THE ROLE OF 53BP1 AND ITS PHOSPHORYLATION IN THE DNA DAMAGE RESPONSE

by

Shane Michael Harding

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Graduate Department of Medical Biophysics
University of Toronto

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Abstract

The tumour suppressor p53-binding protein 1 (53BP1) is phosphorylated following DNA double strand breaks (DSBs); however, little is understood about the upstream signaling pathways that control this phosphorylation. Additionally, it is not known how these processes combine with 53BP1 to control the survival of cells following DNA damage such as that imparted by ionizing radiation (IR), which is the basis of radiotherapy. In this thesis, I have shown that 53BP1 is phosphorylated specifically in S-phase cells, but not relocalized to intranuclear foci, in response to severe oxygen stress. This occurs with only partial dependence on the ATM kinase (Chapter 2). Following IR, I find that both ATM and DNA-PKcs contribute to intranuclear phosphorylated 53BP1 foci, but that this phosphorylation is independent of proximal signaling molecules that control the localization of 53BP1 to initial DSBs (Chapter 3). Furthermore, I show that 53BP1 loss confers sensitivity to IR and this can be further augmented by inhibition of ATM and DNA-PKcs kinases suggesting that there are both 53BP1-dependent and -independent pathways of survival from IR (Chapter 4). These findings may have important implications for molecular pathology and personalized medicine as 53BP1 has recently been found to be activated or lost in subsets of human tumours. I have collaborated to initiate the development of a novel system to interrogate the implications of 53BP1 loss as traditional siRNA approaches in human cancer.
cells were not feasible (Chapter 5 and Appendix 2). This system can be used *in vivo* as tumour xenografts to further understand how 53BP1 and the tumour microenvironment interact endogenously and in response to IR. I also present the possibility and proof of concept for the use of 53BP1 as a biomarker in primary human prostate cancer tissue where little is known about 53BP1 biology (Chapter 5).
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Graduate school is a large undertaking not only for the student but also for everyone surrounding them. I’ve been fortunate to have a very supportive group of people helping me over the last 6(ish) years. We really are taught by the village and at every stage in my life I have had the opportunity to learn from so many people that I can’t possibly give them the credit they deserve. All I have accomplished is thanks to this network, but there are some special people that have really been instrumental in this part of my life.

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53BP1—p53 binding protein 1
aCGH—array comparative genomic hybridization
AT—ataxia telangiectasia
ATF—AT-fibroblasts
ATLD—ataxia telangiectasia-like disorder
ATMi—ATM inhibitor
ATM—kinase mutated in ataxia telangiectasia
ATP—adenosine triphosphate
ATR—ataxia telangiectasia and RAD3-related
ATRIP—ATR interacting protein
BLM—Bloom syndrome
BRCA1/2—breast cancer associated proteins 1 and 2
BRCT—BRCA1 C-terminus domain
BrdU—5-bromo-2’-deoxyuridine
BSA—bovine serum albumin
CDK—cyclin dependent kinase
CENP-F—centromere protein F
CHK1/2—checkpoint proteins 1 and 2
CSR—class switch recombination
CTIP—C-terminal binding protein interacting protein
DAPI—4’,6-diamidino-2-phenyindole
DDR—DNA damage response
DMEM—Dulbecco’s modified eagles medium
DMSO—dimethylsulfoxide
DNA—deoxyribonucleic acid
DNA-PKcs—DNA-dependent protein kinase catalytic subunit
DNA-PK—DNA-dependent protein kinase holoenzyme
DNA-PKi—DNA-PK inhibitor
DOX—doxycycline
DSB—DNA double strand break
dsDNA—double stranded DNA
EdU—5-ethynyl-2’-deoxyuridine
EF5—pentafluorinated derivative of etanidazole [2-(2-nitro-1-H-imidazole-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)acetamide]
ELISA—enzyme linked immunosorbent assay
EME1—essential meiotic endonuclease 1
EXO1—exonuclease 1
FA—Fanconi’s Anaemia
FANCD2—Fanconi’s Anaemia complementation group D2
FCS—fetal calf serum
GAR—glycine-arginine rich domain
GEN1—gen homologue 1
GS—gleason score
Gy—Gray
H3P—histone 3 phosphorylated at serine-10
HR—homologous recombination
HU—hydroxyurea
i.p.—intraperitoneal
ICGC—international cancer genome consortium
IGRT—image guided radiotherapy
IL-6—interleukin 6
IP—immunoprecipitation
IRIF—ionizing radiation-induced immunofluorescent foci
IR—ionizing radiation
KAP1—Krab associated protein 1
KBD—kinetochore binding domain
LFS—Li Faumeni’s Syndrome
LIGIV—DNA ligase IV
MDC1—mediator of DNA checkpoint 1
MDM2—mouse double minute 2
MEC—microenvironmental chamber
MEF—mouse embryonic fibroblast
MEM—modified eagles medium
MMC—mitomycin C
MRE11—meiotic recombination 11 protein
MRN—MRE11-NBS1-RAD50 complex
mtMRE11.3—nuclease deficient mutant MRE11
MMS—methyl methanesulfonate
MTT—3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Myb—myeloblastosis domain
NBS1—Nijmegen breakage syndrome protein 1
NBS—Nijmegen breakage syndrome
NHEJ—non-homologous end joining
NHF—normal human fibroblasts
NIR—non-irradiated
NLS—nuclear localization signal/sequence
OD—oligomerization domain
P53—tumour protein 53
PARPi—PARP inhibitor
PARP—poly(ADP)-ribose polymerase
PIAS4—protein inhibitor of activated STAT protein 4
PIKK—phosphoinositide 3-kinase-like kinase
PI—propidium iodide
PML-NB—promyelocytic leukemia nuclear bodies
PNKP—polynucleotide kinase phosphatase
PRMT1—protein arginine methyltransferase 1
PSA—prostate specific antigen
RAD50—radiation 50 protein
RAD51—radiation 51
RAD52—radiation 52
RB—retinoblastoma
RIDDLE—radiosensitivity, immunodeficiency, dysmorphic features and learning difficulties
RNA—ribonucleic acid
RNF168—ring finger protein 168
RNF8—Ring finger protein 8
RPA—replication protein A
RS—radiosensitivity
SA-βGal—senescence associated β-galactosidase
SD—standard deviation
SDS—sodium dodecyl sulfate
SEM—standard error of the mean
SER—sensitization enhancement ratio
SF2—surviving fraction at 2Gy
shRNA—short hairpin RNA
siRNA—small interfering RNA
SMC1—structural maintenance of chromosomes 1
ssDNA—single stranded DNA
SUMO—small ubiquitin-related modifier
TCGA—The Cancer Genome Atlas
TCD50—Tumour control dose to give 50% cure
TCGA—The cancer genome atlas
TEMED—Tetramethylethylenediamine
TetO—tetracycline operon
TetR—tetracycline repressor
UBC13—ubiquitin conjugating enzyme 13
UV—ultraviolet
WB—western blot
XLF—XRCC4-like factor
XRCC4—x-ray cross-complementation protein 4
YFP—yellow fluorescent protein
γH2AX—Histone variant H2A.X phosphorylated at serine-139
CHAPTER 1
INTRODUCTION
1.1 INTRODUCTION

Cells and tissues have many ways to protect against genetic instability given the latter is a fundamental characteristic of most cancers. Indeed, Hanahan and Weinberg define genetic instability as the most prominent “enabling” characteristic for the development and progression of cancer (Hanahan and Weinberg, 2011). Cells continually encounter challenges to genetic material, both from normal metabolic processes that cause oxidation and alkylation of the DNA, and from external insults such as ultraviolet (UV) light and ionizing radiation (IR) that cause DNA strand breaks (van der Kogel and Joiner, 2009). If left unchecked, these DNA lesions can lead to mutations and subsequently cellular carcinogenesis and tumour progression (Tannock et al., 2004). To overcome these potentially deleterious events cells have evolved molecular mechanisms for slowing cell cycle progression to provide time for DNA repair mechanisms to remove damage and to prevent propagation of unstable genetic material to progenitor cells. In this way DNA damage sensing and repair acts as a barrier to genetic instability and therefore to development of malignancy.

During tumourigenesis, many cancer cells overcome these barriers to genomic instability and acquire defects in the DNA damage response (DDR) and/or cell cycle checkpoints (Bartkova et al., 2005; Gorgoulis et al., 2005; Bartkova et al., 2006; O'Donovan and Livingston, 2010). Some of these defects can be exploited during fractionated radiotherapy or selected chemotherapy agents by increasing lethal DNA damage in cancer cells relative to normal cells (Tannock et al., 2004). This concept is defined within as the “therapeutic ratio” in which malignant cells are differentially targeted over normal cells and tissues as part of cancer treatment (van der Kogel and Joiner, 2009). Ideally, to improve upon this concept with molecularly targeted agents one
would identify a compound that specifically sensitizes tumour cells to radiation, but does not alter the survival of normal tissues. Alternatively, one would identify particular genetic or proteomic signatures of a tumour indicating sensitivity of specifically the tumour cells to a given treatment. In either case the development of such personalized medicine requires understanding of the underlying principles of the cellular response to the most lethal lesion of radiotherapy—the DNA double-strand break (DSB).

1.2 THE DNA DAMAGE RESPONSE AND CANCER

1.2.1 Cancer Susceptibility Syndromes

The importance of genetic stability is underscored by the rarity and severity of human syndromes caused by mutation of key DDR proteins (O'Driscoll et al., 2004). Syndromes that occur as a result of these mutations and influencing DSB repair specifically have been summarized in Table 1.1. These syndromes generally result from consanguineous families and lead to severe clinical phenotypes including immunodeficiency (see below), developmental delays and neurodegeneration. The causes of these latter two symptoms are still unknown, but the study of cells from these patients has been invaluable in our understanding of the DDR response. These syndromes also cause susceptibility to cancer; most commonly lymphomas, and carriers of mutations often have increased risk of developing solid cancers (Kastan, 2008; de Miranda et al., 2011). For example, ATM carriers have increased risk of developing early breast cancer (Renwick et al., 2006).
Table 1.1: Selected cancer susceptibility syndromes involving the DDR

<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Defective Protein</th>
<th>Cancer predisposition</th>
<th>DDR Defect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ataxia telangiectasia (AT)</td>
<td>ATM</td>
<td>Lymphomas</td>
<td>DSB sensing and repair</td>
</tr>
<tr>
<td>AT-like disorder (ATLD)</td>
<td>MRE11</td>
<td>Unknown</td>
<td>DSB sensing (repair)</td>
</tr>
<tr>
<td>Nijmegen breakage syndrome (NBS)</td>
<td>NBS1</td>
<td>Lymphomas</td>
<td>DSB sensing (repair)</td>
</tr>
<tr>
<td>RIDDLE syndrome</td>
<td>RNF168</td>
<td>Unknown</td>
<td>DSB sensing</td>
</tr>
<tr>
<td>Fanconi’s Anaemia (FA)</td>
<td>Fanconi’s</td>
<td>Leukemia</td>
<td>DNA interstrand crosslink sensing and repair</td>
</tr>
<tr>
<td></td>
<td>Complementation</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>group (i.e. FANCD2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Li Fraumeni</td>
<td>Mutations in p53</td>
<td>Sarcomas</td>
<td>Checkpoint signaling</td>
</tr>
<tr>
<td>BRCA1/2</td>
<td>BRCA1 or BRCA2</td>
<td>Breast, ovarian</td>
<td>Homologous recombination</td>
</tr>
</tbody>
</table>

This table has been adapted from The Basic Science of Oncology (Tannock et al., 2004).

1.2.2 DSB sensing and signaling

Regardless of whether a DSB is induced by endogenous or exogenous process, its sensing and subsequent repair can be conceptualized as a three-stage cascade. A number of the proteins involved in these cascades are also cancer susceptibility proteins as described above. This signaling pathway starts with sensing proteins, proceeding to mediator/adaptor proteins and finally to effector proteins, a process diagrammed in Figure 1-1 (Jackson and Bartek, 2009). When a DSB is induced by ionizing radiation the MRE11-NBS1-RAD50 (MRN) complex localizes to the DNA ends and contributes to the trans-activation by autophosphorylation of the inactive dimer of the kinase mutated in ataxia telangiectasia (ATM) (Maser et al., 1997; Nelms et al., 1998; Bakkenist and Kastan, 2003; Lee and Paull, 2005). Simultaneously, in cases where
FIGURE 1-1: Diagram of the initial molecular responses leading to 53BP1 localization at IR-induced DNA double strand breaks. Upon induction of a DSB the ATM dimer autophosphorylates on Serine-1981 and dissociates into active monomers. This activation occurs with partial dependence on the MRE11-RAD50-NBS1 (MRN) complex which also localizes ATM to the site of the DSB. Simultaneously DNA-PKcs is activated by autophosphorylation and in association with the KU70-KU80 heterodimer localizes to the DSB. Together ATM and DNA-PKcs phosphorylated H2A.X at Serine-139 (forming γH2AX) in megabase domains spanning the break. This serves as a platform for the assembly of MDC1, RNF8-UBC13 and subsequently RNF168-UBC13. This induces ubiquitin chain formation on histones that are thought to cause chromosomal conformational changes exposing constitutively methylated residues of histones H3 and H4. This leads to 53BP1 recruitment and the facilitation of checkpoint and DSB repair functions.
non-homologous end joining (NHEJ; see below) is to be used as a repair pathway, the KU70/KU80 heterodimer binds with high affinity to DNA ends and contributes to activation of the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to form the DNA-PK holoenzyme (Lees-Miller and Meek, 2003; Weterings and Chen, 2008). Together active ATM and DNA-PK phosphorylate the histone variant H2A.X at serine-139 (forming γH2AX) surrounding the break site (Rogakou et al., 1998; 1999; Stiff et al., 2004). The activation of γH2AX serves as a platform for the recruitment of various mediator proteins including mediator of DNA damage checkpoint 1 (MDC1) and the E3-ubiquitin ligase ring finger protein 8 (RNF8) which ubiquitylates H2A and H2A.X molecules via the E2-ubiquitin conjugating ligase UBC13 (Stewart et al., 2003; Stucki et al., 2005; Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007). These ubiquitylated histones recruit RNF168 that further stimulates extension of the ubiquitin chains (Doil et al., 2009; Stewart et al., 2009). It is proposed that these histone modifications drive initial chromatin changes that expose constitutively dimethylated lysine-79 and lysine-20 on H3 and H4 histones, respectively (Huyen et al., 2004; Botuyan et al., 2006). The 53BP1 protein recognizes these exposed methylated residues and accumulates around the break site (Schultz et al., 2000; Huyen et al., 2004; Botuyan et al., 2006). Multiple roles of 53BP1 have been described (see below). One of these roles it to concentrate effector proteins, such as Checkpoint protein 1/2 (CHK1/2), p53, LigaseIV/XRCC4, BRCA1/2 etc., around the DSB to aid in their activation by ATM and DNA-PK for further functional outcomes of DNA repair and checkpoints (Wang et al., 2002; Rashid et al., 2011).

The activation of γH2AX occurs in megabase domains surrounding the DSB and it is thought that the extent of this response is what permits the visualization of these molecules by immunofluorescent microscopy (Nakamura et al., 2010). Figure 1-2 shows an example of
FIGURE 1-2: Immunofluorescent microscopy to detect DNA double strand breaks. A) 2-dimensional images of normal human fibroblasts (GM05757) either untreated (non-irradiated, NIR) or irradiated with 2Gy and fixed at 30 minutes post-IR. Cells were stained for phosphorylated H2A.X (γH2AX) and total-53BP1. IR induces γH2AX intranuclear foci whereas 53BP1 redistributes from a pan-nuclear non-nucleolar pattern into discrete foci that colocalize with γH2AX in the DAPI stained nucleus as shown by yellow colouration. Scale bar is 10µm. B) 3-dimensional modeling of colocalized regions of cells in A); colours are maintained. Cells are shown at equal scale.
untreated cells and cells treated with 2Gy of ionizing radiation. After 30 minutes following the radiation dose ionizing radiation-induced immunofluorescent foci (IRIF) can be clearly seen as distinct spots within the nucleus. The number of γH2AX IRIF corresponds to DSB induction as measured by multiple other methods and is therefore a quantitative readout of DSB induction (Sedelnikova et al., 2002; Banáth et al., 2004; Löbrich et al., 2010). Similarly, a pre-existing pool of 53BP1 redistributes following IR into distinct IRIF that colocalize with γH2AX indicating recognition of DSBs (Schultz et al., 2000). This has become a key method in characterizing putative DSB sensing proteins and by various methods most proteins in Figure 1-1 have been found to form IRIF.

