in the zinc finger domain structure, zinc finger motifs are well known for binding DNA, RNA and proteins. The majority of them are found in transcription factors. ZC3H14 has been reported to be a polyadenosine binding protein, first identified in yeast (Kelly et al., 2014). A role for ZC3H14 in the DNA damage response (DDR) has not been reported. Here we identify a new biological function for ZC3H14. We provide evidence of a role for ZC3H14 in the DDR, in particular we show a function for ZC3H14 in the recruitment of DDR proteins that function downstream of MDC1 to sites of DNA damage.
Although we could confirm the interaction between ATM and ZC3H14 and assess a role for the relevance of this interaction in the DDR we could not detect ZC3H14 at ionising radiation induced foci (IRIF). This last data could lead to different hypothesis. ZC3H14 was found to localise to Nuclear Speckles which are interchromatin granules. In intact cells it is possible that only a small fraction of the abundant ZC3H14 protein associates with chromatin which prevents its visualisation to site of DNA damage upon ionising radiation, while the majority of ZC3H14 localises to nuclear speckles. This could also explain our failure to detect ZC3H14 at sites of DNA DSBs using the more sensitive U2OS-DSB reporter cell line system (Shanbhag et al., 2010; Tang et al., 2013). Although Nuclear Speckles are interchromatin granules, they might get disrupted during chromatin fractionation explaining the reason why we see ZC3H14 precipitating with the chromatin fraction. The importance of ZC3H14 in the DDR was confirmed by looking at two important markers in the DNA damage response such as H2AX-S139p, termed γH2AX, and ATM-S1981p. Interestingly, cells depleted for ZC3H14 displayed persistent γH2AX and ATM-S1981p foci compared to control cells suggesting that this zinc finger containing protein is required for efficient DNA repair. We also showed that cells are more sensitive to IR in absence of ZC3H14, as its depletion in both U2OS and RPE1 cells resulted in comparable sensitivity to ionising radiation as observed for inhibition of ATM kinase activity. This suggests an important role for this zinc finger protein in the DNA damage response pathway.

ZC3H14 depletion in U2OS cells also resulted in sensitivity to ICRF-193, but little if any sensitivity to the doses of Olaparib used. It is been previously shown that ICRF-193 inhibits topoisomerase II resulting in hypersensitivity of cells defective in the nonhomologous DNA-end joining machinery (Adachi et al., 2003), whereas Olaparib inhibits the poly (ADP-ribose) polymerase (Caldecott, 2014). The inhibition of the poly (ADP-ribose) polymerase leads to
an accumulation of single-strand DNA breaks (SSB) which are converted into DSB during DNA replication, which in turns are repaired by HR-dependent mechanism (Hellday, 2011). Our data is consistent with ZC3H14 being required for the NHEJ repair pathway. Further, support for this hypothesis was obtained using GFP reporter assays specific to either NHEJ or HR in polyclonal HEK-293T (Bindra et al., 2010; Vriend et al., 2014). Using these reporter assays we could detect a significant defect in the NHEJ, but not in the HR repair, pathway when ZC3H14 was depleted.

Upon establishing a role for ZC3H14 in the DDR we started dissecting this pathway to see where this protein might function. We first examined recruitment of 53BP1 into IRIF upon ZC3H14 depletion. Defective recruitment of 53BP1 into these sites of DNA damage was observed upon ZC3H14 depletion compared to control cells. Similar results were obtained with RIF1, consistent with its role downstream of 53BP1. Next, we examined BRCA1 recruitment to IRIFs. BRCA1 is required for resection and is also recruited into focal structures during S phase. To distinguish BRCA1 IRIF from these S phase foci we co-stained cells for PCNA and only scored PCNA-negative cells. We observed that PCNA negative cells were defective for BRCA1 focal recruitment when ZC3H14 was depleted. Since we could not detect a defect in repair by gene conversion upon ZC3H14 depletion, the defective BRCA1 IRIF in PCNA negative cells, together with a slightly accumulation of RPA2 S4p/S8p foci when ZC3H14 is depleted might suggest a much more important role for this zinc finger protein in single strand annealing (SSA) than gene conversion (GC); for instance depletion of ZC3H14 might promote error prone HR due to excessive resection. It would be interesting to see whether RAD52 focal recruitment is affected when ZC3H14 is knocked down since RAD52 plays a major role in SSA (Ma et al., 2017). All together the data above strongly suggests a role for ZC3H14 in upstream DNA repair pathway choice, that is upstream the
53BP1 and BRCA1 focal recruitment. All additional markers of DSBs we examined FK2, RNF168 and RNF8 were defective upon depletion of ZC3H14, whereas the upstream markers γH2AX, ATM-S1981p and MDC1 were not. Our data suggests a potential role for ZC3H14 between MDC1 focal recruitment and the RNF8 focal recruitment upon damage. RNF8 recruitment to sites of damage is triggered by the presence of phosphorylated MDC1 at the DSBs (Kolas et al., 2007). It is known that RNF8 specifically binds to MDC1-Thr719p and MDC1-Thr752p via its forkhead-associated (FHA) domain (Kolas et al., 2007). MDC1 is mainly phosphorylated by ATM (Stewart et al., 2003; Kolas et al., 2007), while MDC1 is recruited to DSB via its interaction with NBS1 (Melander et al., 2008). Specifically, phosphorylation of the so called SDTD region of MDC1 by Casein kinase 2 (CK2) is required for the MDC1-NBS1 interaction (Spycher et al., 2008). MDC1 recruitment to sites of damage triggers its sumoylation and ubiquitination (Luo et al., 2012), which in turn leads to MDC1 degradation. These important MDC1 modifications upon damage do not seem to affect its recruitment to DSBs. MDC1 migrates on SDS-PAGE gels as two strong bands of similar intensity, one upper and one lower, with a middle band that is much less intense (Goldberg et al., 2003; Stewart et al., 2003). These bands migrate at an apparent molecular weight of between 250 kDa and 148 kDa, and all three bands shift upon DNA damage due to phosphorylation. When we examined the MDC1 pattern upon ZC3H14 depletion in the absence of exogenous DNA damaging agents, in U2OS and RPE1 h-TERT cells, we could detect the three MDC1 isoforms, upper, middle and lower, but the bottom one was much stronger than the top one compared to control cells. Even at time points after IR when less MDC1 can be detected in control cells and ZC3H14 depleted cells, the MDC1 lower band was always more abundant than the upper band in ZC3H14 depleted cells compared to control cells. Nothing is known about these three MDC1 isoforms. They could be different isoforms which are generated by alternative splicing. As a poly(A) binding protein, ZC3H14 might
regulate the length of the poly(A) tail of transcripts allowing a longer 3’ extended transcripts with extra exons. Since MDC1 is subject to different modifications, the three MDC1 bands detectable by western blot, could be modified forms of MDC1 with the lower band of MDC1 being the actual protein and the upper band being a modified form of MDC1. It has been reported that both upper and lower MDC1 bands are phosphorylated on Thr719 upon damage and that only the phospho-form, which resulted to be the upper band, disappears upon ATMi treatment (Kolas et al., 2007). Interestingly, we detected an interaction between MDC1 and ZC3H14. Because ZC3H14 is also found to interact with ATM and MDC1 is an actual ATM substrate, this last data might suggest that ZC3H14 could act as bridge between MDC1 and ATM and be required for the MDC1 ATM-dependent phosphorylation. Based on the fact that ZC3H14 is required for RNF8, but not MDC1, recruitment to DSBs we tested whether ZC3H14 is important for the MDC1-RNF8 interaction in U2OS WT and ZC3H14 KO cells. However, the MDC1-RNF8 interaction was not affected in ZC3H14 KO cells.

It is known that RNF8 binds phosphorylated MDC1 and its recruitment to DSBs is ATM-dependent (Kolas et al., 2007). When we saw defective RNF8 recruitment to sites of DNA damage, but not MDC1, upon ZC3H14 depletion we hypothesised that this C3H1-type zinc finger protein might be involved in the regulation of the MDC1-RNF8 interaction. However, this was not the case. RNF8 binds MDC1 on pThr719 and pThr752 and its recruitment to DSBs is dependent on ATM (Kolas et al., 2007). The ATM-dependent focal recruitment of RNF8 is likely explained by the fact that ATM is required for the MDC1 phosphorylation on the Thr719 and Thr752, although there is no currently evidence of a direct defect of RNF8 focal recruitment when those MDC1 residues are mutated. This does not exclude a few additional hypotheses. RNF8 might bind other MDC1 phosphorylated sites, which might be phosphorylated by ATM, or other PIKKs upon damage in order to be recruited to DSBs.
(Goldberg et al., 2003). In fact, MDC1 presents many TQXF consensus sites that can be phosphorylated upon damage (Kolas et al., 2007) and might be necessary for the MDC1-RNF8 interaction. In this case, the mutation of Thr719 or Thr752 of MDC1 might not affect the RNF8 recruitment to sites of damage, although a direct relationship between the RNF8 recruitment to DSBs and these phospho-MDC1 sites has never been shown. RNF8 could be itself an ATM substrate, this would mean that the RNF8 recruitment to sites of damage is not exclusively MDC1-dependent and explain the reason why its recruitment to DSBs is affected upon ATM kinase activity inhibition. (Kolas et al., 2007).