1.2.3 DSB Repair and Cell Death

If a cell is unable to respond to and/or repair DSBs this genetic instability can lead to cell death. Indeed, the number of unrepaired (residual) DSBs correlates with cell death following ionizing radiation (Jeggo and Lavin, 2009). In this context cell death is measured by colony forming assays. In this assay single cells are plated in known numbers, treated accordingly and allowed to grow such that surviving cells form colonies derived from greater than 5 cellular divisions (Puck and Marcus, 1956; van der Kogel and Joiner, 2009). These colonies result from single “clonogenic” cells, or what radiobiologists define as “tumour clonogens” which are capable of re-growing the tumour and are a critical target when developing strategies for improved local control and cure (Baumann et al., 2008). Long-term clonogenic survival can encompass multiple types of cell death; even those that may manifest long after (2-5 days) the initial lethal dose of IR (Brown and Wouters, 1999; Forrester et al., 1999). Major types of cell death are outlined in Table 1.2. It is important to note that certain cell types have a propensity to undergo death by different mechanisms. For example, rapidly proliferating cells such as lymphocytes or
spermatogonia die quickly following IR through apoptotic mechanisms whereas fibroblasts and epithelial cells typically die following multiple attempts at cell division following unsuccessful DSB repair (van der Kogel and Joiner, 2009). Other assays, such as 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), use relative metabolic rates to measure cell death. These assays are problematic for measurement of fibroblasts or epithelial cell killing where the cells may remain metabolically active, but clonogenically dead (Bromfield et al., 2003; Brown and Attardi, 2005). The colony-forming assay avoids these pitfalls and encompasses all modes of cell death giving a more accurate estimate of cell killing following a particular treatment. A schematic of survival curves from radiosensitivity syndromes listed in Table 1.1 is shown in Figure 1-3 and depicts 3 broad groups of radiosensitivity. No human syndrome for 53BP1 is described, but based on murine studies and RIDDLE syndrome a prediction of 53BP1 deficiency in Group II is suggested.

A number of other surrogate assays for survival following IR have been developed to provide a more rapid analysis of the cell death response to DSBs. One such assay is the micronucleus assay where cytokinesis is blocked by cytochalasin-B and unrepaired DSBs that are carried into mitosis are identified by the staining of fragments in binucleated cells that would be lost in subsequent generations. The correlation of micronuclei and clonogenic survival is complicated in tumour cells that may be aneuploid and more tolerant to loss of this genetic information and generally is a poor surrogate for clonogenic survival (Bush and McMillan, 1993; Villa et al., 1994; Champion et al., 1995). Direct measures of DSBs by single-cell electrophoresis (Comet assay) or pulsed field gel electrophoresis (PFGE) can correlate with clonogenic survival following IR, but these techniques rely on doses much higher (>5-10Gy) than those typically
FIGURE 1-3: Relative survival from ionizing radiation in human DNA repair syndromes. Cell lines fall into one of 3 broad categories of sensitivity to ionizing radiation (Arlett and Harcourt, 1980; Joubert et al., 2008). The most resistant, group I includes normal cells and p53-deficient LFS cells (Jalali and Bristow, Unpublished observations; Slichenmyer et al., 1993). The most sensitive group III includes ATM and ATM-pathway deficient (i.e. NBS1, ATLD), which show profound sensitivity similar to NHEJ deficient cell lines (i.e. LIG IV) (Kühne et al., 2004; Riballo et al., 2004; Darroudi et al., 2007). Intermediate sensitivities in Group II are displayed by cell lines from FA and RIDDLE patients (Xia et al., 2001a; Djuzenova and Flentje, 2002; Stewart et al., 2007).
used clinically and within the clonogenic assay (0-10Gy) (Olive et al., 1994; Sarkaria et al., 1998; Eastham et al., 1999). More recently, the “residual γH2AX” assay has been used to monitor DSB repair and correlated to clonogenic survival. Here, 24h following damage γH2AX is stained by immunofluorescence and levels are monitored either microscopically by foci counting or by flow cytometry (MacPhail et al., 2003; Macphail et al., 2003; Goodarzi and Jeggo, 2011). This assay is much more sensitive than the Comet and PFGE and can detect residual breaks following doses below 1Gy (Redon et al., 2011). Of the “short-term” assays currently in use the residual γH2AX assay shows the most promise as a surrogate for clinical scenarios, but ultimately it is long-term survival that is most relevant for measuring cell-death following ionizing radiation and DSB repair (Ivashkevich et al., 2011).

<table>
<thead>
<tr>
<th>Cell Death Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td>Programmed cell death controlled by molecular signaling</td>
</tr>
<tr>
<td>Necrosis</td>
<td>Passive death resulting from membrane breakdown by poorly understood mechanisms</td>
</tr>
<tr>
<td>Autophagy</td>
<td>“Self-eating” referring to cellular digestion of macromolecules to make up for nutrient deprivation. The role of this “death” pathway following IR is not well understood and under active study.</td>
</tr>
<tr>
<td>Mitotic catastrophe</td>
<td>Multiple abortive mitoses and mitotic spindle abnormalities may take place when lethally irradiated cells attempt to divide with unrepaired DNA damage</td>
</tr>
<tr>
<td>Terminal growth arrest (senescence)</td>
<td>Molecularly controlled exit from the cell cycle where cells do not maintain capacity to divide but remain metabolically active.</td>
</tr>
</tbody>
</table>

Table adapted from Brown and Attardi (Brown and Attardi, 2005)
1.2.4 Radiobiology in cancer treatment

The ultimate goal of any cancer treatment is to cause cell death within tumour cells but not in normal tissue; this is called the *therapeutic ratio*. In radiobiology a framework of “5 R’s” forms the basis for tumour response and the therapeutic ratio (Tannock et al., 2004; van der Kogel and Joiner, 2009). *Radiosensitivity* refers to the intrinsic sensitivity of cells to IR and refers mainly to the difference between tumour and normal cell radiosensitivity. *Recovery* involves two facets: 1) repair where cells are able to recover the function of macromolecules and to repair DNA and 2) direct recovery of the tissues where cell survival increases or radiation damage decreases over time following the dose. Radiosensitivity also changes throughout the cell cycle (Figure 1-4; Rothkamm et al., 2003)). An irradiated asynchronous population of cells within a tumour attains some degree of synchrony and a temporal delay in dose delivery provides *redistribution* within the cell cycle to allow more radioresistant cells to progress to more sensitive cell cycle phases. The toxic effects of IR are highly dependent on oxygen and regions of solid tumours are often hypoxic (Chan et al., 2009). Irradiation of a tumour kills a greater fraction of oxic than hypoxic cells and over time following the dose previously hypoxic cells are *reoxygenated* which sensitizes them to subsequent doses. *Repopulation* occurs as cells re-enter the cell cycle following IR and begin to regrow the tumour.

The impact of the “5 R’s” on the therapeutic ratio relies on timing following the treatment. Clinically this is exploited by fractionating radiation doses. Multiple smaller doses limit normal tissue toxicity by providing time for normal tissues to recover following IR (van der Kogel and Joiner, 2009). Simultaneously this time allows for redistribution and reoxygenation that sensitize cells to the next fraction of IR. The exact fractionation schedule is determined by practical means and also by the repopulation rate of a given tumour type. This is a balancing act with a goal of maximizing tumour kill (i.e. reducing repopulation effects) while providing enough time for the
FIGURE 1-4: Depiction of DSB repair pathway and radiosensitivity throughout the cell cycle. The cell cycle is presented with segments corresponding to $G_0$, $G_1$, $S$, $G_2$ and $M$ phases. The propensity for usage of NHEJ and HR is indicated by the two outer circles, with darker areas indicating activity. The inner most circle represents radiosensitivity (RS) with the darkest red regions corresponding to the most radiosensitive cell cycle stages. Figure is modified from Rothkamm et al (Rothkamm et al., 2003).
normal tissues to recover, the *therapeutic ratio*. These fractionation regimes are under active study.

### 1.3 53BP1 AND ITS ROLE IN DNA REPAIR

#### 1.3.1 The 53BP1 protein

53BP1 is located at chromosome position 15q15-21 and was originally identified in a yeast 2-hybrid screen as a binding partner of the DNA-binding domain of p53 (Iwabuchi et al., 1994; 1998). It is a 1972 amino acid protein with an N-terminal S/TQ phosphorylation cluster representing consensus phosphorylation sites for the phosphatidylinositol 3-kinase related kinase-like (PIKK) family of proteins including ATM and DNA-PKcs (Anderson et al., 2001; Rappold et al., 2001; Xia et al., 2001b). The majority of these sites are in the N-terminus and those shown to be induced by IR have been highlighted in Figure 1-5 along with another relevant site in the p53-binding domain of 53BP1 at Serine 1778 (Ward et al., 2003a; Jowsey et al., 2007). Amino acids 1235-1616 contain a kinetochore binding domain (KBD) that is responsible for the localization of 53BP1 to kinetochores in prophase, however a functional outcome of this localization has not been described (Jullien et al., 2002). The region required for localization of 53BP1 to IRIF has been mapped to amino acids 1480-1616 which contains a tandem tudor domain that is involved in binding methylated histone residues as described above and a myb domain which is broadly involved in DNA-binding (Iwabuchi et al., 2003; Morales et al., 2003; Ward et al., 2003a). A glycine-arginine rich domain of 53BP1 lies upstream of the tudor domains and is methylated by protein arginine methyltransferase 1 (PRMT1) in vivo, but independently of IR and is not required for IRIF (Adams et al., 2005; Boisvert et al., 2005).
**FIGURE 1-5: Domain organization of p53 Binding Protein 1.** The 1972 amino acid 53BP1 is diagrammed with major domains identified. See text for a detailed description of each domain and its putative function. This diagram is not to scale. Residues of the kinetocore binding domain and minimum focus forming region are derived from mapping studies and may not represent precise definitions of these domain boundaries.
Recent evidence has shown that sumoylation of 53BP1 within residues 1050-1483 is modulated by protein inhibitor of activated STAT4 (PIAS4) and is required for efficient localization of 53BP1 to DSBs (Galanty et al., 2009). Finally, the C-terminus of 53BP1 contains a tandem BRCA1 C-terminus domain (BRCT) (Derbyshire et al., 2002; Joo et al., 2002; Manke et al., 2003; Mochan et al., 2004). BRCT domains are a common feature of DSB-responsive proteins and generally direct protein-protein interactions through a phosphopeptide binding capacity (Manke et al., 2003).

A number of DSB gene mutations confer cancer susceptibility in patients and mice, including ATM, NBS1 and Checkpoint protein 2 (CHK2) (Kastan and Bartek, 2004; Hakem, 2008). Mice engineered to express truncated forms of 53BP1, or harbouring knockouts of the 53BP1 gene display increased spontaneous chromosomal abnormalities that are exacerbated upon IR treatment, increased polyploidy, are growth retarded, radiosensitive and mildly tumour prone when compared to wild-type littermates (Morales et al., 2003; Ward et al., 2003b). Furthermore, it was found that intercrossing p53 -/- mice with 53BP1 -/- generated double knockout mice with a synergistic phenotype that accelerated tumourigenesis (lymphoma, B-cell tumours, sarcomas and teratomas) (Ward et al., 2005; Morales et al., 2006). Currently, no human patients have been described with mutations or loss of 53BP1 in the germline, but recent evidence has correlated loss of 53BP1 to tumourigenesis (see below).

1.3.2 53BP1 in NHEJ

Non-homologous end-joining (NHEJ) is the dominant repair pathway in non-cycling mammalian cells and is active throughout the cell cycle (Figures 1-4 and 1-6). When a DSB is incurred the
FIGURE 1-6: Two distinct DSB repair pathways. Two pathways contribute to repair of DSBs. The first, NHEJ is predominant in mammalian cells and operates through all cell cycle stages and is error prone. The DNA-PK holoenzyme (KU70/80-DNA-PKcs) controls rejoining via the Ligase IV/XRCC4/XLF proteins. The DSB ends may require processing in a slow pathway as described in the text. These events may lead to deletions or insertions and reduce the fidelity of DNA repair. Error-resistant HR occurs during S and G2 phases where a sister chromatid is present and is initiated by tracks of ssDNA coated by RPA. End resection leads to strand invasion and synthesis of a new strand using the sister chromatid as a template. These Holliday junctions are resolved into repaired daughter strands as described in the text.
Ku70/Ku80 heterodimer forms at DSB ends and recruits DNA-PKcs inducing autophosphorylation and activation (Mahaney et al., 2009). This results either in the direct rejoining of the DSB ends by the XRCC4/Ligase IV/XLF complex or by this same complex following processing of the ends to make them competent for repair. The processing step is, at least partially, ATM-dependent and can involve the addition or removal of nucleotides (by MRN or Artemis nucleases and polymerases such as Polμ/λ), 5’-phosphate addition and 3’ phosphate removal (by polynucleotide kinase phosphatase (PNKP)) (Hiom, 2010). Some evidence indicates that chromatin compaction states impact significantly on the ability to repair DSBs, especially by NHEJ (Goodarzi et al., 2008; Noon et al., 2010). In these models heterochromatic regions are too tightly packed for access to the end-joining machinery and ATM-dependent phosphorylation of KRAB-associated protein 1 (KAP-1), a transcriptional co-repressor, loosens its chromatin binding and, theoretically, decreases local chromatin compaction allowing access of repair complexes to the DNA ends (Fernandez-Capetillo and Nussenzweig, 2008; Goodarzi et al., 2009; 2010). This is a relatively new area of study and it is likely that a number of other factors are involved. Chromatin relaxation may also be necessary for repair by HR, but this is less well understood (Murga et al., 2007).

The contribution of 53BP1 to NHEJ in non-lymphoid cells is not well understood. In early studies it was found that the kinetochore binding domain was necessary for in vitro rejoining of dsDNA by Ligase IV/XRCC4 (Iwabuchi et al., 2003). Subsequent studies using 53BP1-/- mouse embryonic fibroblasts (MEFs) found that resolution of γH2AX foci in G0/G1 was severely impaired implying defective NHEJ (Ward et al., 2006). Importantly, truncation of the N-terminal phosphorylation domain or selective mutation of 15 conserved (S/T)Q sites to unphosphorylatable AQ sites led to equal defects as the knockout as did loss of the
oligomerization domain (Ward et al., 2006). Loss of the BRCT domain did not alter γH2AX foci resolution. Analysis of γH2AX for DSB repair is complicated by a large number of factors and is therefore not an unambiguous marker of DSB repair (i.e. apoptosis, cell cycle etc) (Cleaver et al., 2011; Goodarzi and Jeggo, 2011). Several other lines of evidence have implicated 53BP1 in NHEJ, including chromosome analyses of irradiated 53BP1-/- MEFs that show a marked increase over wild-type in chromosome breaks (Minter-Dykhouse et al., 2008). Elegant live-cell imaging has found that critically shortened telomeres, a substrate for NHEJ, were more mobile in the presence of 53BP1 and were therefore rejoined with dependence on 53BP1 (Dimitrova et al., 2008). Finally, defective NHEJ repair of a stably integrated reporter construct in MEFs was rescued by wild-type but not mutants deficient in foci formation (Xie et al., 2007). In human cells, much less is known about 53BP1 in NHEJ, but recent evidence has shown that 53BP1-dependent localization of phosphorylated KAP1 contributes to chromatin remodeling and increases accessibility of otherwise irreparable heterochromatic DSBs (Noon et al., 2010). From these data it is suggested that the phosphorylation cluster and localization of 53BP1 to DSBs contributes directly to DSB repair and potentially to chromatin remodeling that facilitates this process. How this is manifest as a consequence of upstream pathways required for 53BP1 remains elusive.

In addition to the deficiencies described above, 53BP1 knockout mice showed defects in B- and T-cell development (Morales et al., 2003; Ward et al., 2003b; 2004; Difilippantonio et al., 2008). These studies have made it clear that 53BP1 is involved in both the process of V(D)J recombination and in class switch recombination (CSR) of antibody isotype. This is significant because a number of human disorders of the DDR and mouse models are profoundly immunodeficient as a consequence of defective NHEJ which is required for rejoining
programmed DNA breaks during immune development. In particular, it was found that long range V(D)J joining in T-cell receptor diversification did not occur in 53BP1-/- mice and a model was proposed whereby oligomerization of 53BP1 tethers the DNA in a configuration such that efficient long-range rejoining can occur (Difilippantonio et al., 2008). Similarly, class switching that occurs in B-cells during antibody maturation is markedly defective when these programmed breaks must be rejoined over larger distances (Bothmer et al., 2011). Importantly, Bothmer et al also showed that oligomerization and N-terminal phosphorylations are required for this long-range CSR and also for protecting the ends from resection (Bothmer et al., 2011). Together these effects have formed the basis for our understanding of how 53BP1 can act in the repair of DSBs.

1.3.3 53BP1 in HR

Homologous recombination (HR) is a process by which DSBs are repaired using a template, usually the sister chromatid, and is therefore considered to be error-free and restricted to the S- and G2-phases of the cell cycle (see Figure 1-6) (Helleday, 2010). HR is initiated by tracts of ssDNA generated during stalling or collapse of the replication fork due to normal delays in replication or due to DNA damage (DSBs, interstrand crosslinks, etc.) (Helleday et al., 2008). These ssDNA tracts are coated by RPA and cause activation and localization of the ataxia telangiectasia and RAD3-related (ATR) kinase and its co-activator, ATR-interacting protein (ATRIP) to the ssDNA (Cimprich and Cortez, 2008). Activated ATR-ATRIP complexes contribute to a number of processes including cell-cycle checkpoints and stabilization of stalled replication forks, and likely to initiation of HR by phosphorylation of multiple targets including BRCA1/2 (Cimprich and Cortez, 2008). Through undefined mechanisms RPA is exchanged for RAD51 with dependence on the RAD52 epistasis group (RAD51A, B, C, D) and on BRCA2
(Davies et al., 2001; Tannock et al., 2004; Roy et al., 2012). This RAD51-ssDNA filament then
invades the sister chromatid where DNA polymerases catalyze addition of complementary
oligonucleotides in an “extended D-loop” (Helleday, 2010). This recombination intermediate is
either directly resolved, or can lead to crossover events with dependence on the GEN1 and/or
MUS81-EME1 complexes before joining ends with DNA ligase (Abraham et al., 2003;
McPherson et al., 2004; Osman and Whitby, 2007; Ip et al., 2008; Fekairi et al., 2009; Svendsen
and Harper, 2010). Although considered to be error-free, it is more accurate to describe this as an
error resistant pathway since the DNA-polymerases have an associated error-rate, and the sister
chromatid may not contain the same information as the strand being repaired. This can lead to
“loss of heterozygosity” (Berger et al., 2011).

The role of 53BP1 in HR has not been well studied and conflicting evidence remains on how
53BP1 contributes to HR. MEF studies have indicated that spontaneous chromosome
abnormalities are increased in 53BP1/- cells as compared to wild-type (Ward et al., 2003b;
Minter-Dykhouse et al., 2008). Many of these defects were chromosome type breaks indicating
repair defects during G$_0$/G$_1$ (i.e. NHEJ), but a substantial fraction were chromatid-type breaks
that are associated with S- or G$_2$-phase repair (i.e. HR) and both types were increased following
IR (Minter-Dykhouse et al., 2008; Nagasawa et al., 2010). Induction of replication arrest by
hydroxyurea led to an increase in chromosome abnormalities significantly higher in 53BP1/-
MEFs compared to wild-type (Tripathi et al., 2007). In this study a loss of 53BP1 led to
increased RAD51 nucleofilament formation, which is a limiting step in HR. Furthermore,
CHK1-mediated phosphorylation of 53BP1 in the KBD contributes to its association with BLM
and p53, the latter of which has antirecombinogenic properties (Sengupta et al., 2004; Tripathi et
al., 2008). From a functional standpoint in human cells, 53BP1 siRNA increased levels of HR-
mediated plasmid integration (Xie et al., 2007). Although not part of the core-HR machinery, these studies suggest that 53BP1 is able to negatively regulate HR and may be a determinant of whether NHEJ or HR is used for DSB repair.

1.4 53BP1 AND CELL CYCLE CHECKPOINT CONTROL

1.4.1 Multiple Cell Cycle Checkpoints

When DSBs are induced the DDR leads also to cell cycle checkpoint activation allowing the cell time to repair the damage and to prevent damage propagation to daughter cells or mitotic cell death. A simplified diagram of the molecular mechanisms controlling cell cycle checkpoints is shown in Figure 1-7. It is important to understand that this checkpoint response, the repair responses (Figures 1-4 and 1-6), and the sensing response (Figure 1-1) are overlapping and cooperative. The loss of cell cycle checkpoints is a key event in tumourigenesis, and generally occurs through mutation of checkpoint proteins (i.e. p53, RB etc) (Hanahan and Weinberg, 2000; Berger et al., 2011). Checkpoints are largely controlled by ATM and ATR which are activated by the mechanisms detailed above depending on the cell cycle stage in which the damage is recognized. There are also spindle checkpoints that operate during mitosis to ensure that chromosomes are properly segregated to daughter cells, but these are not technically DNA damage checkpoints and have been reviewed elsewhere (Lew and Burke, 2003; Decordier et al., 2008).

_G1-phase Checkpoint:_ The predominant proximal checkpoint kinase activated in G₁ is ATM which acts on many targets, including CHK2, MDM2 and p53 (Lavin and Kozlov, 2007; Matsuoka et al., 2007). Production of p53 is continuous, but rapid turnover mediated by MDM2
FIGURE 1-7: DSB-induced Cell Cycle Checkpoints. DSB induction by IR activates ATM leading to accumulation of p53 and transactivation of its target genes, such as p21. Simultaneous phosphorylations of CHK2, SMC1 and NBS1 lead to G₁/S checkpoints and intra-S phase cell cycle delay by mediating CDKs as described in the text. DNA damage incurred during S-phase (i.e. by IR, UV or hydroxyurea (HU)) may create ssDNA, leading to activation of ATR. This activates CHK1 and likely ATM-mediated targets to enact the G2/M checkpoint and G₂-accumulation as described in the text.
prevents its accumulation in the cell (Wu et al., 1993). When ATM is activated MDM2 is phosphorylated and prevented from regulating p53-turnover and activated CHK2, itself a kinase, also phosphorylates p53 (Caspari, 2000). These events and ATM-phosphorylation of p53, stabilize p53 to act as a transcription factor to a multitude of genes, including p21\textsuperscript{WAF} (Beckerman and Prives, 2010). Inhibition of CDC25A by p21\textsuperscript{WAF} blocks the dephosphorylation of CDK2 which is required for hyperphosphorylation of RB to drive initiation of S-phase (Saha et al., 1997). Simultaneously, CHK2 phosphorylates CDC25A and prevents its activity in a more rapid fashion (Falck et al., 2001). Together, these two pathways lead to accumulation of cells near the G\textsubscript{1}/S boundary (Tannock et al., 2004; Weinberg, 2006).

\textit{S-phase Checkpoint:} When damage is recognized during S-phase the proximal kinase activated is ATR, and recently it has become clear that this contributes to ATM activation and each of these proteins acts on similar targets (Jazayeri et al., 2006; Stiff et al., 2006; Matsuoka et al., 2007). These kinases activate structural maintenance of chromosomes protein 1 (SMC1) and Nijmegen breakage syndrome protein 1 (NBS1) which transiently slows progression of replication (Bartek et al., 2004). A parallel pathway involves activation of the CHK1 and CHK2 proteins that phosphorylate CDC25A and CDC25C. This leads to CDC25C binding to the 14-3-3\textsigma (a p53 target) and this complex and CDC25A block CDK2 function and prevent further replication origin firing (Cimprich and Cortez, 2008). Together these pathways work to slow S-phase progression but do not stop it outright and even after high radiation doses cells eventually arrest in G\textsubscript{2} with dependence on ATR-CHK1 signaling (van der Kogel and Joiner, 2009).

\textit{G\textsubscript{2} Checkpoints:} There are actually two distinct “checkpoints” enacted during G\textsubscript{2} phase. The first rapid phase is termed the G\textsubscript{2}/M checkpoint and is dependent on ATM but independent of IR-dose
Like the intra-S phase checkpoint ATM-mediated activation of CHK2 induces phosphorylation of CDC25C and its association with 14-3-3σ prevents dephosphorylation of CDC2 (CDK1) and mitotic entry (Matsuoka et al., 1998; Lopez-Girona et al., 1999; Tannock et al., 2004). The second G₂ “checkpoint” is more accurately termed the G₂-accumulation. This particular function represents cells that were in other phases of the cell cycle at the time of irradiation and is ATM-independent and dose dependent (Xu et al., 2002). This G₂-accumulation occurs through a poorly understood ATR-CHK1 mediated pathway and is dominant in mutants that lack G₁/S or intra-S-phase checkpoints. It is proposed that this pathway may act as a final salvage mechanism to prevent mitotic entry of cells that were unable to arrest and remove DNA damage at earlier cell cycle stages.

### 1.4.2 53BP1 Contribution to Cell Cycle Checkpoint Control

The earliest functional studies of 53BP1 loss identified defects in cell cycle checkpoints. Most notably, multiple groups found increased mitotic fractions at low, but not high, IR-doses in 53BP1 siRNA-treated or 53BP1/- MEFs (Ditullio et al., 2002; Fernandez-Capetillo et al., 2002; Wang et al., 2002). The dose-dependence of this particular checkpoint poses a paradox since this G₂/M checkpoint measured here is dose-independent (Xu et al., 2002). Indeed, another study has found no defect of the G₂/M checkpoint in 53BP1/- MEFs, so the precise role of 53BP1 in the G₂/M checkpoint is still controversial (Ward et al., 2003a). Some of these anomalies may stem from poorly targeted siRNA oligos, as the early sequences used did have some affinity for ATM, a key controller of many cell cycle checkpoints (J. Petrini, personal communication). More recently it was found that 53BP1 helps sustain phosphorylation of CHK1/2 and loss of 53BP1 leads to a dose-dependent early release from the G₂/M checkpoint (Shibata et al., 2010). Mild
defects in the G₁/S checkpoint have also been noted by some, but not all groups and have not been systematically examined (Wang et al., 2002; Ward et al., 2003b).

It is worth noting that another predominant phenotype when 53BP1 is lost is a decrease in activation of downstream ATM-targets following DNA damage. It was initially found that 53BP1 siRNA knockdown in human cells reduced the ATM-dependent IR induction of CHK2\textsuperscript{Thr68} phosphorylation (Wang et al., 2002). This effect was also present in isogenic MEF cells as measured by using a general ATM substrate antibody, however CHK2\textsuperscript{Thr68} phosphorylation was not found to be affected by loss of 53BP1, possibly indicating off-target affects in the Wang et al. study (Ditullio et al., 2002; Fernandez-Capetillo et al., 2002). Similarly, ATM-dependent SMC1 phosphorylation was decreased in isogenic MEFs (Ditullio et al., 2002). Importantly, 53BP1 also appears to be involved in the activation of ATM\textsuperscript{Ser1981} autophosphorylation, as 53BP1 stimulates MRN-mediated ATM activation in vitro and siRNA targeting 53BP1 in NBS1-fibroblasts decreases CHK2\textsuperscript{Thr68} and ATM\textsuperscript{Ser1981} activation following IR (Mochan et al., 2003; 2004; Lee et al., 2010). It is worth emphasizing that 53BP1 and NBS1 appear to act in redundant pathways controlling ATM activation by autophosphorylation and may help to explain the G₂/M checkpoint paradox posed above (Mochan et al., 2003). The currently accepted model is one where 53BP1 contributes to proximal ATM activation, likely through a MRN-mediated mechanism. 53BP1 can also contribute to downstream signaling to ATM substrates, such as CHK2, p53 and SMC1, likely by concentrating them near the break site through protein-protein interactions.

Despite all of these findings, little radiosensitivity is noted when any of the three checkpoints discussed here is lost in isolation (Xu et al., 2002). This last point and relatively mild checkpoint-
defects without 53BP1 suggest that the radiosensitivity exhibited by 53BP1 loss is mainly attributed to defects in DNA repair, and not in cell cycle checkpoints.

1.5 53BP1 IN CANCER

1.5.1 Histochemical Loss and Activation of 53BP1 in Tumours

Two studies have found histological loss of 53BP1 protein in multiple tumour sites (lung, breast, kidney, larynx and stomach) which may be correlated with loss of transcript (Bartkova et al., 2007; Nuciforo et al., 2007). Another subset of these tumours showed focal localization of 53BP1 reminiscent of IRIF suggesting activated DDR signaling (Bartkova et al., 2007). One predominant theory is that during carcinogenesis the activation of oncogenes (i.e. cyclin E, E2F1 etc.) leads to replication stress and activation of the DDR to maintain cell cycle checkpoints, senescence or apoptosis to prevent tumour formation (Bartkova et al., 2005; Bartek et al., 2007a). The activation of DDR components (i.e. CHK2, H2AX, focal 53BP1 etc) are often present in pre-neoplastic lesions and are lost during progression to highly proliferative advanced tumours (Bartkova et al., 2005; Gorgoulis et al., 2005; Bartek et al., 2007b; Halazonetis et al., 2008). This may be a basis for development of 53BP1-null tumours in humans, however clinical immunohistochemical data is not conclusive on the matter and examining these phenomena in rodent models is complicated by the relatively rapid development of lymphomas and not solid tumours in constitutive mouse knockouts (Hakem, 2008). Whether this loss of 53BP1 is associated with regions of tumour hypoxia, cell cycle or necrosis/apoptosis is also not clear and careful tumour staging and correlation with 53BP1 status has not been done. The matter of hypoxia-53BP1 interactions will be important to understand as hypoxia itself is a negative prognostic factor in cancer outcome (Bristow and Hill, 2008; Chan et al., 2009). Recently, ATM
has been found to be activated in severe anoxic conditions which may impact 53BP1 through phosphorylations (Bencokova et al., 2009). A deeper understanding of these issues has been made more relevant by recent evidence showing that the most aggressive subtypes of breast cancer are more routinely associated with 53BP1 loss which may have important implications for treatment of such advanced tumours (Bouwman et al., 2010).

1.5.2 53BP1 in Cancer Treatment

The role of 53BP1 in repair has become increasingly important from a therapeutic standpoint. Cells with mutated BRCA1/2, such as found in familial breast cancers, are exquisitely sensitive to Poly(ADP)-ribose polymerase (PARP) inhibitors (Bryant et al., 2005; Farmer et al., 2005; Helleday et al., 2005; McCabe et al., 2006). PARP inhibition leads to accumulation of single stranded breaks that are recognized by the replication fork during S-phase and are repaired by BRCA1/2 mediated HR. When BRCA1/2 is lost in tumours they become sensitive to PARP inhibition through a “synthetically lethal” interaction. Recently, it was found that a loss of 53BP1 in these situations abolished the sensitivity to PARP inhibition (Bouwman et al., 2010; Bunting et al., 2010). In keeping with the above suppressive role for 53BP1 in HR, it is proposed that 53BP1 blocks the ATM-mediated end-resection step needed for HR, and instead promotes NHEJ of these replication-associated DSBs creating further chromosomal abnormalities (Cao et al., 2009; Bunting et al., 2010). Furthermore, 43% of triple negative breast cancers had loss of 53BP1 compared to only 2% in the remaining tumour subtypes and 53BP1 loss was most prevalent in BRCA1/2 mutated cancers (Bouwman et al., 2010). This may limit the utility of PARP inhibition in a certain subset of BRCA1/2 tumours and using 53BP1 status may be an important predictor of response to these molecularly targeted agents.
To date there have been no studies on 53BP1 status and radiosensitivity of either tumour or normal tissue in humans. There have been no descriptions of patients to date with mutations in 53BP1 and the expression pattern of 53BP1 throughout normal human tissue has not been studied. Consequently, mouse models have been key in the understanding of 53BP1 contribution to survival following radioresistance. Mice engineered to express a C-terminal truncation of 53BP1 (lacking >700 amino acids including the focus forming region and the nuclear localization signal) were highly sensitive to 7Gy IR where 100% of animals died 9-15 days following irradiation, whereas only 16% of wild-type littermates died (Morales et al., 2003). Similar results were found in 53BP1 knockout mice where the cause of death was determined to be bone marrow failure and severe intestinal bleeding (Ward et al., 2003b). Radiation sensitivity in relation to other DDR proteins has also not been well established in humans. MDC1 and 53BP1 double knockout mice are radiosensitive to the same degree as either single knockout indicating a common (epistatic) pathway for IR-survival of irradiated mice (Minter-Dykhouse et al., 2008). This final matter is confused by a recent study indicating that MDC1 has HR-promoting effects whereas 53BP1 promotes NHEJ and HR (Xie et al., 2007). There is no clear consensus on how 53BP1 and MDC1 operate with respect to NHEJ/HR and how upstream processes, including phosphorylations, influence this has not been tested. Most fundamentally, the functional outcome measured by clonogenic survival is poorly understood for 53BP1 and upstream processes and these may have important implications for therapeutic decision making.

1.6 GLOBAL HYPOTHESIS

The role of 53BP1 in the DDR has been extensively examined over the last 10+ years and has been implicated in cell cycle checkpoints, NHEJ, HR and maintenance of senescence. However,
little is understood about post-translational modifications of 53BP1 and how they are controlled by upstream signaling components, by localization to chromatin and by a myriad of cellular stresses from IR to low oxygen. The importance for understanding these responses is underscored by the involvement of 53BP1 loss in multiple tumour sites and the impact this may have on treatment responses. I hypothesized that 53BP1 phosphorylation occurs downstream of the canonical MRN-ATM-RNF168 signaling pathway following replication, oxygen and IR-induced stress and would contribute to non-homologous end-joining and cell survival following ionizing radiation. These studies would expand our knowledge of the signaling towards 53BP1 and of the in situ phosphorylation responses of 53BP1. Combined with functional analyses for 53BP1 involvement in survival following ionizing radiation these in vitro studies would serve as a basis for the use of 53BP1 and its phosphorylation as a biomarker in cancer tissue and potentially as a method for evaluating the response of tumours to specific therapies.

1.7 OUTLINE OF THESIS

A general literature review as it pertains to this thesis has been outlined here in Chapter 1. The questions that underlie the following 4 Chapters are diagrammed in Figure 1-8. In Chapter 2 I show published work that severe oxygen stress in vitro activates ATM and this contributes to phosphorylation of 53BP1, specifically in S-phase cells, in absence of exogenously generated DSBs (Harding et al., 2011). In Chapter 3 I have shown that both ATM and DNA-PKcs phosphorylate 53BP1 in response to ionizing radiation and this is independent of the stable localization of 53BP1 to DSBs; this work has also been published (Harding and Bristow, 2012). In Chapter 4 I have found that 53BP1 and two of its phosphoforms contribute to in vitro DSB
rejoining and that cells lacking 53BP1 are more sensitive to ionizing radiation. This radiation sensitivity can be further augmented with inhibitors of kinases that act on 53BP1 suggesting that there are 53BP1 dependent- and independent- pathways for survival from ionizing radiation. Appendix A shows that 53BP1, ATM and p53 physically interact as a complex at the sites of DSBs; I was a joint co-first author on this paper (Rashid et al., 2011). In Appendix B I outline some studies from early in my PhD that indicated siRNA knockdown of 53BP1 expression was not a suitable model for clonogenic survival assays and suggests a possible reason. This prompted our development of a stable shRNA based system that is described in Chapter 5. During the course of my experiments, there were a number of interesting data that in the future may be the basis of further hypotheses relating to the basic and translational biology pertaining to the DDR. I have begun to collaborate on the development of a model system in which these aspects can be queried. These future studies are discussed in Chapter 5.
Does hypoxia-induced ATM-activation signal to 53BP1-phosphorylation?

Do upstream signaling pathways control localization of phosphorylated 53BP1 to IRIF?

Does 53BP1 and phosphorylation contribute to NHEJ and survival from IR?