From the literature and our data we could hypothesise that ZC3H14 might be required for MDC1 phosphorylation on specific residues that are required for the MDC1 activation and subsequent transduction of the signal to the downstream DDR factors. This suggests that ZC3H14 is functioning upon DNA repair pathway choice. In conclusion, here we report that:

- **ZC3H14**, which is found at Nuclear Speckles, interacts with ATM and is required for genome integrity. ZC3H14 depletion results in sensitivity to ionising radiation in U2OS and RPE1 cells. U2OS cells also show persistent γH2AX and ATM-S1981p upon ZC3H14 depletion.
- **U2OS cells** are sensitive to ICRF-193, but not to Olaparib, upon ZC3H14 depletion suggesting a role for this zinc finger protein in the NHEJ repair pathway, but not in the HR repair pathway.
- **RIF1, 53BP1, BRCA1, FK2, RNF168, RNF8 IRIFs** but not MDC1 IRIFs are affected upon ZC3H14 depletion.
- **ZC3H14 depletion results** in more abundant MDC1 lower band compare to control cells.
- **ZC3H14 interacts** with MDC1 but it does not affect the MDC1-RNF8 interaction.
3.7 Future work

Although we narrowed down where in the DDR pathway ZC3H14 acts there are many questions to be answered.

Firstly, does ZC3H14 play a role in resection and SSA? We could look at the recruitment of DDR factors required for efficient SSA when ZC3H14 is depleted. We could examine a GFP reporter assay specific for SSA upon ZC3H14 depletion.

Secondly, is ZC3H14 an ATM substrate? We could test this hypothesis by looking at the phosphorylation of ZC3H14 in presence or absence of ATM kinase activity inhibitor by immunoprecipitating tagged ZC3H14. It would be nice then to mutate the sites identified and investigate whether ZC3H14 activity is increased or reduced in some of these mutants.

Thirdly, does ZC3H14 localise to site of DNA damage? A more sensitive technique might be needed to see ZC3H14 localisation at breaks, such as the laser-striping methodology. If ZC3H14 is found at laser stripes then it would be extremely interesting to see if this hypothetical localisation is dependent on all or some of the zinc finger domains.

Fourthly, Are the MDC1 bands isoforms or different MDC1 modifications? It would be interesting to mass spec these two MDC1 bands to clearly understand what they are. Once this is clear, based on our data, we could investigate the role that ZC3H14 has on regulating MDC1 stabilisation.

Fifthly, is ZC3H14 required for the phosphorylation of MDC1 on the Thr719 and Thr752? We could test whether these MDC1 phosphorylation events are responsible for the RNF8 recruitment to DSBs. RNF8 might also be phosphorylated upon damage and perhaps, ZC3H14 might be responsible for such phosphorylation.
Sixthly, does ZC3H14 act as a bridge between ATM and MDC1? Since MDC1 is phosphorylated by ATM (Stewart et al., 2003; Kolas et al., 2007) and ZC3H14 interacts with both ATM and MDC1, ZC3H14 might work as a bridge between ATM and MDC1 and allow the MDC1 activation by ATM. In this case it would be interesting to map the region of ZC3H14 that interacts with ATM and MDC1.

Considering the published literature and taking all the above data together is clear that ZC3H14 plays more than one role in the human cells. We report here, for the first time, a role for ZC3H14 in the DDR. ZC3H14 acts upstream of DNA double strand break repair pathway choice between the MDC1 and RNF8 proteins. It is still unclear precisely how this C3H1-type zinc finger protein modulates the DNA damage repair.
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Table 2: Antibodies conditions used for Western Blot
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Table 3: Antibodies conditions used for immunofluorescence
### Table 4: siRNA sequences

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<tr>
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<td>eGFP</td>
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<tr>
<td>DNA-PK</td>
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<tr>
<td>DNA ligase 4</td>
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### Table 5: gRNAs sequences and mutations. The PAM sequence is outlined in yellow.

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<td>Deletion of 2 G p149, insertion (A) p143</td>
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<td>Stacking Gel 5% (36:1 cross-linking ratio)</td>
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<tr>
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<td>------------------------------------------</td>
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<tr>
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<td>40% Acrylamide</td>
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Table 6: 6,5% Gel (80:1 cross-linking ratio) recipe.
CHAPTER 4

Publication arises during the PhD

Recruitment of lysine demethylase 2a to DNA double strand breaks and its interaction with 53BP1 ensures genome stability
4.1 Personal contribution to the paper

I worked on this project together with Dr. Murilo T.D. Bueno, who held a Post-Doc position when I started working in Prof Noel F. Lowndes’ lab. Dr. Bueno started this project during his PhD in Canada, he brought it over and at the time he left the project was almost ready for a publication so I took it over and completed it.

My contribution to this paper is listed below.

- Fig 3A-B
- Fig 4A-B-C together with the relative text
- Fig 5C
- Materials and Methods: Immunofluorescence; Microscopy and images analysis, Analysis of ATM Phosphorylation
- I addressed all the reviewer’s suggestions by generating so all the supplementary materials
4.2 Paper

Recruitment of lysine demethylase 2A to DNA double strand breaks and its interaction with 53BP1 ensures genome stability

Murilo T.D. Bueno\textsuperscript{1,2,3,*}, Marta Baldascini\textsuperscript{4,*}, Stéphane Richard\textsuperscript{1,3,3} and Noel F. Lowndes\textsuperscript{4}

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\textsuperscript{2}Department of Medicine, McGill University, Montréal, Québec, Canada
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\* These authors have contributed equally to this work

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Keywords: DNA double strand break repair; genome stability; KDM2A; 53BP1; ubiquitin

Received: November 26, 2017 Accepted: February 27, 2018 Published: March 23, 2018

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ABSTRACT

Lysine demethylase 2A (KDM2A) functions in transcription as a demethylase of lysine 36 on histone H3. Herein, we characterise a role for KDM2A in the DNA damage response in which KDM2A stimulates conjugation of ubiquitin to 53BP1. Impaired KDM2A-mediated ubiquitination negatively affects the recruitment of 53BP1 to DSBs. Notably, we show that KDM2A itself is recruited to DSBs in a process that depends on its demethylase activity and zinc finger domain. Moreover, we show that KDM2A plays an important role in ensuring genomic stability upon DNA damage. Depletion of KDM2A or disruption of its zinc finger domain results in the accumulation of micronuclei following ionizing radiation (IR) treatment. In addition, IR-treated cells depleted of KDM2A display premature exit from the G2/M checkpoint. Interestingly, loss of the zinc finger domain also resulted in 53BP1 focal distribution in condensed mitotic chromosomes. Overall, our data indicates that KDM2A plays an important role in modulating the recruitment of 53BP1 to DNA breaks and is crucial for the preservation of genome integrity following DNA damage.

INTRODUCTION

Lysine demethylase 2A (KDM2A), also known as JHDM1A/FBXL11, was the first Jumonji C (JmjC)-domain-containing histone demethylase to be identified \[1\]. JmjC domains are present in metalloenzymes that function in histone demethylation and characterise the Jumonji family of transcription factors \[2\]. KDM2A has been implicated in the regulation of gene transcription \[1, 3\] by preferentially demethylating Lys36 on histones H3 (H3K36me3), a histone mark commonly associated with gene activation \[4\]. KDM2A also contains a CXXC-type zinc finger domain, a PHD domain, one F-box domain and numerous C-terminal leucine-rich repeats. Both over and under expression of KDM2A have been linked to oncogenesis \[5\] and, although the underlying mechanisms have not been defined, depletion of KDM2A has also been linked to cell survival upon genotoxic damage \[3, 6\].

The p53-binding protein 1 (53BP1) is a major regulator of DNA double-strand break (DSB) repair \[7\]. In particular, by impeding resection, 53BP1 preferentially promotes the non-homologous end-joining DNA repair pathway \[7\]. 53BP1 specifically localizes to DSBs and its recruitment to damaged chromatin is essential for maintenance of genomic integrity \[7\]. The accumulation of 53BP1 at DSBs relies on the recognition of ubiquitinated Lys15 on histones 2A (RD1AK15b) and demethylated Lys20 on histones 4 (H4K20me2) \[8, 9\]. These histone marks are recognized by 53BP1 through its tandem tudor
The localization of 53BP1 to DSBs is directly dependent on the ubiquitination of chromatin that surrounds DNA breaks. The E3 ubiquitin ligases RNF8 and RNF168 play a major role in this process [7, 10, 11]. In fact, RNF8-dependent and K63-linked ubiquitination of the linker histone H1 at DSBs [12], and possibly other substrates, provides a high affinity docking site for RNF168 which in turn is responsible for ubiquitination of H2AK115 and subsequent 53BP1 UDR-dependent recruitment [13]. Furthermore, RAD18 and BRCA1 have similarly been shown to interact with 53BP1 and promote its ubiquitination [14, 15].