Does 53BP1 loss contribute to HR or sensitivity to novel therapeutics?

Does 53BP1 loss in human tumour models sensitize cells to therapies?

Is 53BP1 a predictive or prognostic factor in prostate cancers?

FIGURE 1-8: Diagrammatic outline of Thesis
1.8 REFERENCES


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CHAPTER 2
ATM-DEPENDENT PHOSPHORYLATION OF 53BP1 IN RESPONSE TO GENOMIC STRESS IN OXIC AND HYPOXIC CELLS

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2.1 ABSTRACT

The ATM kinase is activated by chromatin modification following exogenous and endogenous DSBs or cell stress, including acute anoxia. The 53BP1 protein contains multiple ATM-consensus phosphorylation sites in its N- and C-termini and may therefore be a distal read-out of ATM function. We have examined the cellular activation of these phosphorylation sites for the first time in situ following anoxic/hypoxic stress and IR-induced exogenous DSBs. We show that multiple residues of 53BP1 are phosphorylated and that these phosphoforms form discrete nuclear foci following IR or during DNA replication as exogenous or endogenous DNA double strand breaks, respectively. Novel data pertaining to the phosphorylation of 53BP1\textsuperscript{Ser25} in situ supports its dependency on the ATM kinase; but this occurs independently of p53 function. We show that 53BP1\textsuperscript{Ser25} is activated specifically in S-phase cells during anoxia in an ATM-dependent manner. Exogenous DSBs form discrete IR-induced foci whereas oxygen stress induced non-localized 53BP1\textsuperscript{Ser25} activation. Our in vitro data is supported by irradiated xenograft studies in vivo whereby 53BP1\textsuperscript{Ser25} phosphorylation does not occur in sub-regions positive for the hypoxia marker EF5. We propose a model whereby DSBs induce chromatin modification at sites of DNA damage which is tracked by the ATM substrates γH2AX and 53BP1\textsuperscript{Ser25} in a mechanism distinct from p53-mediated cell cycle arrest. Together this work indicates 53BP1\textsuperscript{Ser25}, and possibly other 53BP1 phosphoforms, as a bona fide DSB-biomarker for surveying ongoing DNA-damage related signaling in oxic and hypoxic cells during clinical radiotherapy.
2.2 INTRODUCTION

Tumour hypoxia can modulate the response to radiotherapy or chemotherapy and the capacity for metastasis, and is therefore associated with a poor clinical prognosis (Bristow and Hill, 2008; Rodemann, 2009). Depending on the degree and duration of oxygen deprivation, hypoxic cells acquire decreased numbers of exogenous DSBs following IR or chemotherapy and may have a reduced capacity for DSB repair (Bristow and Hill, 2008; Chan et al., 2009). For example, hypoxic cells can have decreased transcription or translation of DSB-repair proteins (e.g. the HR-related proteins RAD51, BRCA1 or BRCA2) and DNA mismatch repair proteins (e.g. MLH1, MSH2) (Ward et al., 2003; Meng et al., 2005; Chan et al., 2008; Chan and Bristow, 2010). In HR-defective hypoxic cells, this can lead to an increased sensitivity to IR, PARP inhibitors or DNA crosslinking agents due to “contextual” synthetic sickness or lethality in replicating tumour cells (Meng et al., 2005; Chan et al., 2008; 2010).

Hypoxia and exogenous DSBs can also modify chromatin and activate the ATM kinase to drive an ATM-p53 DDR signaling cascade resulting in cell cycle arrest and DSB repair (Kastan, 2008; Bencokova et al., 2009; Jackson and Bartek, 2009). A component of this response is 53BP1 that rapidly re-distributes from a pan-nuclear staining pattern into discrete irradiation IRIF (Schultz et al., 2000). These co-localize with the DSB-marker γH2AX. 53BP1 itself is phosphorylated by the ATM kinase at multiple sites in its N- and C-termini; however, the functional significance of this activity for the tracking of DSBs is unknown as is 53BP1’s precise function during checkpoint control and DSB repair (Adams et al., 2005). In cases of severe acute anoxia (0.02% for 16 hours), the ATM kinase is activated and induces a CHK2-dependent, G2/M cell cycle arrest (Bencokova et al., 2009; Pires et al., 2010a). These anoxic cells can further acquire DSBs
during re-entry into the cell cycle following re-oxygenation or secondary to collapsed replication forks in cells destined to permanently arrest in S-phase (Olcina et al., 2010). During this anoxic response, both the ATM and ATR kinases modulate the phosphorylation of the histone variant H2A.X at Serine-139 (forming γH2AX) in arrested anoxic cells \textit{in vitro} and \textit{in vivo} (Freiberg et al., 2006; Bencokova et al., 2009; Olive et al., 2010). Importantly, recent evidence has shown that 53BP1 is phosphorylated at serine-25 (53BP1$^{\text{Ser25}}$) in response to acute anoxia, independent of DNA-damage (Bencokova et al., 2009).

The initial sensing and repair of both endogenous and exogenous DSBs can be different in oxic versus hypoxic cells to impact on both genetic stability and cancer treatment (Bristow and Hill, 2008). Herein, we have determined the spatial distribution and cell-cycle dependence of 53BP1-phosphoforms in irradiated oxic cells or hypoxic cells \textit{in vitro} and \textit{in vivo}. These responses have been quantified \textit{in situ} for the first time and support the 53BP1$^{\text{Ser25}}$ response as a \textit{bona fide} DSB biomarker directly downstream of ATM. However, this DDR response is independent of the ATM-p53 pathway involved in cell cycle arrest setting up a model of 53BP1 activation by the ATM kinase, independent of cell cycle arrest.

2.3 RESULTS

2.3.1 Multiple residues of 53BP1 are phosphorylated following IR in normal and malignant cells

Previous studies using western blotting had shown that 53BP1 is phosphorylated on multiple N-terminal residues (Serine-166, Serine-176/178, Threonine-302, Serine-452 and Serine-831; see \textbf{Supplemental Figure 2-1A}) in an ATM-dependent manner. Phospho-specific 53BP1 antibodies
were provided by J. Rouse and previously evaluated for specificity (Jowsey, 2007). This study used purified peptides and western blotting of HEK293 cells to show: (i) Phosphospecific antibodies recognized phosphorylated peptides but not peptides corresponding mutation of the specific epitope to alanine. (ii) Ionizing radiation (5Gy 15-120 minutes) induced an ATM-dependent phosphorylation at all examined 53BP1 epitopes. (iii) Phosphorylation of 53BP1 was induced following UV-irradiation with dependence on the ATR kinase. Based on these results, two commercial antibodies, 53BP1\(^{\text{Ser25}}\) and 53BP1\(^{\text{Ser1778}}\) were obtained from Novus and Cell Signaling, respectively. Of these two commercially available antibodies, only 53BP1\(^{\text{Ser25}}\) has been found to be ATM-dependent (Ward et al., 2003). These were validated for their detection of 53BP1 by: (i) showing lack of 53BP1 detection using immunofluorescence in irradiated (2Gy 30m) 53BP1\(-/-\) MEFs and in 53BP1-siRNA treated HCT116 and H1299 human tumour cell lines. (ii) Focal nuclear induction using IF following IR-induction for both phosphospecies in all 53BP1-proficient cell types examined. (iii) Induction of both phosphospecies on western blot in all 53BP1-proficient cell types examined. These data were consistent between the commercial antibodies and those provided by J. Rouse. And finally, (iv) wild-type MEF cell lines were negative for 53BP1\(^{\text{Ser1778}}\) consistent with the absence of this phosphosite from the murine 53BP1 protein.

The putative ATM-dependent phosphorylation epitopes on 53BP1 are diagrammed in Supplemental Figure 2-1A in relation to the tandem tudor domain required for 53BP1 IRIF formation and the BRCT domain which is the motif in which 53BP1 binds to p53 (Adams and Carpenter, 2006). We first examined the \textit{in situ} localization of these phospho-53BP1 epitopes following ionizing radiation and observed that they form similar IRIF in human colorectal carcinoma cells (HCT116; Supplemental Figure 2-1B). As it is readily available commercially
we selected the $53BPI_{\text{Ser25}}$ phosphoform for further analysis and showed that IRIF are also induced in normal human fibroblasts (NHF), indicating that this is not restricted to malignant cells (Supplemental Figure 2-2). We conclude that IRIF of 53BP1 include molecules that are phosphorylated on multiple residues in the N- and C-termini and that this can occur independently of malignant transformation.

### 2.3.2 Phosphorylation of 53BP1 at Serine-25 is an ATM-dependent marker for DSBs.

The canonical marker for DSBs is phosphorylated histone H2A.X$^{S139}$ ($\gamma$H2AX) which forms IRIF that co-localize with 53BP1 (Rogakou et al., 1998; Rappold et al., 2001). Using immunofluorescent microscopy, we showed that in HCT116 colorectal carcinoma cells, like $\gamma$H2AX and 53BP1$^{\text{Total}}$, 53BP1$^{\text{Ser25}}$ formed IRIF within 30 minutes following exposure to 2Gy of ionizing radiation (Figure 2-1A). These foci colocalized with $\gamma$H2AX as evidenced in composite images generated both in 2- and 3-dimensions. We also determined that the number of 53BP1$^{\text{Ser25}}$ IRIF increased with dose and with those of 53BP1$^{\text{Total}}$ and $\gamma$H2AX and that this increase in 53BP1$^{\text{Ser25}}$ foci number correlated with a dose-dependent induction of phosphorylation on western blot (Figures 2-1B-D). Finally, we found that despite the known interaction of 53BP1 and p53, the phosphorylation of 53BP1 did not depend on p53 presence (Figure 2-1E) (Iwabuchi et al., 1994; Rashid et al., 2011). These results indicate that the dose-dependent 53BP1$^{\text{Ser25}}$ phosphorylation induced by IR can be localized to sites of DSBs independent of p53 status.
FIGURE 2-1: 53BP1 is phosphorylated in a dose-responsive manner and tracks DSB repair following IR in HCT 116 cells. A) 53BP1<sup>Ser25</sup> co-localizes with the canonical DSB marker γH2AX at 0.5h following 2 Gy (shown as 2- and 3-D images; scale bar is 10µm). NIR indicates non-irradiated control cells. B) 53BP1<sup>Ser25</sup> phosphorylation occurs in a dose-responsive manner based on Western blotting for protein expression (0-10Gy at 30 minutes). Continued on next page.
FIGURE 2-1 Continued: 53BP1 is phosphorylated in a dose-responsive manner and tracks DSB repair following IR in HCT116 cells. C) 53BP1^{Ser25} foci induction following 0-4 Gy at 30 minutes (scale bar is 10µm). D) Quantification of IRIF is shown based on 3 independent experiments and reported as foci/nucleus incution by subtraction of average foci number in non-irradiated cells (mean +/- SEM; * denotes significant difference for 53BP1^{Total} from 53BP1^{Ser25} and γH2AX at p<0.05 see Materials and Methods). E) 53BP1^{Ser25} foci induction is similar in HCT116 p53+/+ and p53/- isogenic cells at 0.5h following 2Gy (scale bar is 10µm).
Using subnuclear UV-microbeam induced DNA damage we have recently shown that 53BP1\textsuperscript{Ser25} phosphorylation is ATM-dependent (Rashid et al., 2011). We next sought to verify that 53BP1\textsuperscript{Ser25} IRIF were also ATM-dependent, and have shown that when normal human fibroblasts (NHF) synchronized in G\textsubscript{0}/G\textsubscript{1} by contact inhibition were pretreated with an inhibitor of ATM the induction of 53BP1\textsuperscript{Ser25} IRIF was reduced to similar levels as γH2AX (Supplemental Figures 2-2A/2B). Likewise, AT-fibroblasts lacking ATM protein, 53BP1\textsuperscript{Ser25} IRIF were markedly reduced. However, localization of 53BP1\textsuperscript{Total} was largely unaffected as were 53BP1\textsuperscript{Ser25} IRIF when DNA-PK was inhibited (Chapter 3). This was expected as deletion of the 53BP1 N-terminus, containing multiple ATM-consensus phosphorylation sites, did not eliminate redistribution of 53BP1\textsuperscript{Total} into IRIF (Ward et al., 2003). We conclude that phosphorylation and localization to DSBs of 53BP1\textsuperscript{Ser25} depends largely on the ATM kinase.

\textbf{2.3.3 Phosphorylation of 53BP1\textsuperscript{Ser25} occurs during chronic anoxia treatment in S-phase cells.}

Prolonged periods of anoxia (<0.02% O\textsubscript{2}) induce ATM autophosphorylation and ATR-dependent γH2AX activation in S-phase cells (Bencokova et al., 2009). Although 53BP1\textsuperscript{Ser25} was induced by prolonged anoxia treatment by western blotting, the distribution of this phosphoform was not examined, nor was the cell-cycle dependence. Here, using the thymidine nucleoside analogue 5-ethynyl-2’deoxyuridine (EdU) to identify S-phase cells, we show that even without treatment, S-phase cells are positive for γH2AX. This is further augmented by anoxia treatment, but left unchanged by ATM inhibition (ATMi; see Figure 2-2 and control images using hydroxyurea to stall DNA replication in Supplementary Figure 2-3). We verified that ATM was inhibited by examining the phosphorylation of CHK2\textsuperscript{T68} and showed that it was induced specifically in anoxic S-phase cells and that inhibition of ATM blocked the S-phase CHK2\textsuperscript{T68}
FIGURE 2-2: 53BP1^{Ser25} phosphorylation is activated during hypoxic stress in HCT116 cells. A) H2AX phosphorylation is activated both during normoxia and anoxic treatments in S-phase cells independently of ATM kinase activity. B) CHK2 is phosphorylated at Threonine-68 specifically in S-phase anoxic cells with dependence on ATM. All scale bars are 10µm. Asterisks indicate S-phase cells identified by EdU positivity in silico. Continued on next page.
FIGURE 2-2 Continued: 53BP1<sup>Ser25</sup> phosphorylation is activated during hypoxic stress in HCT116 cells. C) 53BP1<sup>Total</sup> displays a focal staining pattern in S-phase anoxic cells dependent on ATM kinase activity. D) 53BP1<sup>Ser25</sup> is activated by anoxia in S-phase cells. All scale bars are 10µm. Asterisks indicate S-phase cells identified by EdU positivity <i>in silico</i>. EdU incubation was at 10µM for 1h before placing in the MEC and maintained until fixation.
phosphorylation (Figure 2-2B). Although not to the same degree as IRIF, 53BP1Total becomes more focal in anoxic S-phase cells and ATMi inhibited this redistribution (Figure 2-2C). When examining 53BP1Ser25 we noticed that when oxic cells were treated with ATMi phosphorylation is induced over baseline (Figure 2-2D). Anoxia activated 53BP1Ser25 in S-phase cells, but did not result in foci formation to the same extent as that following IR. Although not abolished by ATMi, the anoxia-dependent 53BP1Ser25 activation is reduced as compared to vehicle control.

To examine replication stress associated with collapsed replication forks we treated cells for 16 hours with 2mM HU and found that both γH2AX and CHK2T68 were activated (Supplementary Figure 2-3 and Chapter 3). Additionally HU treatment resulted in redistribution of 53BP1Total into foci that are less distinct than those induced by IR. Finally, 53BP1Ser25 foci were induced by HU treatment indicating that replication stress also causes phosphorylation of 53BP1Ser25. We conclude that replication stress induced by anoxia in S-phase cells leads to activation of 53BP1Ser25, and may rely less on the ATM-kinase compared to exogenous DSB responses.

2.3.4 Anoxic regions of tumour xenografts exhibit reduced 53BP1Ser25 phosphorylation.

We next examined the activation of 53BP1Ser25 in 22RV1 human prostate xenografts grown in CD1 nude mice treated with whole-body irradiation. Consistent with phosphorylation of 53BP1Ser25 at DSBs hypoxic regions identified by EF5 staining showed markedly reduced focal staining as compared to oxic regions of the tumour which produced IRIF 1h following 10Gy (Figure 2-3A). Interestingly, no activation of 53BP1Ser25 was noted in non-irradiated EF5-positive areas of the tumour. Further experiments are required across multiple doses and times following irradiation in sections co-stained for proliferation markers, to delineate arrested anoxic
cells from replicating hypoxic cells. However, these preliminary data suggests that \( 53BP1^{\text{Ser25}} \) IRIF form \textit{in vivo} in an oxygen-dependent manner.