More recently, KDM2A and its closest homolog, KDM2B/FBX110, have both been reported to promote ubiquitination of β-catenin and c-Fos, respectively [16, 17]. The levels of KDM2A-induced ubiquitination of β-catenin were shown to depend on the demethylase activity of KDM2A. In addition, both INI1 and PHD domains of KDM2A are necessary to promote β-catenin degradation [16]. KDM2B was reported to form an SCF (SKP2-CUL1-F-box) E3 ubiquitin ligase complex via the interaction between its F-box domain and SKP1/CUL1. Moreover, the C-terminal leucine-rich repeats of KDM2B were shown to mediate the binding between KDM2B and its substrate c-Fos [17]. It remains unknown whether KDM2A functions in an SCF E3 ubiquitin ligase complex. These findings indicate that ubiquitination by KDM2A family of lysine demethylases exerts significant cellular functions.

In this report, we demonstrate that KDM2A associates with and ubiquitines 53BP1. Impaired interphase nucleoporin 2 proteins negatively affected 53BP1 recruitment to DSBs and resulted in increased DNA damage-induced genomic instability. Moreover, we provide data showing that KDM2A accumulates at DSBs via a mechanism that depends on its demethylase activity and zinc finger domain. We also report the occurrence of DNA damage-stimulated 53BP1 nuclear accumulation in mitotic cells expressing a ubiquitination-defective mutant of KDM2A.

RESULTS

KDM2A interacts with 53BP1

53BP1 has been identified as a potential KDM2A interactor by proteomic studies [18, 19]. Moreover, KDM2A was demonstrated to associate with ATM [20, 45], previously shown to interact and phosphorylate 53BP1 [21–23]. To independently validate whether KDM2A associates with 53BP1, we immunoprecipitated wild-type FLAG-tagged KDM2A from HeLa293T cell lysates and probed immunoprecipitates with anti-53BP1 (Figure 1A).

Indeed, we observed that endogenous 53BP1 co-purifies with FLAG-KDM2A. RAD18 was shown to interact with 53BP1 via its zinc finger domain [14]. Thus, we evaluated whether the zinc finger domain of KDM2A also mediates the interaction between this protein and 53BP1. In fact, immunoprecipitation analyses demonstrated that depletion of the amino acid residues comprising the CXXC-ZF domain of KDM2A (KDM2A-ZF) significantly impaired the association between MYC-KDM2A and FLAG-53BP1 (Figure 1A).

However, the lysine demethylase activity of KDM2A did not affect its interaction with 53BP1. The association between 53BP1 and KDM2A-Δ2521A, harbouring mutations H212A/D214A known to abrogate demethylase activity [24, 25], efficiently associated with 53BP1.

In order to identify the region of 53BP1 necessary for its interaction with KDM2A, we analyzed a panel of previously described 53BP1 mutants [26]. Co-expression of HA-tagged 53BP1-phospho-mutant 1A–Q, which has all 15 (S/T) Q sites changed to AQ, with full length MYC-KDM2A revealed that the association between these proteins does not depend on the phosphorylation status of 53BP1 (Figure 1B, lower panel). However, analysis of a 53BP1 fragment corresponding to the N-terminal 1051 amino acids demonstrated that these amino acid residues are insufficient to support an interaction with KDM2A (Figure 1B, lower panel, compare lanes 1 and 2).

In contrast, a C-terminal 53BP1 fragment lacking amino acids 1 to 1051 (A1–1051) associated with KDM2A, indicating that the C-terminal region of 53BP1 is necessary for this interaction (Figure 1B, lower panel, compare lanes 2 and lane 3). Further mapping revealed that amino acid residues 1052–1388 are necessary for the association between 53BP1 and KDM2A (Figure 1B, lane 4), whereas neither the Tudor nor the BRCT domains are required (Figure 1B, lanes 5 and 6).

Little is known about lysine methylation within 53BP1. Only three lysine residues have been reported to undergo methylation, but the relevance of these modifications remains undetermined [27]. Since we confirmed an interaction between 53BP1 and the lysine demethylase KDM2A, we evaluated the methylation status of 53BP1 lysine residues. Given that antibodies specific for lysine methylated 53BP1 are not available, we used a pan-dimethyl lysine antibody (anti-Kma2) which has been successfully utilized to detect lysine methylation of non-histone proteins [28–30]. Lu et al. used this antibody to detect partial demethylation of β-catenin [16] promoted by KDM2A.

Interestingly, immunoprecipitation of 53BP1 followed by immunoblotting with anti-Kma2 suggested that 53BP1 harbours methylated lysines residues (Figure 1C). However, we did not observe a change in the lysine methylation status of 53BP1 when either wild type
Figure 1: KDM2A associates with 53BP1. (A-i) KDM2A interacts with endogenous 53BP1. HEK293T extracts from cells transfected with an empty vector or FLAG-tagged KDM2A were subjected to immunoprecipitation with anti-FLAG beads. Interaction between KDM2A and 53BP1 was observed by immunoblotting with anti-53BP1. (A-ii) Deletion of the zinc finger domain (564-610) of KDM2A disrupts its interaction with 53BP1. Schematic of KDM2A protein structure (top panel). FLAG-tagged 53BP1 co-expressed with wild type MYC-tagged KDM2A (WT), demethylase-deficient KDM2A (DD) or KDM2A lacking the zinc finger domain (AZF) was immunoprecipitated with anti-FLAG beads and immunoblotted with anti-MYC and anti-FLAG. (B) The region comprising residues 1062-1302 of 53BP1 is necessary for its interaction with KDM2A. Schematic of full-length 53BP1 and mutants (right panel). Lysates from cells expressing HA-tagged 53BP1 mutants and MYC-KDM2A-WT were immunoprecipitated with anti-HA and immunoblotted with anti-HA and anti-MYC. (C) 53BP1 interacts methylated lysine residues. Lysates from cells expressing FLAQ-53BP1, co-expressing or not MYC-tagged KDM2A variants, were immunoprecipitated with anti-FLAG beads and immunoblotted with anti-MYC, anti-FLAG and penta-methyl lysine antibody (anti-Kme2).
KDM2A or the demethylase-deficient KDM2A mutant (KDM2A-DD) were expressed, indicating that KDM2A does not affect the methylation of 53BP1 detected using this assay.

KDM2A expression stimulates 53BP1 ubiquitination and modulates its stability

KDM2A has been shown to stimulate ubiquitination of β-catenin and, ultimately, regulate the stability of this protein [16]. Therefore, we next tested whether KDM2A could affect 53BP1 ubiquitination.

In order to estimate a potential role of KDM2A in stimulating overall protein ubiquitination, we co-expressed wild type KDM2A-WT, demethylase-deficient (KDM2A-DD) or zinc finger deletion (KDM2A-ΔZF) mutants with HA-Ubiquitin in HEK293T cells. We used low level expression of HA-Ubiquitin in order to increase the sensitivity of this assay. Immunoblotting analysis with anti-HA antibody revealed a robust increase in total protein ubiquitination in samples co-expressing HA-Ubiquitin and FLAG-KDM2A-WT or FLAG-KDM2A-DD when compared to cells expressing only HA-Ubiquitin (Figure 2A, compare lanes 2 with 3 and 4). However, expression of the zinc finger deletion mutant (KDM2A-ΔZF) displayed significantly less ubiquitination of cellular proteins detected in this assay (Figure 2A, compare lanes 3 and 4 with 5), suggesting that KDM2A-mediated enhanced ubiquitination of multiple proteins is dependent on an intact zinc finger.

Next, we conducted immunoprecipitations to assess the effect of KDM2A on 53BP1-specific ubiquitination. Compared to samples expressing only HA-Ubiquitin, the levels of ubiquitinated endogenous 53BP1 were substantially increased by co-expression of wild type KDM2A-WT (Figure 2B, compare lanes 1 and 2), while the demethylase-deficient mutant (KDM2A-DD) only marginally enhanced 53BP1 ubiquitination. Importantly, the zinc finger deletion mutant (KDM2A-ΔZF) completely failed to stimulate ubiquitination of 53BP1 (Figure 2B, compare lanes 2 and 4). Although the levels of total ubiquitinated proteins look similar in Figure 2B, cells shown in Figure 2A were transfected with 5-fold less plasmid encoding for ubiquitin to increase the sensitivity of the assay and prevent saturation of protein ubiquitination.

To further confirm these results, we performed immunoprecipitations with lysates from cells expressing exogenous FLAG-tagged 53BP1. Immunoblotting analyses confirmed the ability of wild type KDM2A to stimulate 53BP1 ubiquitination (Figure 2C, compare lanes 1 and 2). However, probably due to higher 53BP1 protein levels because of exogenous expression of 53BP1, expression of the KDM2A-DD mutant also resulted in a significant increase in 53BP1 ubiquitination. This result is unlikely due to differences in the amount of soluble and chromatin-associated 53BP1 since all immunoprecipitations were carried out with lysis buffer containing benzamidine, a nuclease that digests both DNA and RNA.

Nevertheless, in support of the previous results, the KDM2A-ΔZF mutant was incapable of stimulating ubiquitination of exogenous 53BP1 (Figure 2C, compare lanes 2 and 3 with 4). Thus, with either endogenous or overexpressed 53BP1, we observed that the zinc finger motif of KDM2A is required for 53BP1 ubiquitination.

Interestingly, the enhanced ubiquitination of 53BP1 caused by KDM2A expression seemed to result in increased degradation of 53BP1, as suggested by smeared bands observed below full-length 53BP1 (Figure 2C, lanes 2 and 3). Thus, we measured the protein stability of 53BP1 upon KDM2A expression using the protein synthesis inhibitor, cycloheximide (Figure 2D). HEK293T cells expressing HA-Ubiquitin with concomitant expression of wild type FLAG-KDM2A or empty vector, were treated with cycloheximide, lysed at the indicated time points and total cell lysates were analysed by immunoblotting. Quantification of 53BP1 levels normalised to Tubulin indicated that decreased 53BP1 stability correlated with enhanced ubiquitination by KDM2A (Figure 2D, lower panel).

KDM2A modulates the recruitment of 53BP1 to DSBs and its depletion results in premature exit from the G2/M checkpoint induced by ionizing radiation

The recruitment of 53BP1 to DNA damage sites is necessary to ensure proper DNA repair [7]. Thus, we evaluated whether KDM2A could influence the formation of 53BP1 foci upon ionizing radiation (IR).

To analyse the effects of down regulation or overexpression of KDM2A on 53BP1 foci formation, we generated U2OS cells stably expressing wild type (WT), demethylase-defective (KDM2A-DD) or zinc finger deleted (KDM2A-ΔZF) MYC-tagged KDM2A. Transfection of siRNA targeting the 3'-UTR region of the KDM2A mRNA resulted in efficient downregulation of endogenous KDM2A, when compared to cells transfected with control siRNA (Supplementary Figure 1A, compare lanes 1 and 2). We stably overexpressed MYC-KDM2A-WT, MYC-KDM2A-DD and MYC-KDM2A-ΔZF mutant in these cells as assessed by immunoblotting (Supplementary Figure 1A, lanes 3–5).

Immunofluorescence analyses showed that depletion of endogenous KDM2A resulted in a significant reduction in 53BP1 IRIF detected in cells 1 hour after treatment with 2Gy IR (Figure 3A). Importantly, this defective recruitment of 53BP1 to DSBs caused by depletion of endogenous KDM2A could be reversed by concomitant expression of MYC-KDM2A-WT. However, cells expressing the demethylase-defective (MYC-KDM2A-
Figure 2: KDM2A modulates 53BP1 ubiquitination and stability. (A) KDM2A expression enhances overall levels of protein ubiquitination. Total cell lysates from HEK293T cells transfected or not with 1 μg of pLPC-Puro-FLAG-KDM2A-WT or KDM2A mutants, and low amount of pRKG-HA-Ubiquitin-WT (0.2 μg) were immunoblotted with anti-HA, anti-FLAG and anti-Tubulin. (B) Stimulation of 53BP1 ubiquitination is dependent on the demethylase activity and zinc finger domain of KDM2A. Lysates from cells transfected with MYC-tagged KDM2A variants (2 μg) and HA-Ubiquitin (1 μg) were immunoprecipitated with anti-53BP1 and endogenous ubiquitinated 53BP1 was detected by immunoblotting with anti-HA. (C) Lysates from cells co-transfected with pDNAAS-FRT/TO-Flag-53BP1 (1 μg), plasmids encoding for MYC-KDM2A (1 μg) variants and HA-Ubiquitin (0.5 μg) were immunoprecipitated with anti-FLAG beads and immunoblotted with anti-HA, anti-FLAG and anti-MYC. (D) KDM2A expression regulates 53BP1 protein stability. Cells transfected with HA-Ubiquitin (1 μg) in the presence or absence of exogenous FLAG-KDM2A WT (2 μg) were treated with cycloheximide (50 μg/ml) and lysed at the indicated time points. Levels of endogenous 53BP1 were detected by immunoblotting with anti-53BP1. Additionally, immunoblotting was performed with anti-Tubulin, anti-FLAG and anti-HA. A graph displaying the ratio between 53BP1 and tubulin protein levels determined with ImageJ is shown at the bottom.
Figure 3: KDM2A modulates the recruitment of 53BP1 to DSBs and entry into mitosis upon IR. (A) KDM2A modulates 53BP1 accumulation at DSBs generated by irradiation. U2OS cells transfected with control siRNA (siCTRL) or siRNA targeting the 3'-UTR region of the KDM2A mRNA (siUTR) and stably expressing MYC-tagged KDM2A variants were irradiated (2 Gy), fixed 1 h later and subjected to immunofluorescence analysis with DAPI, anti-53BP1 (green) and anti-MYC (red). Quantification of immunofluorescence analyses (right panel) shows the average number of 53BP1 IRIF per cell in each condition. Experiments were performed in triplicate. Single (‘), double (""""') and triple (""""""") asterisks indicate p<0.05, two-tailed paired Student t-test. Error bars indicate standard deviation. (B) KDM2A depletion impairs 53BP1 recruitment to DNA breaks generated by FokI endonuclease. U2OS-DSB-reporter cells transfected with siCTRL or siKDM2A were treated with Shield-1 and 4-OHT to promote expression of mCherry-LacI-FokI (FokI) (red). Immunofluorescence analyses were conducted with anti-53BP1 (green) and anti-MYC. Quantification of immunofluorescence analyses (right panel) shows the percentage of cells displaying colocalization between 53BP1 and FokI. Asterisk indicates p<0.05, two-tailed paired Student t-test. Error bars indicate standard deviation. (C) KDM2A depletion results in faster progression into mitosis without proper repair of damaged DNA. U2OS cells transfected with siCTRL or siKDM2A were left untreated or irradiated (2 Gy) and harvested at the indicated time points after irradiation. The percentage of mitotic cells, as indicated by H3pSer10 staining, was determined by flow cytometry. Data shown is representative of experiments replicated three times.
and, in particular, the zinc finger deleted (MYC-KDM2A-ΔZF) mutant forms of KDM2A, displayed significantly less 53BP1 IRIF.

As shown in Figure 2B, while both demethylase and zinc finger domains of KDM2A play a role in stimulating ubiquitination of endogenous 53BP1, deletion of the zinc finger domain resulted in a more severe ubiquitination defect. Thus, our data shows that deficient conjugation of ubiquitin to 53BP1 in the presence of these mutants is consistent with a reduction in the focal recruitment of 53BP1 to sites of DNA damage. In addition, we evaluated whether KDM2A depletion impacted the overall formation of IRIF by proteins harbouring ubiquitin chains. Immunofluorescence analyses using an anti-FK2 antibody (specific to ubiquitin chains), revealed that irradiated KDM2A-deficient cells displayed similar FK2 IRIF when compared to cells transfected with control siRNA (Supplementary Figure 2). To further support these observations, we used a previously described U2OS reporter cell line, U2OS-DSB-reporter [31, 32], that stably expresses a fusion protein termed, ER-m-Cherry-LacI-Foki-DD, which is composed of a modified estrogen receptor fused to mCherry, a lac repressor protein (LacI), the nucleosome of the Foki endonuclease and a destabilization domain (DD). Upon treatment with the Foki-1 ligand and 4-hydroxytamoxifen (4-OHT), ER-mCherry-LacI-Foki-DD is stabilized, re-localized to the nucleus and directed via its LacI moiety to bind to an integrated array of 256 LacO repeats at a single genomic locus. The Foki endonuclease moiety then generates numerous DNA double strand breaks at these LacO repeats. The localization of ER-m-Cherry-LacI-Foki-DD can be directly visualized via its mCherry moiety (Supplementary Figure 1B).