Together our data support a model for ATM-dependent, but p53-independent, phosphorylation of 53BP1 in response to DSBs under oxic or hypoxic conditions. A model for this response is shown in Figure 2-3B.
FIGURE 2-3: Differential activation of $53BP1^{\text{Ser25}}$ in vivo and model for $53BP1$ activation in tumours. A) 22RV1 xenografts were established in CD1 nude mice and irradiated with 10 Gy under air-breathing conditions. Animals were injected with EF5 at 3h prior to sacrifice. Irradiated tumours (IR; excised at 1h post-treatment; lower panel) show increased phosphorylated $53BP1^{\text{Ser25}}$ in non-EF5 (oxygenated) regions whereas no phosphorylated $53BP1^{\text{Ser25}}$ was noted in non-irradiated specimens (NIR, upper panel). The dotted line delineates oxic (Ox) from hypoxic (Hyp) regions of the tumour. Scale bar is 10µm. B) Model of $53BP1$ phosphorylation in response to chromatin modifications secondary to ATM signaling in response to exogenous or endogenous stress. In this model, $53BP1$ phosphorylation by ATM is independent of p53 and is a direct biomarker of DSBs, both in vitro and in vivo.
2.4 DISCUSSION

In this work we have examined, for the first time, the ATM and p53 dependence of \textit{in situ} phosphorylation of 53BP1 during exogenous and endogenous DSBs and under hypoxic conditions, \textit{in vitro} and \textit{in vivo}. Multiple S/T-Q PI-3 like kinase sites are present in the 53BP1 N-terminus leading to IR-induced discrete nuclear and focal phosphoforms. IRIF formation of 53BP1\textsuperscript{Ser25} was not completely eliminated with ATM inhibition and this could reflect the increased sensitivity of this IRIF assay over western blotting that has previously shown ATM-dependent 53BP1\textsuperscript{Ser25} phosphorylation or it could be due to other related PI-3 like kinases such as DNA-PKcs (Ward et al., 2003). The maintenance of 53BP1\textsuperscript{Total} IRIF with ATM inhibition was not entirely unexpected as both phosphomutants and N-terminal truncation mutants of 53BP1 still formed IRIF (Ward et al., 2003). These mutants were without checkpoint defects, but exhibit elevated residual $\gamma$H2AX indicating repair defects (Ward et al., 2006). The fact that this phosphorylation is ATM dependent could have important ramifications for the role of 53BP1 at complex DSBs in locally damaged sites which are difficult to repair and may lead to a senescent phenotype (Noon et al., 2010; Rodier et al., 2011).

ATM-dependent activation of 53BP1\textsuperscript{Ser25} was observed by Western blotting during acute anoxia, and our additional \textit{in situ} results show that 53BP1\textsuperscript{Ser25} is (1) specifically induced in S-phase anoxic cells and (2) is activated without a discrete nuclear focal response (Bencokova et al., 2009). We find that this activation is not as drastic as that following IR, which likely indicates that the pathway is not subject to the feed-forward signaling that occurs after extensive IR-induced DSBs. The anoxia-associated activation likely stems from replication stress associated with disassembly of replisome structures and subsequent replication fork collapse (Pires et al.,
2010b). Supporting this we also find activation of $53\text{BP1}^{\text{Ser25}}$ after treatment with hydroxyurea. It has previously been shown that $53\text{BP1}$ phosphorylation following HU is induced by CHK1 which may also relate to $53\text{BP1}^{\text{Ser25}}$ phosphorylation; the latter requires further investigation (Tripathi et al., 2008).

We also examined $53\text{BP1}^{\text{Ser25}}$ status in both untreated and irradiated human 22RV1-prostate cancer xenografts in preliminary experiments to relate $53\text{BP1}$ staining to oxygen levels as denoted by immunohistochemical staining with EF5. In contrast to in vitro results, we find no induction of $53\text{BP1}^{\text{Ser25}}$ in EF5-positive, untreated xenografts. However, we did observe the expected decreased activation of $53\text{BP1}^{\text{Ser25}}$ in EF5-avid sub-regions consistent with a decreased DSB response under hypoxia. Previous in vitro results indicate that oxygen must be decreased to levels less than 0.5% before DSB-signaling is activated (Bencokova et al., 2009). It may be that the pO$_2$ level in vivo has not reached levels that activate these responses or that S-phase anoxic cells were not captured with our imaging in these tumours; but further defined work is required in vivo to follow-up on these preliminary observations. In particular, $53\text{BP1}$ phosphorylation status may be affected by rounds of anoxia/hypoxia and reoxygenation that impact on ATM-dependent anoxia signaling (Freiberg et al., 2006). More detailed analysis of in vivo tumour tissue using methods that allow the delineation between currently and previously hypoxic regions (reoxygenated) will be required for evaluating these complex $53\text{BP1}$-responses (Dubois et al., 2009).

If, as discussed above, $53\text{BP1}$ phosphorylation actively contributes to DSB repair, then there may be a selective pressure for $53\text{BP1}$ loss during generation of genetically instable and “mutator” phenotypes in aggressive hypoxic-cancers (Chan and Bristow, 2010). Indeed, $53\text{BP1}$
has been found to be lost in subsets of tumours including those of the lung, larynx and breast and has been associated with BRCA1 deficiency in breast cancers (Bartkova et al., 2005; Nuciforo et al., 2007; Bouwman et al., 2010). However, no correlative studies between 53BP1 status and hypoxic/anoxic status have been published to our knowledge and the functional implication of the 53BP1 loss is still not well understood. 53BP1 has been implicated in the repair of DSBs by both NHEJ and HR, and therefore synthetic lethality relating to the loss of 53BP1 might be exploited therapeutically (Iwabuchi et al., 2003; Ward et al., 2006; Tripathi et al., 2007). Interestingly, decreases in HR induced by hypoxia result in sensitivity to PARP inhibition in “contextual synthetic lethality”(Liu et al., 2008; Chan and Bristow, 2010). It will be critical to understand if 53BP1 activation or loss is correlated with oxygen status and radiotherapy response.

Tracking both 53BP1 function and the hypoxic status in tumours may be advantageous in predicting the success of radiotherapy using in situ biomarkers and dynamic contrast-MRI or PET imaging (Busk et al., 2009; Christian et al., 2010; Gulliksrud et al., 2011; Overgaard, 2011). These biomarkers will have to include determinants of cell cycle phase as the role of 53BP1 in HR and NHEJ is modulated by cell cycle, which is turn modulated within sub-regions of hypoxia before and during fractionated radiotherapy (Iliakis, 2009; Koch et al., 2009; Yaromina et al., 2010). Further studies are clearly required to elucidate the biology and function of 53BP1 in vivo during clinical treatment. When taken together, the data suggest that a loss of 53BP1 function specifically in tumours (and particularly in anoxic tumour areas), could improve the therapeutic ratio for tumour cell kill over normal cell kill during radiotherapy for 53BP1-targeted approaches (Bristow et al., 2007; Thoms and Bristow, 2010).
2.5 MATERIALS AND METHODS

2.5.1 Cell line culture and irradiation conditions

All cells were maintained in 5% CO$_2$ at 37°C. Oxic cells were incubated in a standard laboratory incubator at ambient oxygen concentration. Anoxic treatment was performed in a humidified microenvironmental (MEC) chamber (VA500 Microbiology International) with 0.01% O$_2$ for 16h before fixation. HCT116 (wild-type p53 and null-p53) were obtained from Dr Bert Vogelstein and cultured in McCoy’s 5A media supplemented with 10% FBS. GM05757 and GM02052 primary cell lines were obtained from Coriell and maintained in αMEM media with 20% FBS. 22RV1 cells were maintained in RPMI 1640 media with 10% FBS and no phenol red before trypsinizing to inject into mice. Irradiation was performed using a Nordion GammaCell Caesium-137 source at ~1Gy/min. The ATM (KU55933; 10µM) and DNA-PKcs (NU7441; 5µM) were obtained from Tocris Bioscience and were added to cells 1h before introduction of the cells to the microenvironment chamber, or 1h before exposure to ionizing radiation and maintained until fixation. Unless stated otherwise, experiments were repeated at least 3 times and representative images are shown.

2.5.2 Immunofluorescence

Cells were grown on coverslips for 24 hours before treatment. The fixation and staining for nuclear DNA content and IRIF and subsequent foci quantitation has been previously described (Rashid et al., 2005). Briefly, cells were seeded onto #1.5 coverslips 24h before treatments. Following necessary treatment cells were rinsed in PBS, fixed at room temperature (4% paraformaldehyde/0.2% Triton X-100 in PBS pH8.2). Following 20 minutes in fixative, cells were briefly rinsed in PBS and permeabilized in 0.5% NP-40/PBS buffer. EdU staining was
carried out as necessary (see below) and cells were blocked using 2% BSA/1% donkey serum/PBS buffer for at least 1 hour at room temperature. Coverslips were inverted onto parafilm spotted with primary antibody solutions made in 3% BSA and incubated at 4°C in a humidified chamber overnight. Following 3x5 minute antibody washes (0.5% BSA/0.175% Tween-20/PBS) coverslips were inverted onto secondary antibody solution as above. Coverslips were incubated in 0.1µg/mL DAPI after 3 more antibody washes and rinsed in PBS before mounting onto glass slides containing Vectashield (Vector Labs). Unless otherwise indicated all reagents were obtained from Sigma-Aldrich and/or BioShop.

Cells were imaged in 3 dimensions as described using either a 60X or 100X 1.4NA oil immersion objectives and a Photometrics Cascade 512B EM-CCD camera (Fraser et al., 2011; Rashid et al., 2011). Cells were analyzed for foci number using Image Pro Plus (Media Cybernetics) by identifying at least 50 nuclei and manually determining fluorescence intensity thresholds for foci identification which were maintained throughout treatment groups (Rashid et al., 2011). γH2AX (Millipore, JBW301, 1:800), 53BP1Total (Bethyl, A300-272A, 1:1000), 53BP1Ser25 (Novus, NB100-1803, 1:500), CHK2Thr68 (Cell Signaling #2661 1:250). Other 53BP1-phosphospecific antibodies were a kind gift of J. Rouse (Jowsey et al., 2007).

Cells undergoing DNA replication were visualized using EdU-labeling. Staining for EdU was done using a Click-iT EdU 647 Alexa Fluor Imaging kit according to manufacturer’s specifications (Invitrogen). EdU at 10µM was added 1h before fixation of oxic cells or gassing of cells in the MEC.
2.5.3 Western blotting

Western blotting was performed using the Licor Odyssey system as previously described (Rashid et al., 2011; also see Chapter 3). Antibodies used in this study were: 53BP1\textsuperscript{Total} (Bethyl, A300-272A, 1:1000), 53BP1\textsuperscript{Ser25} (Novus, NB100-1803, 1:100) and Ku70 (Santa Cruz, SC5309, 1:10000).

2.5.4 Tumour initiation and immunohistochemistry

22RV1 xenograft tumours were induced in CD1 nude mice (Charles River) treated with i.p. injection of EF5 (Varian) at 30mg/kg 3 hours before sacrifice as previously described (Chan et al., 2010). Mice were whole body irradiated with 10Gy using a Nordion GammaCell Caesium-137 source at -1Gy/min, and sacrificed 1 hour later. Tumours were excised and processed for IHC as previously described (Chan et al., 2010).

2.5.5 Statistics

Quantitative data are show as mean values and associated standard error of the means (SEM). Pairwise comparison of foci quantification was done using the Mann-Whitney non-parametric test using a p-value of less than 0.05 for significance. All statistical analyses were performed using Graphpad software (Prism).
2.6 Supplementary Figures

SUPPLEMENTARY FIGURE 2-1: Multiple 53BP1 epitopes are phosphorylated following DNA damage. A) Schematic of the 53BP1 protein with suggested ATM-dependent epitopes that can be activated following DNA damage. B) Multiple 53BP1 phosphospecies are activated in situ within HCT116 cells 30 minutes following 2Gy (scale bar is 10µm). Antibodies a gift from J. Rouse (Jowsey, 2007). NIR designates non-irradiated cells.
SUPPLEMENTARY FIGURE 2-2: 53BP1<sup>Ser25</sup> phosphorylation is dependent on the ATM kinase in NHF cells. A) 53BP1<sup>Ser25</sup> IRIF inhibition in normal human fibroblasts (NHF) treated with 10μM ATMi or within AT-fibroblasts (ATF; scale bar is 10μm). IRIF of γH2AX and 53BP1<sup>Total</sup> are shown for comparison. Cells were either non-irradiated (NIR) or irradiated with 2Gy and fixed at 0.5h. B) Quantification of foci in (A) for 3 independent experiments (mean +/- SEM; * denotes significance at p<0.05; see Materials and Methods).
SUPPLEMENTARY FIGURE 2-3: Control DNA repair foci images following DNA replication arrest in HCT116 cells. H2A.X phosphorylation, 53BP1^{Total}, CHK2^{Thr68} phosphorylation and 53BP1^{Ser25} phosphorylation are activated by 16h treatment with 2mM HU. Compare to 21% NIR DMSO control images performed and analysed together in Figure 2.2. Scale bar is 10\(\mu\)m.
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CHAPTER 3
DISCORDANCE BETWEEN PHOSPHORYLATION AND RECRUITMENT OF 53BP1 IN RESPONSE TO DNA DOUBLE STRAND BREAKS

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3.1 ABSTRACT

During the DNA damage response chromatin modifications contribute to localization of 53BP1 to sites of DNA double strand breaks. 53BP1 is phosphorylated during the DDR, but it is unclear whether phosphorylation is directly coupled to chromatin binding. In this study, we used human diploid fibroblasts and HCT116 tumour cells to study 53BP1 phosphorylation at Serine-25 and Serine-1778 during endogenous and exogenous DSBs (DNA replication and whole-cell or sub-nuclear microbeam irradiation, respectively). In non-stressed conditions, endogenous DSBs in S-phase cells led to accumulation of 53BP1 and γH2AX into discrete nuclear foci. Only the outright collapse of DNA replication forks following prolonged hydroxyurea treatment initiated 53BP1\textsuperscript{Ser25} and 53BP1\textsuperscript{Ser1778} phosphorylation. In response to exogenous DSBs, 53BP1\textsuperscript{Ser25} and 53BP1\textsuperscript{Ser1778} phosphoforms localized to sites of initial DSBs in a cell cycle-independent manner. 53BP1 phosphoforms also localized to late residual foci and associated with promyelocytic leukaemia nuclear bodies (PML-NBs) during IR-induced senescence. Using isogenic cell lines and small molecule inhibitors, we observed that DDR-induced 53BP1 phosphorylation was dependent on ATM and DNA-PKcs kinase activity, but were independent of MRE11 sensing or RNF168 chromatin remodeling. However, loss of RNF168 blocked recruitment of phosphorylated 53BP1 to sites of DNA damage. Our results uncouple 53BP1 phosphorylation from DSB localization and support parallel pathways for 53BP1 biology during the DDR. As relative 53BP1 expression may be a biomarker of DNA repair capacity in solid tumours, the tracking of 53BP1 phosphoforms in situ may give unique information regarding different cancer phenotypes or response to cancer treatment.
3.2 INTRODUCTION

The 53BP1 protein was originally identified in a yeast 2-hybrid screen as a binding partner of the p53 tumour suppressor protein (Iwabuchi et al., 1994). Subsequently, 53BP1 was shown to localize to sites of DSBs during the initial stages of the DDR (Schultz et al., 2000). During exogenous DSB induction, the MRE11-RAD50-NBS1 (MRN) complex localizes to the break and contributes to activation of phosphoinositide 3-kinase-like kinases (PIKKs) that include ATM and DNA-PKcs (Lavin, 2004). Simultaneously, DNA-PKcs and ATM are activated and induce the phosphorylation of the histone variant H2A.X (forming γH2AX) at megabase domains spanning the break (Stiff et al., 2004; Bonner et al., 2008; Redon et al., 2011). This leads to a cascade where MDC1, also phosphorylated by ATM, is recruited to the chromatin and facilitates ubiquitylation of H2A.X and H2A by the RNF8-UBC13 and RNF168-UBC13 ubiquitin ligase complexes leading to extensive K63-linked chains within DSB-associated chromatin (Stewart, 2009). These chromatin modifications expose constitutively dimethylated K79 and K20 residues on histones H3 and H4, respectively, to concentrate 53BP1 at the DSB break site (Huyen et al., 2004; Botuyan et al., 2006).

Immunofluorescent microscopy techniques using whole cell or microbeam irradiation support the binding of 53BP1 to DSBs as discrete nuclear foci or subnuclear microbeam tracks, respectively (Wang et al., 2002; Bekker-Jensen et al., 2005; Redon et al., 2011). Localized 53BP1 is thought to then contribute to DDR checkpoint functions through further stimulation of ATM activity towards downstream signaling proteins involved in G2/M checkpoint responses (e.g. CHK2, p53) (Wang et al., 2002; Mochan et al., 2004; Adams and Carpenter, 2006; Shibata et al., 2010; Rashid et al., 2011). 53BP1 may also facilitate the specific repair of DSBs through
direct stimulation of NHEJ and by suppressing HR (Iwabuchi et al., 2003; Xie et al., 2007; Dimitrova et al., 2008; Bunting et al., 2010). Unresolved DSBs can be visualized as late foci (unrepaired DSBs) at 24 hours or more following activation of the DDR, and lead to DDR-induced senescence with 53BP1 protein juxtaposed to PML-NBs (Rodier et al., 2011). These late- or non-repairing foci are thought to be associated with heterochromatic regions and ATM-mediated signaling relaxes such chromatin and/or allows processing of otherwise unligatable DNA ends (Goodarzi et al., 2010).

53BP1 is phosphorylated at multiple motifs during the DDR, but the role of this post-translational modification is unclear (Jowsey et al., 2007). The understanding of 53BP1 phosphorylation is important, as it may be a valuable readout of replication stress and response to chemotherapy or radiotherapy. Increased activation of the DDR (i.e. γH2AX, ATM, 53BP1 foci, etc.) has previously been observed for pre-malignant tissues over malignant tissues (Bartkova et al., 2005; Gorgoulis et al., 2005). Subsequently, 53BP1 staining in multiple tumour types, including lung and breast carcinoma, was altered in a subset of tumours. In some of these tumours and pre-neoplastic lesions 53BP1 was focal, indicating activation of DDR signaling, while immunohistochemical staining of 53BP1 was lost in a subset of tumours (~25%) (Bartkova et al., 2007; Nuciforo et al., 2007). It remains to be determined whether 53BP1 phosphorylation occurs throughout the carcinogenic axis.

Herein, we describe the in situ analysis of 53BP1 phosphorylation following whole-cell and UV-microbeam-generated DSBs in relation to ATM, DNA-PKcs and ATR kinase activity and the chromatin modifications that are dependent on the MRN complex and RNF168 ubiquitylation.
Our data are consistent with parallel, rather than serial, DDR pathways for 53BP1 phosphorylation and argue that the binding of 53BP1 to DSBs can be uncoupled from 53BP1 phosphorylation. These novel findings provide a more detailed understanding of the molecular pathways that lead to 53BP1 phosphorylation during exogenous and endogenous damage and may have utility as biomarkers during cancer progression and individual cancer therapy response.

### 3.3 RESULTS

#### 3.3.1 Cell cycle dependency of 53BP1 phosphorylation response to exogenous and endogenous DSBs

The phosphorylation of 53BP1 in the N-terminus at Serine-25 (53BP1$^{\text{Ser25}}$) is activated under conditions of severe oxidative stress specifically in S-phase cancer cells (Harding et al., 2011). To study 53BP1 phosphorylation in response to endogenous or exogenous DNA damage across the cell cycle, we utilized primary normal human fibroblasts (NHF) and characterized staining for 53BP1 in concert with two cell cycle markers: 5-ethynyl-2’-deoxyuridine (EdU) for S-phase cells and the centromere protein F (CENP-F) for G2 phase cells. Together these two markers facilitate *in situ* cell cycle identification (see Methods). We found the histone variant H2A.X was phosphorylated in S-phase as previously reported, consistent with DNA break induction during DNA replication (Figure 3-1A) (Macphail et al., 2003). Total 53BP1 (53BP1$^{\text{Total}}$) redistributed from a pan-nuclear to a punctate staining pattern in S-phase cells. Using two different phospho-specific 53BP1 antibodies (53BP1$^{\text{Ser25}}$ and 53BP1$^{\text{Ser1778}}$) we did not observe 53BP1
FIGURE 3-1: 53BP1 is focal but not phosphorylated during unperturbed S-phase. A) Asynchronously growing normal human fibroblasts (NHF s) were incubated with 10µM EdU for 1h before fixation and staining with EdU, CENP-F and either γH2AX, 53BP1Total, 53BP1Ser25 or 53BP1Ser1778 as indicated. G1 phase cells were identified as being negative for both EdU and CENP-F, S-phase as EdU positive and weakly positive for CENP-F and G2-phase as EdU negative and strongly CENP-F positive. Mitotic cells were excluded for this analysis. Scale bars are 10µm. Continued on next page.
FIGURE 3-1 Continued: 53BP1 is focal but not phosphorylated during unperturbed S-phase. B) As in (A) cell cycle stage was identified in cells stained 30 minutes following 2Gy; EdU treatment was for 1h before IR and through the recovery incubation. C) HCT116 cells were treated for 24h with 2mM HU to induce replication fork collapse and stained as indicated with DAPI counterstain. Scale bars are 10µm.
phosphorylation during DNA replication in unstressed, asynchronously growing cells (Figure 3-1A). We confirmed that these 53BP1 Total foci colocalized with γH2AX in 3-dimensions (Supplementary Figure 3-1A).

In contrast, exogenous DSBs generated by IR, formed discrete intranuclear foci of γH2AX, 53BP1 Total, 53BP1 Ser25 and 53BP1 Ser1778 in all cells independent of cell cycle phase (Figure 3-1B). These findings were confirmed using human colorectal cancer cells (HCT116) and replication stress by HU treatment induced 53BP1 phosphorylation and foci formation (Figure 3-1C and Supplementary Figure 3-1B). We conclude that severe DNA replication arrest by HU, which may result in collapsed replication forks, and exogenous IR-induced DSBs lead to 53BP1 Ser25 and 53BP1 Ser1778 phosphorylation, whereas this phosphorylation is not induced by normal DNA replication.

3.3.2 Unresolved DSBs lead to persistent phosphorylation of 53BP1 and corresponds to DNA damage induced senescence

We next characterized expression of 53BP1 Ser25 and 53BP1 Ser1778 as a function of dose and time following exogenous DSBs. Consistent with previous biochemical data, our in situ cell staining showed that 53BP1 phosphorylation is nuclear, focal and dose-dependent at 30 minutes (initial DSBs) and then decreases until reaching background levels at 24h, similar to the kinetics of γH2AX (Figure 3-2A and Supplementary Figures 3-2A-C) (Jowsey et al., 2007; Harding et al., 2011). These phosphorylated 53BP1 foci colocalized with 53BP1 Total (Supplementary Figure 3-2D). The specificity of recruitment of 53BP1 Ser25 and 53BP1 Ser1778 to sites of DSBs was
FIGURE 3-2: 53BP1 is phosphorylated in the N- and C-termini in a time-dependent manner and is recruited to UV-microbeams and senescence-associated IRIF. A) Normal human fibroblasts (NHF) were synchronized in G₀/G₁ by contact inhibition and either mock irradiated (NIR) or irradiated with 2Gy of ionizing radiation and fixed at indicated time points then stained as indicated. DAPI counterstain is overlaid in blue. The number of foci induced per nucleus was quantified by subtracting the mean foci number in NIR cells. Error bars represent SEM for 3 independent experiments. B) Asynchronous NHFs were UV-microirradiated and fixed 30 minutes later. 3D reconstructions of the data were created using Imaris Software. Scale bar is 10µm. C) Quantification of the fraction of SA-βGal positive NHFs and of IL-6 induction as measured by ELISA following 20Gy and recovery times as indicated. Error bars represent SEM of at least 2 independent experiments. Continued on next page.
FIGURE 3-2 Continued: Normal human fibroblasts (D) or Ataxia-telangiectasia mutated (ATF) (E) Mock irradiated or irradiated with a senescence-inducing dose of 20Gy were fixed at 1 and/or 8days as indicated and stained with PML and 53BP1 antibodies. Large 3D renderings are shown in Supplementary Figure 3-4.
confirmed using sub-nuclear UV microbeams as previously described (Figure 3-2B) (Fraser et al., 2011; Rashid et al., 2011). Recent evidence has shown that residual DSBs reside in regions neighboring heterochromatin (Goodarzi et al., 2008). We examined association of residual 53BP1 and its phosphoforms in relation to heterochromatin (as marked by trimethylated Lysine-9 of histone H3) and observed ~50% of these foci adjacent to the edges of heterochromatin following DNA damage (Supplementary Figure 3-3).

Lethal DNA damage can induce terminal senescence with the association of PML-NBs and 53BP1 at 8 days following IR. This phenotype associates with the induction of senescence associated-βgalactosidase (SA-βGal) activity and increased expression of the cytokine IL-6 (Rodier et al., 2011). We observed that 53BP1 phosphoforms and γH2AX also associated with PML-NBs under these IR-induced senescence conditions (Figure 3-2C-E and Supplementary Figures 3-2E-F). Importantly, we find that although phosphorylated 53BP1 staining was weaker than in normal fibroblasts (Figure 3-2D), 53BP1\textsuperscript{Total} and both phosphoforms associate with PML bodies in AT-cells in an ATM-independent manner (Figure 3-2E). Three-dimensional renderings confirm that these associations include rare frank co-localization and frequent associations without complete overlap of foci (Figure 3-2D-E and Supplementary Figure 3-4). Together these data indicated that phosphorylated 53BP1 is a marker for induction and resolution of DSBs and persists in a hyper-phosphorylated state at chromatin regions with non-resolved DSBs in IR-induced terminally arrested senescent cells. This is distinct from the “pseudo-DNA damage response” that occurs with ectopic expression of p16 which results in activation of γH2AX but not in localization of 53BP1 (Pospelova et al., 2009).
3.3.3 N- and C-termini 53BP1 Phosphorylation is catalyzed by both ATM and DNA-PKcs

Using G0/G1 synchronized fibroblasts to eliminate contribution of endogenous DNA replication-associated DNA breaks and ATR activity, we examined the effect of inhibiting DNA-PKcs, ATM, or both kinases towards 53BP1 phosphorylation (Rashid et al., 2005). The kinase activity of ATM or DNA-PKcs was effectively blocked biochemically by the small molecule inhibitors KU55933 and NU7441, respectively, using CHK2 phosphorylation and DNA-PKcs autophosphorylation as readouts (Supplementary Figure 3-5A). We then functionally examined phosphorylation in situ both using UV-microbeam and in whole-cell irradiation. The use of ATM or DNA PKcs inhibition alone did not eliminate the activation of γH2AX or recruitment of 53BP1Total to UV-microbeams (Figure 3-3A). Phosphorylation of 53BP1Ser25 was markedly reduced in UV-microbeams by ATMi, but not DNA-PKi and neither inhibitor eliminated phosphorylation at 53BP1Ser1778. These results were confirmed using AT-fibroblasts (Figure 3-3B).

To quantify the differential kinase response, we used whole-cell irradiated cultures and quantitative immunofluorescence microscopy (Figure 3-3C and 3-3D and images Supplementary Fig 3-5B and 3-5C). The γH2AX response was significantly reduced with ATMi inhibition, while DNA-PKi had no effect. Inhibition of both kinases effectively abolished γH2AX foci, as previously reported (Figure 3-3C) (Stiff et al., 2004). There was no effect on 53BP1Total by treatment with either inhibitor alone and even after dual kinase inhibition discernable IRIF were still detectable (Figure 3-3C and Supplementary Figure 3-5B). Similar to the UV-microbeam data, 53BP1Ser25 foci were reduced significantly by ATMi. DNA-PKcs
FIGURE 3-3: Both ATM and DNA-PKcs contribute to 53BP1 phosphorylation following DSB induction. A) NHFs were pre-treated for 1h with either vehicle (DMSO), 10µM KU55933 (ATMi) or 5µM NU7441 (DNA-PKi) inhibitors and stained 30 minutes following microirradiation for the indicated antibodies (see Materials and Methods). B) NHFs or ATFs were microirradiated as in A). All scale bars are 10µm. Continued on next page.
FIGURE 3-3 Continued: Both ATM and DNA-PKcs contribute to 53BP1 phosphorylation following DSB induction. Whole-cell irradiated NHFs (C) or ATF (D) were treated with inhibitors, irradiated with 2Gy and fixed 30 minutes later. IRIF of indicated proteins were quantified as in 2B and presented as fold-change of vehicle control. Error bars represent SEM for 3 independent experiments, asterisk indicate p<0.05 by Mann-Whitney non-parametric t-test to DMSO control for each fluor. E) Seckel cells were either mock-irradiated or treated with 2Gy and fixed 30 minutes later and followed by IF staining for indicated antibodies. All scale bars are 10µm.
inhibition had no effect, but both inhibitors further reduced $53\text{BP1}_{\text{Ser25}}$ foci. The $53\text{BP1}_{\text{Ser1778}}$ focal response was significantly reduced by ATMi and further by inhibition of both kinases. This was confirmed using AT-fibroblasts and DNA-PKi, except that in this case $53\text{BP1}_{\text{Total}}$ foci were abolished with DNA-PKi (Figure 3-3D and Supplementary Figure 3-5C). Using ATR-deficient, asynchronous Seckel cells, we observed that IR-induced foci formation and phosphorylation of 53BP1 in all cell cycle phases is ATR-independent (Figure 3-3E). Taken together, our data supports the concept that, like H2A.X, both ATM and DNA-PKcs redundantly phosphorylate 53BP1 (Stiff et al., 2004).

### 3.3.4 Recruitment of phosphorylated 53BP1 to DSBs is independent of DSB Sensing by MRE11

MRE11 acts as a proximal sensor of DSB ends and contributes to ATM activation and downstream signaling to repair and checkpoint responses (Uziel et al., 2003; Lee and Paull, 2005). Using ATLD cells, we determined whether 53BP1 phosphorylation and recruitment was dependent on MRE11, or its nuclease function. Figure 3-4A shows that 30 minutes following UV-microbeam induced damage, $53\text{BP1}_{\text{Total}}$ recruitment is maintained in MRE11-deficient ATLD cells and in ATLD cells reconstituted with a mutant (mt) form of MRE11 that lacks nuclease function (Figure 3-4A). Both $53\text{BP1}_{\text{Ser25}}$ and $53\text{BP1}_{\text{Ser1778}}$ are activated within the microirradiated region as evidenced by colocalization with $\gamma\text{H2AX}$ (Figure 3-4A). As a control, CHK2 phosphorylation (an established ATM target downstream of MRE11) was decreased in microirradiated ATLD cells and rescued by either wtMRE11 or mtMRE11.3 (Supplementary figure 3-6A).
FIGURE 3-4: 53BP1 is phosphorylated and recruited to DSBs independently of MRE11. A) ATLD2 fibroblasts or those reconstituted with nuclease deficient (mtMRE11.3) or wild-type (wtMRE11) MRE11 were microirradiated and stained for indicated antibodies 30 minutes later. B) ATLD2 and ATLD2+wtMRE11 cells were fixed 30 minutes post 2Gy or mock irradiation and stained with indicated antibodies and DAPI counterstain. All scale bars are 10µm. C) ATLD and reconstituted cells lines were irradiated with 10Gy and collected at indicated times and subjected to western blot analysis.
We next examined whether a similar phenomenon was observed when ATLD cells were whole-cell irradiated with ionizing radiation. Figure 3-4B indicates that, like UV-microirradiated cells, 53BP1\textsuperscript{Total} localizes to DSBs as discrete IRIF. Furthermore, mirroring the micro-irradiated case we find that γH2AX, 53BP1\textsuperscript{Ser25} and 53BP1\textsuperscript{Ser1778} form IRIF in ATLD cells, although to a lesser intensity and number than those rescued with either wtMRE11 or mtMRE11 (Figures 3-4B and Supplementary Figure 3-6B and 3-6C). Using Western blotting we confirmed that the levels of 53BP1 phosphoforms were decreased in ATLD relative to either of the reconstituted cell lines (Figure 3-4C). From these data we conclude that the recruitment of 53BP1\textsuperscript{Total} and 53BP1 phosphorylation is largely maintained in MRE11-deficient cells.

3.3.5 53BP1 phosphorylation is independent of RNF168-mediated chromatin ubiquitylation: dissociation of recruitment and phosphorylation

Using RNF168-deficient RIDDLE cells, we confirmed defective recruitment of 53BP1\textsuperscript{Total} to DSBs generated by UV-microbeam (Stewart et al., 2007; Fraser et al., 2011). This was reversed with RNF168 reconstitution (Figure 3-5A). As a control, neither MRE11 nor MDC1 are affected in recruitment to the break site, nor is CHK2\textsuperscript{Thr68} phosphorylation altered (Supplementary figure 3-7A). Both 53BP1\textsuperscript{Ser25} and 53BP1\textsuperscript{Ser1778} were found within the microbeam as defined by γH2AX staining, but were also distributed throughout the nucleus in RIDDLE cells reconstituted with empty vector (Figure 3-5A). Reconstitution with wild-type RNF168 reestablished 53BP1 phosphoforms localization to the γH2AX-marked regions. In whole-cell irradiated cultures, the upstream γH2AX response was unaffected by the presence or absence of RNF168 (Figure 3-5B). The 53BP1\textsuperscript{Total} IRIF were not observed in RIDDLE cells, but were rescued by wtRNF168 expression as expected. As in UV-microbeams, we found that 53BP1\textsuperscript{Ser25} and 53BP1\textsuperscript{Ser1778} were
FIGURE 3-5: 53BP1 is phosphorylated throughout the nucleus independently of RNF168-mediated DSB recruitment. A) 15-9BI (RIDDLE) fibroblasts reconstituted with either empty vector (+ Vector) or wild-type RNF168 (+ RNF168) were subjected to UV-microirradiation and fixed 30 minutes later and stained with indicated antibodies. B) Whole-cell irradiated RIDDLE cultures were subjected to immunofluorescent staining with indicated antibodies following mock irradiation or 30 minutes post-2Gy. All scale bars are 10µm. C) RIDDLE+Vector and RIDDLE+RNF168 cultures were treated with 10Gy and lysed either 0.5 or 2h later and subjected to western blotting analysis.
activated throughout the nucleus of RIDDLE cells. This phenotype was rescued in RNF168-reconstituted cells (Figure 3-5B). Indeed, quantification of total nuclear fluorescence indicated no differences in 53BP1 phosphorylation, despite the lack of IRIF (Supplementary Figure 3-7B). We verified that on a population basis, RIDDLE cells irradiated with 10Gy did not show appreciable defects in 53BP1 phosphorylation on western blot as compared to RIDDLE+wtRNF168 controls (Figure 3-5C). We conclude that 53BP1 phosphorylation is RNF168-independent and our cumulative results are summarized in Table 3.1.

Table 3.1: Summary of staining patterns of 53BP1 and phosphoforms.

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<td>ATM deficient</td>
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<td>DNA-PKcs deficient</td>
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<td>MRE11 deficient</td>
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<td>RNF168 deficient</td>
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* Denotes potential decreased overall intensity

3.4 DISCUSSION

The extent to which 53BP1 phosphorylation is modified by the ability of MRE11 to initially sense DSBs, and whether this signals directly to subsequent chromatin modifications, such as RNF168-dependent ubiquitylation, was previously unknown. Here we show that recruitment of 53BP1 to the sites of DSBs can be uncoupled from MRE11 sensing and from 53BP1
phosphorylation by DDR kinases. Therefore, our novel data suggest that it is unlikely that 53BP1 localization to sites of DNA damage is the result of direct and serial signaling involving the MRE11-ATM-RNF168 pathway; instead our data suggest the existence of alternative RNF168-dependent pathways for 53BP1 DSB localization.