As expected, immunofluorescence analyses of U2OS-DSB-reporter cells treated with Shield-1 and 4-OHT and pre-extracted with cytoskeletal buffer (CSK), revealed co-localization between ER-m-Cherry-LacI-Foki-DD and 53BP1 (Figure 3B). However, upon depletion of endogenous KDM2A, and consistent with the 53BP1 IRIF data (Figure 3A), the recruitment of 53BP1 to DSBs induced within the LacO array was lost (Figure 3B). Thus, two independent approaches revealed that KDM2A depletion impairs the recruitment of 53BP1 to DSBs.

To further understand the role of KDM2A in the DNA damage response we evaluated its role in entry into and exit from the G2/M checkpoint upon IR by monitoring the levels of a mitotic marker (phosphorylation of Histone H3 on Serine 10, termed H3S10ph). Importantly, prior to irradiation, control and KDM2A-depleted cells showed similar cell cycle profiles (Supplementary Figure 3). Entry into the G2/M checkpoint was unaffected by depletion of KDM2A as these cells entered the checkpoint with similar kinetics to control cells (Figure 3C). However, relative to control cells, KDM2A-depleted cells exited the G2/M checkpoint between 3 and 4 hours earlier than control cells. This observation indicated that irradiation of cells depleted of KDM2A results in faster progression into mitosis without proper repair of damaged DNA. Noteworthy, immunoblotting performed with cell lysates obtained from different time points following irradiation did not reveal any striking difference in ATM phosphorylation in cells depleted of KDM2A relative to control cells (Supplementary Figure 4). This observation suggests that KDM2A depletion does not significantly impair checkpoint activation (and see also Figure 3C). However, in support of our findings, depletion of KDM2A has been shown to increase the sensitivity of breast cancer cells to cisplatin-induced DNA damage [33].

The demethylase activity and zinc finger domain of KDM2A mediate its accumulation at DSBs and are required for the recruitment of 53BP1 to DNA breaks

Although KDM2A forms focal structures in undamaged cells that are sensitive to treatment with CSK buffer, recruitment of KDM2A into IRIF was not detectable (Supplementary Figure 5). We performed immunofluorescence analyses in the U2OS-DSB-reporter cell line using antibodies against 53BP1, γH2AX and KDM2A (Figure 4A). Induction of DSBs in U2OS-DSB-reporter cells resulted in 53BP1 and γH2AX focal accumulation at DSBs induced within the LacO array (Figure 4A, top panel). Interestingly, the signal corresponding to ER-m-Cherry-LacI-Foki-DD was less diffuse than the signal obtained for either γH2AX or 53BP1 consistent with the fusion endonuclease being located directly at the LacO array rather than the surrounding chromatin. Immunofluorescence analyses using anti-KDM2A revealed that KDM2A also co-localised with the ER-m-Cherry-LacI-Foki-DD nucleus at DSBs induced within the LacO array but within the signal corresponding to γH2AX modified chromatin (Figure 4A, lower panel). Notably, post-extraction with cytoskeletal buffer to remove nucleoplasmic and weakly chromatin-bound KDM2A, was required for the visualisation of KDM2A localization at DSBs within the LacO array.

Immunofluorescence performed without CSK pre-extraction revealed a pan-nuclear distribution of KDM2A with numerous spots that did not change significantly upon Shield-1 and 4-OHT treatment (Supplementary Figure 1C and also Figure 3B).

Next, we used the U2OS-DSB-reporter system to assess the importance of the lysine demethylase activity and zinc finger domain of KDM2A in mediating its recruitment to DSBs. Endogenous KDM2A was depleted in U2OS-DSB-reporter cells that were also transfected with an empty plasmid or plasmids encoding MYC-tagged (MYC-KDM2A-WT), demethylase deficient (KDM2A-ΔZF) or zinc finger deleted (KDM2A-ΔZF). Immunofluorescence was performed with U2OS-DSB-
reporter cells in which DSBs were induced by addition of Shield-1 and 4-OHT and pro-extracted with CSK (Figure 4A). Similar to endogenous KDM2A (Figure 4A, lower panel), MYC-tagged wild type KDM2A was recruited to nuclease-induced DSBs as evidenced by co-localisation between MYC-KDM2A-WT and ER-mCherry-Lac-FokI-DD (Figure 4B). Importantly, unlike WT, the MYC-KDM2A-DD and MYC-KDM2A-ΔZF mutants failed to accumulate at DSBs. In agreement with Tanaka et al. [34], we observed that MYC-tagged wild-

Figure 4: Recruitment of KDM2A and 53BP1 to DSBs: depends on its demethylase activity and intact zinc finger domain. (A) Endogenous KDM2A is recruited to DSBs. Detectable KDM2A at the FokI-induced breaks upon CSK treatment. U2OS-DSB-reporter cells were treated or not with Shield-1 and 4-OHT to induce expression of ER-mCherry-Lac-FokI-DD (FokI) (red). Cells were immunostained with anti-γH2AX, anti-53BP1 (green, upper panel) and anti-KDM2A (green, lower panel). Immunofluorescence analyses were conducted using cells that were pro-extracted with CSK buffer. (B) The demethylase activity and zinc finger domain of KDM2A are necessary for its recruitment to DSBs. U2OS-DSB-reporter cells were transfected with siCTL or siUTR and plasmids encoding for MYC-tagged variants of KDM2A. Following transfection, cells were treated with Shield-1 and 4-OHT to induce expression of ER-mCherry-Lac-FokI-DD (FokI) (red). Immunofluorescence analyses were conducted with anti-MYC (green) and anti-γH2AX. (C) Focal accumulation of 53BP1 at DSBs is modulated by the demethylase activity and zinc finger domain of KDM2A. U2OS-DSB-reporter cells were transfected with siCTL or siUTR and plasmids encoding for MYC-tagged variants of KDM2A. Cells were treated with Shield-1 and 4-OHT to induce expression of ER-mCherry-Lac-FokI-DD (FokI) (red). Immunofluorescence analyses were conducted with anti-MYC and anti-53BP1 (green). The percentage of cells displaying colocalization between 53BP1 and FokI fusion protein was quantified in each condition (right panel). Experiments were repeated four times. Single (*), double (**) and triple (***)) asterisks indicate p < 0.05, two-tailed paired Student t-test. Error bars indicate standard deviation.
type KDM2A appeared to localise within nucleoli in the absence of DNA damage (Supplementary Figure 6). Similarly to WT KDM2A, the demethylase-deficient and zinc finger deletion mutants of KDM2A also localised within nucleoli. However, after treatment with ionising radiation, rather than localising within nucleoli, WT KDM2A was either localised to the nuclear periphery or appeared to be excluded from nucleoli. Interestingly, the zinc finger deletion mutant of KDM2A remained localised to the nucleoli, albeit more diffusely than WT, while the demethylase-deficient KDM2A behaved more similarly to the WT. As previously demonstrated in Figure 3A-3B, depletion of endogenous KDM2A resulted in impaired recruitment of 53BP1 to DSBs. Moreover, Figure 4B shows that KDM2A itself is recruited to DSBs in a process that depends on its demethylase and zinc finger domains. Consequently, we next evaluated the ability of the KDM2A mutants MYC-KDM2A-DD and MYC-KDM2A-DZF to regulate the accumulation of 53BP1 at DNA breaks. R immortalisation of wild type MYC-KDM2A into U2OS-DSB-reporter cells depleted of endogenous KDM2A allowed normal co-localization between 53BP1 and the DSBs induced within the LacO array (Figure 4C). However, the percentage of cells displaying co-localization between 53BP1 and the fusion endomucin class was significantly reduced upon expression of MYC-KDM2A-DD or the fusion endomucin class. In line with the 53BP1 IRIF data (Figure 3A), depletion of the zinc finger domain of KDM2A had a more drastic effect on preventing 53BP1 recruitment to induced DSBs. Interestingly, within the population of cells displaying efficient siRNA-mediated depletion of KDM2A, we observed that the majority (87%) of the cells displayed defective 53BP1 recruitment to Fok1-generated DSBs in the U2OS-DSB reporter cell line, while 13% of the KDM2A-depleted cells still displayed residual 53BP1 recruitment. This likely reflects different extents of KDM2A-depletion within the treated population. (Supplementary Figure 7).

In summary, our data are consistent with the lysine demethylase and zinc finger domains of KDM2A being necessary for the recruitment of KDM2A to DSBs where it is required for efficient 53BP1 focal accumulation at sites of DNA damage.

The zinc finger motif of KDM2A is required for genome stability by preventing both the accumulation of micronuclei and 53BP1 foci in mitotic chromosomes upon ionizing radiation

Defective repair of DNA double strand breaks is known to generateacentric chromosomes and chromatin fragments that in turn give rise to micronuclei [35]. Interestingly, and consistent with premature exit from the IR-induced G2/M checkpoint (Figure 3C), depletion of KDM2A resulted in a significant increase in the number of cells harbouring micronuclei 24h after irradiation (Figure 3A). Furthermore, we observed micronuclei displaying 53BP1 foci in KDM2A-depleted cells, suggesting that these structures indeed contained unrepair DNA (Figure 3A).