The relay of signals from the sensing of DSBs to repair is generally considered linear, with MRN sensing the break and contributing to activation of ATM, which leads to recruitment of RNF168 and subsequently 53BP1 localization (Harper and Elledge, 2007; Kastan, 2008). We hypothesized that cells with defects in any of these components would not be able to recruit 53BP1 to DSBs. Instead, we found that neither loss of ATM kinase activity nor MRE11 abolished 53BP1 recruitment, nor did it eliminate phosphorylation within foci of 53BP1, however both phenotypes were reduced. Conversely, loss of RNF168, thought to be the distal signaling component of the pathway leading to 53BP1, did abolish recruitment of the total protein. Importantly, this did not eliminate the phosphorylation of 53BP1, but rather resulted in phospho-53BP1 disseminated throughout the nucleus. This is consistent with the finding that mutation of 53BP1 at serines 6, 25/29 and 784 to alanine does not ablate recruitment of 53BP1 to DSB foci, and deletion of the TUDOR domain required for 53BP1 IR-induced foci formation did not prevent phosphorylation of 53BP1 (Iwabuchi et al., 2003; Ward et al., 2003).

Multiple N-terminal S/T-Q consensus sites for the phosphorylation by ATM are present in 53BP1. The best characterized of these sites, Serine-25, was found to be phosphorylated by ATM following IR (Ward et al., 2003). Multiple other residues (i.e. S6, S29, S166, S176/178, T302, S452, S784 and S831) have also been shown to be ATM-dependent (Jowsey et al., 2007).
It has been suggested that two other PIKKs, ATR and DNA-PKcs, do not contribute to 53BP1 phosphorylation following IR (Ward et al., 2003; Jowsey et al., 2007). We found that blocking of DNA-PKcs and ATM led to additive decreases in 53BP1 phosphorylation. This was most notable in the C-terminus at 53BP1<sup>Ser1778</sup>, which had not previously been examined and relies on both DNA-PKcs and ATM equally. Like γH2AX, 53BP1<sup>Ser25</sup> appears to be a less efficient DNA-PKcs target, but is phosphorylated by both kinases (Stiff et al., 2004). This is consistent with recent findings in lymphocytes that suggest 53BP1<sup>Ser25</sup> is a target of both ATM and DNA-PKcs (Callen et al., 2009). It is important to note that combined inhibition of ATM and DNA-PKcs caused additive defects in class-switch recombination and suppression of aberrant DSB joining (Callen et al., 2009). Together with our results this suggests that phosphorylation of 53BP1 by both ATM and DNA-PKcs may have important implications for DSB repair across many cell types (see below). Interestingly, unlike when both kinases are inhibited in NHFs, the inhibition of DNA-PK in AT-fibroblasts led to abolition of 53BP1<sup>Total</sup> foci. This did not eliminate focal 53BP1 phosphorylation, but the levels of such were sufficiently low de novo to make evaluation difficult. Nevertheless, this suggests that the ATM protein itself, and not activated ATM<sup>Ser1981</sup> per se, may be an important component in localization of 53BP1 to the DSB. This suggestion is consistent with the recent finding that AT-fibroblasts may adapt to ATM loss and have distinct phenotypes from normal fibroblasts subjected to short-term ATM inhibition with small molecules (White et al., 2010).

The contribution of DNA-PKcs to 53BP1 phosphorylation could be relevant for NHEJ where DNA-PKcs is the dominant kinase (Dobbs et al., 2010). Indeed, loss or mutation to alanine of 53BP1 N-terminal phosphorylation sites led to defects in γH2AX foci resolution following IR and mutation of increasing numbers of these sites led to corresponding decreases in class-switch
recombination (Ward et al., 2006; Bothmer et al., 2011). Both of these phenotypes suggest roles for phosphorylated 53BP1 in NHEJ. ATM contributes to NHEJ through chromatin modifications that facilitate access to late-repairing DSBs and DNA-PKcs is a part of the core NHEJ machinery (Goodarzi et al., 2008). The relative contribution of these two kinases to different 53BP1 phosphorylation sites and the consequences for NHEJ will be an important area of future studies. It is clear that the use of 53BP1^{Ser25} as the prototype for 53BP1 phosphorylation may be naïve as the data shown here indicate differing kinase dependencies at different SQ sites of 53BP1.

We also observed that the phosphorylation of 53BP1 is cell-cycle dependent. Activation of γH2AX occurs during DNA replication and we observed these foci co-localized with focal 53BP1^{Total}, but 53BP1^{Ser25} and 53BP1^{Ser1778} residues were not phosphorylated (Macphail et al., 2003). However, 53BP1 was phosphorylated in the N- and C-termini during HU-induced replication stress. We speculate that this phosphorylation may be due to a requirement for prolonged ATR-signaling at collapsed replication forks and may be enacted through ATR-dependent ATM signaling (Stiff et al., 2006; Petermann et al., 2010). Recent evidence has shown that 53BP1 is excluded from γH2AX foci in a dynamic process during progression through mitosis, however staining throughout the stages of interphase was not examined (Nelson et al., 2009; Nakamura et al., 2010). Interphase dependence on cell cycle stage (G1, S, and G2) was not observed following exogenous DSBs as we found no difference in 53BP1 or γH2AX response to IR-induced DSBs. Consistent with this finding, we did not detect differences in 53BP1 phosphorylation in ATR-deficient Seckel cells treated with IR. Recent evidence has suggested that ATR contributes at a low level to IR-induced 53BP1 phosphorylation (Tomimatsu et al., 2009). Inhibition of both ATM and DNA-PKcs effectively eliminated 53BP1 phosphorylation
confirming that in our system any potential ATR-mediated phosphorylation occurs at very low levels.

Our results also have implications for molecular pathology as shown in Table 3.1. Loss of 53BP1 expression has been associated with breast and lung cancer carcinogenesis and tumour progression and loss of $53BP1^{\text{Total}}$ in BRCA1-associated breast cancer abrogates cell killing by PARP inhibition (Bencokova et al., 2009; Bouwman et al., 2010; Bunting et al., 2010; Harding et al., 2011). We suggest that it will be possible to use 53BP1 staining patterns (negative, focal and/or phosphorylated) as a predictive indicator for therapy response or to monitor patient response to particular therapies. However, we and others, recently demonstrated that 53BP1 phosphorylation is activated in S-phase cells in response to severe oxygen stress with partial dependence on ATM (Rashid et al., 2005; Bencokova et al., 2009; Harding et al., 2011). When taken together with the results in this paper, we believe that the use of 53BP1 as a biomarkers could be confounded by cell cycle stage, tumour hypoxia, germline mutations and potentially the dynamics of dephosphorylation (Shreeram et al., 2006; Kang et al., 2009; Moon et al., 2010). Nonetheless, further studies using a panel of antibodies in primary human cancers treated with different DNA-damaging agents may be useful predictors of individualized therapeutic response and should be actively studied for their utility within the context of personalized cancer medicine.

### 3.5 MATERIALS AND METHODS

#### 3.5.1 Cell lines and treatments

Normal (GM05757), Seckel (GM18366) and ataxia telangiectasia (GM02052) fibroblasts were obtained from the Coriell Institute, cultured in $\alpha$-Modified Eagles Medium ($\alpha$MEM)
supplemented with 20% fetal calf serum (FCS) and used within 5 passages from receipt. Where indicated normal fibroblasts were synchronized in G₀/G₁ by contact inhibition as previously described (Uziel et al., 2003; Rashid et al., 2005). HCT116 cells were obtained from B. Vogelstein and cultured in McCoy’s 5A medium supplemented with 10% FCS. ATLD fibroblasts and complemented cells were a gift from Y. Shiloh and cultured in Dulbecco’s MEM (DMEM) with 20% FCS and 1mM Glutamine (Uziel et al., 2003; Stewart et al., 2009). RIDDLE (15-9BI) fibroblasts were a gift from G. Stewart and D. Durocher and were cultured in DMEM with 20% FCS and 1mM Glutamine (Stewart et al., 2009; Fraser et al., 2011). All cells were incubated in a humidified atmosphere at ambient oxygen concentrations with 5% CO₂. All cells were verified to be mycoplasma free and were subcultured with antibiotics.

Hydroxyurea was obtained from Sigma-Aldrich and freshly prepared to a concentration of 2mM and incubated on the cells for 24h before fixation. Inhibitors of ATM (KU55933) and DNA-PKcs (NU7441) were obtained from Tocris Biosciences and used at 10µM and 4µM, respectively for 1 hour before further treatment or fixation. Irradiations were carried out using a ¹³⁷Cs irradiator (Nordion) at a dose rate of ~1Gy/minute at ambient oxygen concentrations. After irradiation cells were returned to the incubator for the necessary recovery time. Initial DSBs as marked by γH2AX foci were defined as those present 30 minutes following IR, and residual breaks were defined as those present at 24 hours or later following damage.

3.5.2 Immunofluorescence and Microscopy

Immunofluorescence was performed as described (Fraser et al., 2011). Briefly, cells were seeded onto #1.5 coverslips 24h before treatments. Following necessary treatment cells were rinsed in PBS, fixed at room temperature (4% paraformaldehyde/0.2% Triton X-100 in PBS pH8.2). Following 20 minutes in fixative, cells were briefly rinsed in PBS and permeabilized in 0.5%
NP-40/PBS buffer. EdU staining was carried out as necessary (see below) and cells were blocked using 2% BSA/1% donkey serum/PBS buffer for at least 1 hour at room temperature. Coverslips were inverted onto parafilm spotted with primary antibody solutions made in 3% BSA and incubated at 4°C in a humidified chamber overnight. Following 3x5 minute antibody washes (0.5% BSA/0.175% Tween-20/PBS) coverslips were inverted onto secondary antibody solution as above. Coverslips were incubated in 0.1µg/mL DAPI after 3 more antibody washes and rinsed in PBS before mounting onto glass slides containing Vectashield (Vector Labs). Unless otherwise indicated all reagents were obtained from Sigma-Aldrich and/or BioShop.

Cells were imaged in 3 dimensions as described using either a 60X or 100X 1.4NA oil immersion objectives and a Photometrics Cascade 512B EM-CCD camera (Fraser et al., 2011; Rashid et al., 2011). For experiments requiring quantification maximum intensity projections of at least 50 nuclei were counted in three separate experiments using Image Pro Plus software (Media Cybernetics) and manually determined thresholds for foci identification were used and maintained throughout each experiment. The fold foci-induction was calculated as the ratio of foci number for the IR-treated cells to the foci number for unirradiated cells, errors presented are SEM. 3D renderings were produced using Imaris software (Bitplane). For qualitative experiments at least 2 replicates were performed and representative maximum intensity projections are shown. Images were prepared for publication using ImageJ software (NIH). Data was graphed and statistics calculated using GraphPad software (Prism).

Primary antibodies and dilutions used were as follows: γH2AX (Epitomics #2212 1:800 or Millipore JBW301 05-636 1:800), 53BP1 Total (Bethyl A300-272A 1:1000 or BD Biosciences
#612522 1:1000), 53BP1\textsuperscript{Ser25} (Novus NB100-1803 Lot A1 1:500), 53BP1\textsuperscript{Ser1778} (Cell Signaling #2675 1:200), CENP-F (Novus NB500-101 1:1000 or BD Biosciences #610768 1:750), RPA (AbCam 9H8 ab2175 1:1000), PML (Gift from D. Bazzett-Jones 1:1000), H3-K9(Me)\textsubscript{3} (AbCam ab10812 1:300), CHK2\textsuperscript{Thr68} (Cell Signaling #2661 1:250), MRE11 (Genetex 12D7 GTX70212 1:400), MDC1 (Sigma MDC1-50 M2444 1:1000).

Secondary antibodies were: Alexa Fluor 488 Donkey Anti-Rabbit (A21206), Anti-Mouse (A21202) or Anti-sheep (A11015); Alexa Fluor 568 Donkey Anti-Rabbit (A10042) or Anti-Mouse (A10037); Alexa Fluor 647 Donkey Anti-Rabbit (A31573) or Anti-mouse (A31571). All were used at a dilution of 1:500.

### 3.5.3 Subnuclear UV-microbeams

Subnuclear UV-microbeams were generated as previously described (Beucher et al., 2009; Rashid et al., 2011; see Appendix A). Briefly, a 355nm laser set at a power of 4mW was directed through the 100X 1.4NA objective and onto the cells grown on #1.5 coverslips and pre-sensitized for 24h by incubation with 10\(\mu\)M BrdU (Sigma-Aldrich). Following UV-microirradiation cells were returned to the incubator and fixed 30 minutes later according to the protocol above, and imaged as described.

### 3.5.4 Cell Cycle analyses

Cells to be analyzed for cell cycle were incubated for 1h before fixation with 10\(\mu\)M EdU according to company protocol (Invitrogen). Following NP-40 treatment as described above, incorporated EdU was labeled using the Click-iT EdU Alexa Fluor 647 kit according to company protocol with the following modification. Instead of staining coverslips in 6-well dishes, they were inverted on parafilm containing Click-iT reaction cocktail as was done for antibody
incubation. Following this labeling the standard Immunofluorescence protocol was followed starting with the blocking step. To delineate G₁/G₀ from S from G₂ cells an antibody against centromere protein F (CENP-F) was used in combination with EdU such that cells negative for both EdU and CENP-F are in G1, with low CENP-F levels but positive for EdU are S-phase, and those negative for EdU, but strongly positive for CENP-F are in G2 (Beucher et al., 2009). The DAPI staining pattern and distinct CENP-F structures associated with mitosis were eliminated from these analyses.

### 3.5.5 Senescence assays

Doses greater than 10Gy induce senescence and correlate with increased senescence-associated β-galactosidase (SA-βGal) activity and increased secretion of IL-6 (Debacq-Chainiaux et al., 2009; Rodier et al., 2009). For SA-βGal assessment asynchronous cells were treated as indicated and fixed in a solution of 2% paraformaldehyde/0.2% glutaraldehyde in PBS for 5 minutes at room temperature. Cells were rinsed 2X in PBS and incubated in staining solution (40mM citric acid-sodium phosphate buffer/5mM K₄[Fe(CN)₆]/5mM K₃[Fe(CN₆)]/150mM NaCl/2mM MgCl₂/1mg mL⁻¹ X-gal) for 16-24 hours at 37°C. Cells were rinsed in PBS and in Methanol for storage for up to one week (Debacq-Chainiaux et al., 2009; Harding et al., 2011). Cells were manually counted and the fraction of SA-βGal positive cells was computed for 3 independent experiments.

To measure IL-6 secretion serum free media was added to the cells 24h before collection at which point media was collected, centrifuged to remove cellular debris and stored at -80°C. Cells were trypsinized and counted to compute the picograms of IL-6 secreted per cell over a 24h
period as measured by the Quantikine ELISA assay (R&D Systems) according to company protocol. Three replicates were performed and the SEM was computed using GraphPad software (Prism).