As previously shown (Figure 2B), KDM2A-DD displayed reduced ability to stimulate 53BP1 ubiquitination while KDM2A-DZF was incapable of promoting conjugation of ubiquitin to 53BP1.

Furthermore, cells expressing KDM2A-DD or MYC-KDM2A-DZF also displayed defective recruitment of 53BP1 to DSBs (Figure 3A and 4C). Thus, we evaluated the role of these mutants with respect to genome stability using a micronuclear assay. Endogenous KDM2A was depleted in U2OS cells stably overexpressing MYC-tagged KDM2A-WT, KDM2A-DD or MYC-KDM2A-DZF and micronuclei were quantified by immunofluorescence (Figure 5B). Unirradiated cells expressing endogenous MYC-KDM2A-WT or MYC-KDM2A-DD displayed similar percentages of cells with micronuclei to the percentage observed upon depletion of endogenous KDM2A (compare Figure 5B with Figure 5A, right panel). However, expression of MYC-KDM2A-DZF with concomitant depletion of endogenous KDM2A resulted in a significant increase in the percentage of cells harbouring micronuclei both in the absence and after IR treatment (Figure 5B). Interestingly, irradiation promoted a further increase in micronuclei frequency.

While monitoring micronuclei in these experiments, we noticed that mitotic cells expressing MYC-KDM2A-DZF displayed numerous 53BP1 foci within condensed mitotic chromatin 24h after irradiation (Figure 5C). This suggests the possibility that KDM2A downregulation could result in the recruitment of 53BP1 to chromatin during mitosis using a mechanism dependent upon its zinc finger domain. Interestingly, 53BP1 phosphorylation has been shown to modulate its recruitment to DNA breaks in mitotic cells [36, 37]. Intriguingly, depletion of endogenous KDM2A alone was not sufficient to produce this phenotype.

**DISCUSSION**

Thus far, lysine demethylation of both histones [24] and non-histone proteins [58] has been assigned as the primary function of KDM2A. However, KDM2A has also been shown to play additional biological roles such as regulation of β-catenin stability by promoting its ubiquitination [16]. Similarly, KDM2B, the closest homolog of KDM2A, has also been demonstrated to stimulate protein ubiquitination [17]. Thus, emerging evidence indicates that KDM2A executes some of its functions via mechanisms beyond its lysine demethylase activity.

Consistent with these novel findings, we report that KDM2A plays an important role in the mechanisms
Figure 5: KDM2A modulates genome stability upon IR. (A-i) KDM2A knockdown sensitizes cells to IR leading to micronuclei formation. U2OS cells transfected with siCTL or siKDM2A were irradiated (2Gy) and fixed 24 hrs later. Cells were stained with DAPI and micronuclei observed by fluorescence microscopy. The percentage of cells harboring micronuclei before and after IR was quantified in each condition (right panel). Experiments were performed in triplicates. Asterisk indicates p<0.05, two-tailed paired Student t-test. Error bars indicate standard deviation. (A-ii) 53BP1 foci formation in micronuclei found in KDM2A knockdown cells. Cells transfected with siKDM2A were irradiated (2Gy) and, 24 hrs later, subjected to immunofluorescence with anti-53BP1(green) and DAPI (blue). (B) The zinc finger domain of KDM2A is crucial for the maintenance of genome stability. Endogenous KDM2A was downregulated in U2OS cells stably expressing wild type MYC-tagged KDM2A-WT, -DD or -ΔZF mutants by transfection with siUTR. Cells were irradiated (2Gy) or left untreated and the percentage of cells showing micronuclei was determined 24 hrs after IR treatment. Single (*) and double (**) asterisks indicate p<0.05, two-tailed paired Student t-test. Error bars indicate standard deviation. Experiments were performed in triplicates. (C) 53BP1 localization in mitotic cells upon IR. U2OS cells transfected with siCTL or siUTR and, stably expressing or not, wild type MYC-KDM2A (WT), DD or ΔZF mutants were irradiated (2Gy) and fixed 24 hrs later. Cells were subjected to immunofluorescence with anti-53BP1 (green), anti-Tubulin (red) and DAPI (blue).
involved in DNA damage repair and maintenance of genome stability. Our data demonstrates that KDM2A interacts with 53BP1 via a process predominantly mediated by the zinc finger domain of KDM2A, which is reminiscent of the reported association between RAD18 and 53BP1 [14]. This finding suggests that associations between 53BP1 and its molecular partners are frequently mediated by zinc finger domains. Moreover, we observed that amino acid residues spanning positions 1052-1302 of 53BP1 are necessary for its interaction with KDM2A. Notably, these residues have already been shown to play a role in the recruitment of 53BP1 to DNA breaks [26].

Our data indicate, for the first time, that KDM2A stimulates ubiquitination and regulates the stability of 53BP1. While demethylase-deficient KDM2A showed reduced ability to promote 53BP1 ubiquitination, deletion of the zinc finger domain of KDM2A abrogated its capacity to stimulate conjugation of Ubiquitin to 53BP1. Notably, we provide data demonstrating that both endogenous and overexpressed KDM2A are recruited to DSBs. Further analysis of KDM2A mutants revealed that its zinc finger domain and demethylase activity are both necessary for the accumulation of KDM2A at DNA double strand breaks. Interestingly, other lysine demethylases have been shown to accumulate at DNA damage sites [39–43] consistent with enzymes involved in lysine demethylation playing active and important roles in the process of DNA repair.

In addition, we demonstrate that the association between KDM2A and 53BP1 is necessary for the proper recruitment of 53BP1 to sites of DNA double strand breaks ascertained either by ionizing radiation or by the endonuclease activity of FokI. As expected, depletion of endogenous KDM2A followed by complementation with wild type MYC-tagged KDM2A resulted in normal 53BP1 localization to DSBS. However, complementation with demethylase-deficient or zinc finger deleted KDM2A mutants revealed a significant reduction in the recruitment of 53BP1 to DNA double strand breaks. Nevertheless, cells expressing the KDM2A-dZF mutant displayed a more severe defect in 53BP1 recruitment to DSBS. Given that this mutant is also unable to stimulate ubiquitination of 53BP1, our findings suggest that lack of KDM2A-driven ubiquitination of 53BP1 prevents its accumulation at DSBS perhaps by affecting the dynamics of processes involved in the recruitment of 53BP1 to DNA breaks. Such processes include a distinctive type of ubiquitination mediated by RNF168 which has been shown to actively modulate the focal accumulation of 53BP1 at DSBS [44]. Alternatively, defective demethylination or ubiquitination of another KDM2A target, rather than 53BP1, could exert an indirect negative effect on the recruitment of 53BP1 to DSBS.

The recruitment of 53BP1 to DSBS is known to be a rapid process. RNF168-mediated ubiquitination of 53BP1 has been shown to promote the initial recruitment to DNA double strand breaks [44]. Of note, 53BP1 degradation observed upon KDM2A expression was mild and exhibited slow kinetics. Thus, similarly to RNF168, we propose that KDM2A-stimulated ubiquitination of 53BP1 could be necessary for the initial recruitment of 53BP1 to breaks and, eventually, a small portion of ubiquitinated 53BP1 would be subjected to degradation. Alternatively, we hypothesize that KDM2A mostly stimulates the attachment of ubiquitin chains on 53BP1 (e.g. K63 linkages) that do not necessarily result in protein degradation.

Overexpression of KDM2A was linked to reduced accumulation of MRE11 at DNA breaks leading to a potential decrease in DNA repair [20, 45]. However, recruitment of 53BP1 upon overexpression of KDM2A was not evaluated in this study [20, 45]. Our data demonstrates that depletion of KDM2A, in contrast to its overexpression, prevents the recruitment of 53BP1 to DSBS. In support of our findings, 53BP1 focal recruitment to DNA double strand breaks has been reported by at least two other studies to occur independently of MRE11 [46, 47]. In further support of our data, KDM2A depletion was shown to increase the sensitivity of breast cancer cells to cisplatin-induced DNA damage [33].

A previous study by Frasca et al [3] reported that KDM2A depletion reduces mitotic fidelity and genome stability due to augmented H3K27me2 levels at pericentric satellite repeats. In contrast, Bergmann et al. showed more recently that elevated levels of H3K27me2 at centromeric chromatin are actually necessary for chromosomal stability [48]. Thus, the link between KDM2A deplation and reduced genome stability proposed by Frasca et al [3] is unlikely to be a result of alterations in H3K27me2 levels at pericentric satellite repeats. We note that Frasca et al [3] did not perform complementation experiments using KDM2A-depleted cells expressing wild type or KDM2A mutants as a strategy to establish a clear connection between KDM2A-regulated expression of pericentric repeats and mitotic fidelity.

Indeed, our data confirm that depletion of KDM2A leads to genome instability [3, 6]. Nevertheless, we observed that this decreased genomic stability due to KDM2A depletion is directly related to impaired recruitment of 53BP1 to DSBS and exacerbated upon DNA damage. In addition, following irradiation, KDM2A-depleted cells displayed premature entry into mitosis upon exit from the G2/M checkpoint, suggesting that KDM2A depletion results in progression into mitosis with unrepaired DSBS. Of note, unrepaired double-stranded DNA breaks that arise from defects in the NHEJ pathway are known to be a significant source of micronuclei formation [35]. Therefore, we propose that the genome instability observed in KDM2A-depleted cells, which is exacerbated upon IR, is due to defective 53BP1 recruitment to DSBS and not a result of elevated levels of H3K27me2 at centromeric as proposed by Frasca et al [3].
Specific phosphorylation of 53BP1 was shown to prevent its recruitment to DSBs during mitosis [36, 37]. Expression of 33BP1 that cannot be phosphorylated on residues threonine 1609 and serine 1618 (T1609A/S1618A) results in 53BP1 recruitment to DNA breaks in mitotic cells and genomic instability [36]. Moreover, Ortmann et al. [37] showed that, during mitosis, 53BP1-T1609A/S1618A can promote DSB repair that ultimately results in telomere fusions. Interestingly, our findings show that cells concomitantly depleted of endogenous KDM2A and expressing KDM2A-AZF displayed 53BP1 focal accumulation on metaphase chromosomes observed in cells undergoing mitosis 24 hrs after irradiation. It remains unknown whether these 53BP1 foci correspond to DNA breaks or to another structure. Of note, these regions of 53BP1 focal accumulation were not observed in mitotic cells expressing wild-type or demethylase-deficient KDM2A.

This observation suggests that expression of the zinc finger deletion mutant KDM2A may affect the phosphorylation status of 53BP1 on residues threonine 1609 and serine 1618 in mitotic cells. Therefore, the incidence of micronuclei observed in cells expressing KDM2A-AZF may be directly related to the occurrence of telomere fusions. In fact, the high incidence of chromosomes bridges reported upon KDM2A depletion [3] suggests that these structures potentially originate from telomeres to telomeres and fusions.

Altogether, we report several novel findings: KDM2A associates with 53BP1 and modulates its ubiquitination; KDM2A depletion leads to defective recruitment of 53BP1 to DSBs; KDM2A itself is recruited to DNA double strand breaks through a mechanism that requires an intact zinc finger domain and demethylase activity; deletion of the KDM2A zinc finger domain disrupts its capacity to associate and ubiquitinate 53BP1 which leads to increased genomic instability and results in 53BP1 focal accumulation in mitotic cells following irradiation.

MATERIALS AND METHODS

Plasmids and siRNA

The plasmid pLPC-Puro-FLAGKDM2A-WT was constructed by cloning PCR-amplified FLAG-tagged KDM2A from pcDNA3FLAGKDM2A (a gift from Dr. Yi Zhang, Harvard Medical School) into pLPC-Puro. A MYC-tagged KDM2A vector was created by PCR amplification of KDM2A from pLPC-Puro-FLAGKDM2A-WT and cloning into pLPC-Puro-MYC. A demethylase-deficient KDM2A mutant, pLPC-Puro-MYC-KDM2AADD, was created by site-directed mutagenesis of pLPC-Puro-MYC-KDM2A-WT. This mutant contains the histidase and aspartic acid residues at position 212 and 214 mutated to alanine. A plasmid encoding for the MYC-tagged KDM2A mutant lacking the zinc finger domain (pLPC-Puro-MYC-KDM2AΔZF) was obtained by deletion of the amino acid residues spanning positions 564-610 of pLPC-Puro-MYC-KDM2A-WT. The plasmid pRK3-HA-Ubiquitin-WT was a gift from Ted Dawson (Addgene plasmid # 17608) [49] and pcDNA3-FK7/70-Flag-53BP1 was a gift from Daniel Durack (Addgene plasmid # 52307) [50]. Plasmids encoding for HA-tagged 53BP1 wild type (pCMH6K53BP1WT) and mutants (pCMH6K53BP1Δ1-1051, pCMH6K53BP1Δ1-1051, pCMH6K53BP1Δ1052-1302, pCMH6K53BP1ΔTuker and pCMH6K53BP1ABRCT were a gift from Dr. Michael Hsuan (The University of Hong Kong) and Dr. Junjie Chen (The University of Texas MD Anderson Cancer Center). These plasmids have been previously described [26].

Antibodies

The following primary antibodies were used in this study: anti-53BP1 Novus Biologicals (NB100-904), anti-Tubulin (B512 clone) Sigma, anti-MYC 9E10 clone, anti-HA 12CA5 clone, anti-FLAG M2 Sigma (F3165), anti-H3pSer10 Millipore (06-570), anti-pas methyl lysine Abcam (ab7315), anti-JHDMA1/KDM2A Bethyl (A301-475A), anti-phospho H2AX Ser139 (γH2AX) Abcam (ab2893), anti-MYC Bethyl (A190-105A), anti-ubiquitylated proteins FK2 Millipore (04-263) and anti-phospho-ATM Ser1981 R&D Systems (AF1655). Fluorescent-labeled secondary antibodies conjugated to FITC or TRITC were obtained from Jackson ImmunoResearch.

Cell culture and stable cell lines

Human osteosarcoma (U2OS) and human embryonic kidney (HEK293T) cell lines were purchased from LGC Standards, UK. U2OS-DSB reporter cell line was a gift from Dr. Roger A. Greenberg (University of Pennsylvania, Philadelphia). These cells were cultured in Dulbecco’s modified Eagle high glucose medium (Lonza) supplemented with 10% fetal bovine serum (FBS) (Lonza) and 1% Pancreatin-Stratysynomicin (Sigma). U2OS and HEK293T cells stably expressing MYC-tagged KDM2A were generated by transfection of pLPC-Puro-FLAGKDM2A-WT, pLPC-Puro-FLAGKDM2AADD or pLPC-Puro-FLAGKDM2AΔZF and further selection with Puromycin (2μg/ml) for 15 days. Control cell lines were generated by transfection of pLPC-Puro-MYC. All plasmid transfections were performed with Lipofectamine 2000 (Thermo Fisher).

Immunofluorescence

U2OS stable cell lines expressing or not MYC-tagged KDM2A constructs were transfected with 40 nM of siRNA targeting GFP (siCILT) or siKDM2A-UTR.
targeting the 3'-UTR region of the KDM2A mRNA. This siRNA was used to downregulate the expression of endogenous KDM2A but not exogenously expressed MYC-tagged KDM2A variants. Forty-eight hours after transfection, cells were gamma-irradiated (IR) with 2 Gy and harvested 1h and 24h later. Cells were then fixed for 10 min at room temperature (RT) in 4% paraformaldehyde (PFA), paraaffilinized for 2 min at RT with 0.125% Triton X-100 diluted in phosphate-buffered saline (PBS) and later blocked for 1h at RT in 10% FBS/PBS. Cells were then washed 3 times with 1x PBS and incubated with 3% FBS diluted in PBS containing primary antibody for 1h at RT. Later, cells were washed 3 times with 1x PBS and incubated with fluorescent-labeled secondary antibody diluted in 5% FBS/PBS for 1h at RT. Cellular nuclei was stained with DAPI mounting media (Vectorshield). The sequence of siCTL was GCCACAAAGUCUAAUAUCAU and siKDM2A-UTR was GUAUUGUUUCUCGUAGAAGAAU. All siRNA transfections were performed with Oligofectamine (Thermo Fisher).

For the analysis of endogenous KDM2A accumulation at DSBs, 1.5x10^6 U2OS-DSB-reporter cells were plated on 35mm dishes and, 24h later, treated or not for 5h with Shield-1 (1uM) and 4OHT (1uM). Cells were then processed for immunofluorescence as described above. Immunofluorescence analyses for the identification of DSBs in the presence or not of endogenous KDM2A was conducted by plating 1.5x10^6 U2OS-DSB-reporter cells on 35mm dishes and, 24h later, transfecting them with 50nM of siCTL or siKDM2A (UGCUUGUAUCUGCAAGAGA). Forty-eight hours after transfection, cells were treated or not for 5h with Shield-1 (1uM) and 4OHT (1uM) and immunostained with the indicated antibodies.

The recruitment of MYC-tagged wild type KDM2A and mutants to DSBs was evaluated by plating 2x10^5 U2OS-DSB-reporter cells on 35mm dishes and, 24h later, transfecting them with 50nM of siCTL or siKDM2A-UTR. On the next day, cells were transmisionally transfected with 3 ug of an empty vector (pLPC-Puro) or pLPC-Puro-MYC-KDM2Awt, pLPC-Puro-MYC-KDM2Aadd and pLPC-Puro-MYC-KDM2Aatt. Cells were washed once with 1x PBS and fresh media was added 6h after plasmid DNA transfection. On the next day, cells were treated or not for 5h with Shield-1 (1uM) and 4OHT (1uM) and immunostained with the indicated antibodies. Where mentioned, cells were pre-extracted for 10min at room temperature with CSE buffer (100mM NaCl, 300mM Sucrose, 10mM Pipes pH6.7, 3mM MgCl2, 0.2% Triton X-100) before fixation.

Microscopy and image analysis

Images were acquired with an Olympus IX73 inverted microscope (Inviiva, Imaris system). Each image was taken as Z-stacks (0.5um thickness) which were then deconvolved with the Huygens software (Scientific Volume Imaging). Quantification of 33BP1 foci was performed with the ImageJ software. The parameters for the 33BP1 foci quantification were set on the positive control (siCTL+IR) and kept constant throughout the entire analysis. Images of 16-bit were converted to 8-bit and split into three channels creating three individual images. The DAPI channel was used to adjust the threshold in order to highlight the nuclei. The FITC channel, corresponding to 33BP1 staining, was used to highlight the 33BP1 foci as single points (black dots on a white background). Equal noise tolerance was then kept constant throughout the images analyzed. The number of foci per cell was then calculated by dividing the raw integrated density by the maximum gray value for each cell.

G2/M checkpoint assay

U2OS cells were transfected with 40 nM of siCTL or previously validated siKDM2A (UGCUUGUAUCUGCAAGAGA) [16]. After 24 hrs, cells were split and re-plated on 7 new 35mm culture dishes and incubated for another 24 hours. This procedure was followed to ensure that the efficiency of the siRNA transfection was similar across all the time points collected. Cells were then irradiated or not with 2Gy and harvested at specific time points. Staining with anti-H3pSer10 and propidium iodide/RNase treatment followed by flow cytometry analysis was conducted as previously described [31].

Immunoblotting and immunoprecipitations

HEK293T cells were lysed in lysis buffer (0.5% Triton-X100, 150 mM NaCl, 50 mM Tris-Cl pH 8.0 and 20% Glycerol) containing protease (Leupeptin, Pepstatin, PMSF) and phosphatase (Na2VO4, NaF) inhibitors and benzamidine (1:1000) Sigma. Lysates were incubated on ice for 40 min and later cleared by centrifugation at 14,000 rpm for 10 min at 4°C. Supernatant was saved and protein concentration determined by Bradford assay. Proteins were then resolved by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, incubated with the appropriate antibodies and exposed to X-ray films. For immunoprecipitations, equal amounts of total cell extracts obtained with lysis buffer were used for each sample. Extracts were incubated on ice with primary antibody for 2 hrs. Protein G Saporhose beads (GE healthcare) were then added to lysates which were further incubated for 30 min at 4°C under rotation. Beads were washed 3 times with lysis buffer and proteins bound to beads were eluted in 1x Laemmli sample buffer. Alternatively, immunoprecipitations were conducted with anti-FLAG M2 agarose beads (Sigma).
Analysis of ATM phosphorylation

Levels of ATM phosphorylation on Serine 1981 were evaluated by transfecting 50nM of siGFP or siKDM2A into 1x10^6 U2OS cells plated in 35mm dishes. Forty-eight hours later, cells were irradiated with 2Gy and harvested at the indicated timepoints. Subsequently, cells were lysed for 1 hour on ice with 1X lysis buffer containing protease and phosphatase inhibitors and benzonase. Supernatants were recovered after spinning down the samples at 14000 rpm for 10min at 4°C. Protein concentration was determined by Bradford protein assay. A total of 40μg of crude cells extract was resolved by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and incubated, sequentially, with anti-phospho ATM Ser1981, anti-ATM and anti-KDM2A. Following incubation with HRP-conjugated secondary antibody, the membrane was washed and exposed to X-ray films.

Cell cycle analysis

U2OS cells were plated 24 hours prior siRNA transfection of GFP and KDM2A. Forty-eight hours after transfection, cells were irradiated with 2Gy and harvested at the indicated timepoints. Cells were washed twice in 1X PBS and then fixed overnight at 4°C in 70% ice-cold ethanol. Subsequently, cells were washed once in 1X PBS and resuspended in 1X PBS containing 40μg/ml of Propidium Iodide and 250μg/ml of RNase A and incubated for 30 minutes in the dark prior to flow cytometry analysis.

Author contributions

M.T.D.B., M.B., S.R. and N.F.L. designed the experiments. M.T.D.B. and M.B. performed the experiments and analysed the data. M.T.D.B wrote the paper with M.B. and N.F.L. The study was supervised by S.R. and N.F.L. All authors were involved in discussion of results and critical reading of the manuscript.

ACKNOWLEDGMENTS

We thank Dr. Roger A. Gesenburg (University of Pennsylvania, Philadelphia) for the U2OS-DSB-reporter cell line. We thank the Flow Cytometry Core Facility, Biomedical Sciences Building (National University of Ireland, NUI Galway).

CONFLICTS OF INTEREST

The authors declared no competing financial interest.

FUNDING

This work was supported by awards from Science Foundation Ireland (10/1/I1594) to N.F.L. and from the Canadian Institute of Health Canada (MOP-93811) to S. R.

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4.3 Supplementary materials

Supplementary Figure 1: (A) U2OS cells stably expressing MYC-tagged KDM2A variants were transfected with siCtl or siUTR. Efficient downregulation of endogenous KDM2A and expression of MYC-tagged KDM2A variants was observed by immunoblotting with anti-KDM2A. Relative loading was shown by immunoblotting with anti-Tubulin. (B) Schematic of the U2OS-DSB-reporter cell line. Upon treatment with Shield-1 and hydroxytamoxifen (4-OHT), unstable and cytoplasmic ER-nCherry-LacI-FokI-DD (FokI) (light red) is stabilized and translocated to the nucleus where it is recruited to LacO repeats (dark red) and promotes DSBs. (C) U2OS-DSB-reporter cells were treated (+) or not (-) with Shield-1 and 4-OHT to induce expression of ER-nCherry-LacI-FokI-DD (FokI) (red). Cells were immunostained with anti-pH2AX and anti-KDM2A (green). Immunofluorescence analyses were conducted with cells that were not preextracted with cytoskeleton buffer (CSK).
**Supplementary Figure 2:** (A) Protein ubiquitination upon IR was evaluated in U2OS cells transfected with siCTL or siKDM2A and immunostained with anti-FK2 (green), anti-phH2AX (red) and DAPI. (B) Data shown in (A) was quantified. Graph representing the number of FK2 IRIF in cells transfected with the indicated siRNA. (C) KDM2A knockdown efficiency in cells shown in (A) was determined by immunoblotting of total cell extracts with anti-KDM2A and anti-ACTIN.
Supplementary Figure 3: (A) Flow cytometry analyses demonstrating the cell cycle profile of U2OS cells transfected with siCTL. Cells were collected at the indicated timepoints after irradiation and stained with propidium iodide in the presence of RNase. (B) Cells transfected with siKDM2A were analyzed as described in (A).
**Supplementary Figure 4:** ATM phosphorylation at the specified timepoints following irradiation was assessed by immunoblotting total cell lysates from siCTL- and siKDM2A-transfected U2OS cells with anti-ATM, anti-ATM S1981 and anti-KDM2A.
**Supplementary Figure 5: KDM2A does not colocalize with γH2AX IRIF.** U2OS cells irradiated or not, were immunostained with anti-γH2AX (green), anti-KDM2A (red) and DAPI in the absence or presence of CSK buffer.
Supplementary Figure 6. Nucleolar localization of KDM2A variants in irradiated and non-irradiated cells. U2OS cells were immunostained with anti-MYC (red) and DAPI (green). Nucleoli were identified by lack of DAPI staining. White arrows indicate KDM2A localization within the nucleolus.
Supplementary Figure 7: Colocalization between FokI and 53BP1 in U2OS-DSB-reporter cells transfected with siCTL or siKDM2A. The efficiency of KDM2A silencing via siRNA transfection plays a partial role in 53BP1 recruitment to DSBs. Immunostaining analyses of cells transfected with siCTL showed colocalization between FokI (red) and 53BP1 (green) (A). The cell population displaying effective depletion of KDM2A via siKDM2A transfection revealed that 53BP1 still colocalized with FokI in 13% of these cells (compare panel B with C).
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