### 3.5.6 Western Blotting

Protein lysates were collected using a NETN butter (50 mM Tris-HCl pH 7.5/150mM NaCl/1 mM EDTA/1% NP40/1unit mL$^{-1}$ benzonase (Novagen)) and incubation with rotation at 4°C for 20 minutes. The following protease (1mM Benzamidine hydrochloride hydrate/1µg mL$^{-1}$ antipain/5µg mL$^{-1}$ aprotinin/1µg mL$^{-1}$ leupeptin/1mM Phenylmethanesulfonyl fluoride) and phosphatase (10mM sodium fluoride/2mM imidazole/1.15mM sodium molybdate/4mM sodium tartrate/2mM Sodium Pyrophosphate/2mM β-Glycerophosphate/2mM sodium orthovanadate) inhibitors were used; all chemicals were from Sigma-Aldrich. Western blotting was performed according to standard protocols and blots were imaged using the Odyssey scanner (LICOR) as previously described (Harding et al., 2011). For 53BP1 western blots either pre-cast tris-acetate gels were used according to company protocol (Invitrogen) or self-cast tris-acetate gels were run according to a modified protocol by Cubillos-Rojas et al. (Cubillos-Rojas et al., 2010). Briefly, a 6% running gel was cast (6% 37.5:1 acrylamide:bisacrylamide/200mM pH 7.0 Tris-acetate buffer/0.75mg mL$^{-1}$ ammonium persulfate/0.125% TEMED) and overlaid with a 3% stacking gel (as above). Gels were run at ~100V in Tris-acetate running buffer (50mM Tricine/50mM Tris/0.1% SDS/5mM sodium bisulfite) and wet-transferred to PVDF in 1X NuPage transfer buffer (Invitrogen) supplemented with 10% methanol and 5mM sodium bisulfite at 350mA for 4 hours at 4°C. Primary antibodies and dilutions used were as follows: 53BP1$^{Total}$ (Cell Signaling #4937 1:1000), 53BP1$^{Ser25}$ (Novus NB100-1803 Lot A1 1:1000), 53BP1$^{Ser1778}$ (Cell Signaling #2675 1:1000), CHK2$^{Thr68}$ (Cell Signaling #2661 1:1000), DNA-PKcs$^{Ser2056}$ (A kind gift of
David Chen 1:200), Actin (Sigma A2066 or A5316 1:10000), β-Tubulin (Sigma T4026 1:10000) or Ku70 (Santa Cruz sc5309 1:10000). Secondary antibodies were: IRDye 680 (Li-cor Biosciences 926-32221 or 926-32222) or IRDye800CW (Li-cor Biosciences 926-32212 or 926-32213), and were used at 1:1000.
SUPPLEMENTARY FIGURE 3-1: A) Asynchronously growing NHFs were co-stained for EdU, γH2AX and 53BP1\textsuperscript{Total}. 3D rendering was done with Imaris software. B) HCT116 cells were stained and cell cycle phases were identified as in Figure 1. A dose of 2Gy was administered 1h after addition of EdU and cells fixed 30 minutes later. Specific antibody staining was performed as indicated with DAPI costain. All scale bars are 10\(\mu\)m.
SUPPLEMENTARY FIGURE 3-2: A) NHFs were subjected to 0(NIR)-4Gy and fixed 30 minutes later and stained for 53BP1<sub>Ser1778</sub>. Scale bar is 10µm. Quantification in B) indicates dose-dependent foci induction of 53BP1<sub>Ser1778</sub> phosphorylation. Error bars represent SEM for 3 independent experiments. C) Time dependent phosphorylation of 53BP1 was analysed by western blotting of protein lysates collected at indicated times following 10Gy. D) Colocalization of 53BP1-phosphoforms with 53BP1<sub>Total</sub> and 53BP1<sub>Total</sub> with γH2AX at 30 minutes following 2Gy.
SUPPLEMENTARY FIGURE 3-2 Continued: E) Colocalization of 53BP1 and phospho-53BP1 with γH2AX was analysed in mock-irradiated or in cells treated with a senescence-inducing dose of 20Gy at 1 or 8 days. Scale bar is 10µm. F) Representative images of SA-βGal staining in NHFs show induction by 8 days following IR. The arrow identifies a senescent cell. Scale bar is 100µm.
SUPPLEMENTARY FIGURE 3-3: Foci of 53BP1 and its phosphoforms do not preferentially associate with regions of heterochromatin. At indicated dose and time cells were fixed and stained with H3-K9(Me)\textsubscript{3} to mark heterochromatic regions and indicated antibodies. Scale bars are 10\(\mu\)m. Select cells are expanded in 3D renderings to show foci in relation to heterochromatic staining.
SUPPLEMENTARY FIGURE 3-4: Residual $53BP1^{\text{Total}}$, $53BP1^{\text{Ser25}}$ and $53BP1^{\text{Ser1778}}$ are shown in Red and PML-NB are shown in green with regions of colocalization in yellow as rendered in 3-dimensions from A) NHF and B) ATF nuclei irradiated with 20Gy and allowed to recover for 8 days as presented in Figures 2D and 2E, respectively. All cells are presented at the same scale.
SUPPLEMENTARY FIGURE 3-5: 53BP1 is phosphorylated by both ATM and DNA-PKcs kinases following IR. A) Western blot verifying inhibition of ATM and DNA-PKcs by inhibitor treatments as used throughout the thesis. B) Representative images used for quantification in Figure 3C. Scale bar is 10µm. Continued on next page.
SUPPLEMENTARY FIGURE 3-5 Continued: C) Representative images used for quantification in Figure 3D. Scale bar is 10µm.
SUPPLEMENTARY FIGURE 3-6: A) Control images showing reduced CHK2$^{\text{Thr68}}$ phosphorylation in response to UV-microbeam treatment in ATLD2 cells compared to rescued ATLD2. Untreated cells were stained for MRE11 to show reconstitution of wtMRE11 or mtMRE11.3. Scale bars are 10$\mu$m. B) Quantification of total nuclear intensity relative to nuclear area for proteins as indicated on x-axis 30 minutes pre- and post-2Gy of IR in ATLD2 and ATLD2+wtMRE11 as shown in Figure 4B. Error bars represent SD. C) Whole-cell irradiated ATLD2+mtMRE11.3 show no appreciable difference to ATLD+wtMRE11 shown in Figure 4B. Scale bar is 10$\mu$m.
SUPPLEMENTARY FIGURE 3-7: A) Controls showing RIDDLE+Vector and RIDDLE+RNF168 cells treated by UV-microbeam as in Figure 5A that have normal accumulation of MRE11 and MDC1 and CHK2<sup>Thr68</sup> phosphorylation. Scale bar is 10μm. B) Quantification of total nuclear intensity relative to nuclear area for proteins as indicated on x-axis 30 minutes pre- and post-2Gy of IR in RIDDLE and RIDDLE+RNF168 as shown in Figure 5B. Error bars represent SD.
3.7 REFERENCES


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Cell Cycle 9, 389–397.


CHAPTER 4
53BP1-DEPENDENT AND INDEPENDENT CONTROL OF NHEJ AND IONIZING RADIATION SURVIVAL
4.1 ABSTRACT

The cellular response to DNA double strand breaks is of critical importance in determining cell survival following ionizing radiation. A mediator of the DNA damage response, 53BP1, has been implicated in DNA repair by promotion of NHEJ and suppression of HR. As 53BP1 expression is lost in a subset of human cancers, this might affect the ability for cellular DSB repair. Using small molecule inhibitors and paired isogenic mouse embryonic fibroblasts (MEFs), differing only in their 53BP1 status, we examined how the ATM or DNA-PKcs kinases combine with 53BP1 to promote survival following ionizing radiation. We find that 53BP1 loss results in radiosensitivity associated with defective NHEJ \textit{in vitro} and increased residual $\gamma$H2AX foci. We also find that 53BP1 loss results in a defective G$_2$/M checkpoint; but this is insufficient to explain the radiosensitivity in 53BP1-null cells. Finally, we show that the effect of 53BP1 expression on radiosensitivity is not epistatic with ATM and DNA-PKcs kinase inhibition. Together these results suggest that loss of 53BP1 in tumours may lead to increased radiosensitivity and this may be further augmented with the use of inhibition of upstream kinases. This argues for the examination of 53BP1 as a prognostic factor and a biomarker for novel cancer therapies.
4.2 INTRODUCTION

The 53BP1 protein is a tumour suppressor that is rapidly phosphorylated and recruited to chromatin at sites of DSBs during the DDR (Harding and Bristow, 2012). The mechanism of tumour suppression by 53BP1 is partly due its role in G2-cell cycle checkpoint control during the DDR and suppression of genetic instability based on proposed roles in DSB repair (Adams and Carpenter, 2006; FitzGerald et al., 2009). 53BP1 has recently been implicated in both NHEJ and HR pathways of DSB repair (Xie et al., 2007; Dimitrova et al., 2008; Bouwman et al., 2010; Bunting et al., 2010). The 53BP1-deficient mouse models display immune defects, particularly in B- and T-cell development due to defective class-switch recombination and V(D)J recombination, respectively (Manis et al., 2004; Ward et al., 2004; Difilippantonio et al., 2008; Dimitrova et al., 2008). In particular, these studies suggested a defect in the rejoining phase of these processes, a characteristic of NHEJ deficient cells (Sekiguchi et al., 1999; Stavnezer et al., 2008; Kotnis et al., 2009; Gapud and Sleckman, 2011). Most recently the contribution of 53BP1 to class switching was shown to be dependent on 53BP1 oligomerization, ATM-mediated phosphorylation and chromatin binding (Bothmer et al., 2011). It has also been found that in vitro rejoining by the Ligase IV/XRCC4 required 53BP1, particularly the domain necessary for relocalization to chromatin (Iwabuchi et al., 2003). MEFs lacking 53BP1 were defective in resolution of γH2AX immunofluorescence foci, a surrogate marker for DSB repair however this was not examined in the context of DNA-PK dependent NHEJ (Ward et al., 2006). Importantly, deletion of the N-terminus of 53BP1, which contains multiple S/TQ consensus phosphorylation sites for the ATM and DNA-PKcs kinases important for NHEJ repair, were also unable to efficiently resolve γH2AX (Ward et al., 2006). Finally, and perhaps most convincingly, 53BP1/- MEFs have an increased number of chromosomal breaks at first mitosis following ionizing
radiation than MEFs derived from wild-type littermates (Morales et al., 2003; Ward et al., 2003; 2005).

We have found 53BP1 is phosphorylated by both ATM and DNA-PKcs following IR by overlapping pathways (Harding and Bristow, 2012). It remains to be determined whether 53BP1 and its phosphorylation are required for DSB rejoining, and whether ATM and/or DNA-PKcs operate in the same pathway with 53BP1 for survival of cells following IR. In this study we examine how DNA-PKcs inhibition in combination with 53BP1 loss contributes to NHEJ DSB repair. Our data suggests that at least two pathways exist for DNA-PKcs-mediated NHEJ: one dependent on 53BP1 and one independent of 53BP1. These novel findings have important implications for the role of 53BP1 in the DDR response and the use of 53BP1 function as a biomarker of differential clinical responses following cancer treatment with DNA damaging agents.

4.3 RESULTS

4.3.1 Deficiency in 53BP1 leads to cellular radiosensitivity

53BP1 expression is heterogeneous amongst primary human tumours and selected tumours have been reported to lack 53BP1 expression (Bartkova et al., 2007; Nuciforo et al., 2007). This suggests that tumours lacking 53BP1 function would have altered sensitivity to DNA damaging agents used in the clinic (e.g. radiotherapy or chemotherapy). We first verified that 53BP1-/- MEFs were deficient in 53BP1 protein relative to wild-type 53BP1+/+ cells with western blotting (Figure 4-1A). Figure 4-1B shows the relative clonogenic survival of 53BP1-proficient versus –deficient MEF cell lines relative to the effect of ATM or DNA-PKcs inhibition. 53BP1-
deficient cells were significantly more sensitive than 53BP1-proficient (SER value of 1.636±0.075; see summary of SER values in Table 4.1). Of interest, the increased radiosensitivity was similar to that achieved with ATM inhibition alone in wild type 53BP1 cells (SER of 1.721±0.135). DNA-PKcs-inhibition had the greatest effect on sensitivity with a SER value of 2.105±0.305. To explore the ramifications of these differences in survival during a protracted radiotherapy regimen of multiple 2 Gy fractions, we calculated final surviving fraction based on differential surviving fraction at 2Gy (SF2) for total radiotherapy doses up to 80 Gy (Figure 4-1C). Assuming equal cell kill per fraction, this analysis suggests that a lack of 53BP1 function could lead to a difference of up to 8 logs of final survival in oxic cells (see Supplementary Figure 4-1).

Table 4.1: Sensitization Enhancement Ratios (SER) for MEF cells and inhibitors calculated when SF=0.2

<table>
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<th>SER (±SEM)</th>
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<tr>
<td><strong>53BP1+/+</strong></td>
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<tr>
<td>ATMi</td>
<td>1.721±0.135</td>
</tr>
<tr>
<td>DNA-PKi</td>
<td>2.105±0.305</td>
</tr>
<tr>
<td><strong>53BP1-/-</strong></td>
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</tr>
<tr>
<td>ATMi</td>
<td>1.321±0.055</td>
</tr>
<tr>
<td>DNA-PKi</td>
<td>2.079±0.170</td>
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FIGURE 4-1: **53BP1 loss confers radiosensitivity.** A) Western blots confirming lack of 53BP1 in knockout MEFs relative to wild-type. B) 53BP1 +/- MEFs were assayed for clonogenic survival from IR with and with ATM and DNA-PKcs inhibitors and compared to 53BP1-/- MEFs. Error bars are geometric SEM of 3 independent experiments. C) Surviving fractions at 2Gy (SF2) were calculated from the experiments in B). Error bars are SEM for 3 experiments. * denotes significant difference from wild-type DMSO control with p<0.001 as measured by Kruskall-Wallis 1-Way ANOVA.
4.3.2 53BP1-deficient MEFs Have a Defective G<sub>2</sub>/M DDR Cell Cycle Checkpoint

To study 53BP1-dependent cell cycle checkpoint control after DNA damage, we compared the relative proliferation of the MEF cell lines before and after DNA damage. Figure 4-2A shows the relative growth of cells with or without 53BP1 expression in which we observed that a loss of 53BP1 expression leads to a reduction in overall cell proliferation. Independently of 53BP1 status, we observed a defective G<sub>1</sub>/S checkpoint using flow cytometry as shown in Figure 4-2B in both cell lines. This may be a reflection of the immortalization procedure which led to defects in the function of p16INK4A, p53 or other genes involved in the IR-induced G1 checkpoint. This is currently under investigation. From this analysis we also find that both cell types accumulate in G<sub>2</sub> at late times following IR, suggesting that this process remains intact (Figure 4-2B). We next measured mitosis following ionizing radiation by flow cytometry for Serine-10 phosphorylated Histone 3 (H3P). This suggested 53BP1-null cells have a defective G<sub>2</sub>/M checkpoint following low (2Gy), but not high (10Gy), doses of IR (Figure 4-2C). This is consistent with a 53BP1-dependent G<sub>2</sub>/M checkpoint at low IR doses as suggested by earlier studies using cells treated with siRNA to 53BP1 (Ditullio et al., 2002; Fernandez-Capetillo et al., 2002; Wang et al., 2002).

4.3.3 Total and Phosphorylated 53BP1 contribute to NHEJ in vitro

The ability to repair DSBs is a key determinant of survival following IR and a defective G<sub>2</sub>/M checkpoint could lead to unresolved DSBs progressing into mitosis (Ward, 1988; Jackson and Bartek, 2009; Jeggo and Lavin, 2009). To examine the effects of 53BP1 and its phosphorylation on biochemical NHEJ, we first determined the role of 53BP1 for DSB rejoining activity in vitro.
FIGURE 4-2: 53BP1 MEFs have a deficient G1/S checkpoint, intact G2-accumulation and a dose dependent G2/M checkpoint defect. A) 53BP1+/+ and -/- MEFs were seeded at 50 000 cells/well of a 6-well plate in triplicate and harvested each day for 4 days in panel A. Shown are means +/- SD. B) Dual parameter (BrdU/PI) flow cytometry of 53BP1 +/- MEFs show a defective G1/S checkpoint but accumulation in G2 at 16h following 6Gy. C) As in B) cells were labeled with PI and phospho-H3 (Ser10) to identify mitotic cells 1h follow 2 or 10Gy. ATMi is shown as a control in 53BP1+/+. 
In this assay, purified linear plasmid fragments are mixed with cell-free extract in a buffer containing ATP for the required energy and magnesium as a cofactor for Ligase IV activity (Diggle et al., 2003). Following incubation, RNA and proteins are removed, and then rejoining is visualized by agarose gel electrophoresis. Efficient joining results in multimeric linear plasmid fragments on the stained gel that appear as a ladder (see schematic in Figure 4-3A). We first determined whether DSB end fragments activate 53BP1 phosphorylation as a function of dose and time in HCT116 colorectal cancer cells (Figure 4-3B). DNA ends were joined within 2 hours in a dose-dependent manner. Western blotting indicated that 53BP1 Ser25 and 53BP1 Ser1778 were also phosphorylated in a dose-dependent manner similar to DSB end fragment induced DNA-PKcs Thr2056 and ATM Ser1981 activation (Figure 4-3B). Using specific antibodies to block DSB end fragment rejoining, we observed that blocking of 53BP1 Total or one of the other of the two 53BP1-phosphoforms reduced the efficacy of rejoining. Incubating the lysates with a non-specific IgG control antibody had no effect (Figure 4-3C). Blocking of XRCC4 reduced rejoining to a level similar to ATP or magnesium depletion. Small molecule inhibition of ATM did not affect joining, but DNA-PKcs inhibition markedly reduced it. Interestingly, KU70 depletion also did not affect this assay, which is likely due to the inability to titrate the antibody to a high enough concentration as this is one of the more abundant proteins in the nucleus (A. Kiltie, personal communication). The fidelity of these joints was largely maintained as the fragments can be re-cut by the original restriction endonuclease used to create the DNA fragments. Rejoining in MEF cells was less sensitive than in the HCT116, however we determined that 53BP1-null MEF extracts were not able to rejoin these DSB ends to the extent of wild-type cells (Figure 4-3D). Together, these results indicate that 53BP1 contributes to NHEJ in vitro and that selected phospho-epitopes of 53BP1 may contribute to this phenotype. This is a possible mechanism for the enhanced radiosensitivity observed in the 53BP1-/- MEFs.
FIGURE 4-3: 53BP1 and phosphorylation contribute to NHEJ in vitro. A) Schematic of NHEJ assay for detection of DSB rejoining of cohesive DNA ends. B) Increasing dose of DNA was used in the NHEJ assay from cellular extracts of HCT116 cells and rejoined DNA was collected following 1 and 2h of incubation and subjected to agarose gel electrophoresis. Simultaneously protein was collected at 2h and western blotting was performed to identify activated phosphorylation events. ATP was omitted as a negative control for phosphorylation studies. In C) assays using 40ng of DNA fragments were analysed at 2 hours. As indicated assays were spiked with antibody or small molecule inhibitor and rejoining was analyzed as in B). Fragments were recut with the original restriction enzyme as a measure of fidelity of repair. D) Cellular extracts of 53BP1 +/- and 53BP1 -/- MEFs were analyzed for NHEJ. Images shown are representative of at least 2 independent experiments.
4.3.4 Residual γH2AX foci suggest 53BP1-dependent and independent NHEJ

To further examine the contribution of NHEJ inhibition in the context of 53BP1 loss to resolution of DSBs in vivo, we next quantified residual γH2AX nuclear foci in our cell lines at 24h following damage in the presence and absence of a DNA-PKcs inhibitor (DNA-PKi; Figure 4-4A). We counterstained cells with EdU to delineate S-phase cells and eliminated them from our analysis since S-phase may confound interpretation of DNA repair based on exogenous DSBs tracked by nuclear γH2AX foci (Löbrich et al., 2010; Goodarzi and Jeggo, 2011; Redon et al., 2011). We observed that the number of initial DSBs at 30 minutes following 2Gy was identical between the two cell lines when corrected for the initial number of foci in undamaged cells (NIR; Figure 4-4B). However, the residual number of γH2AX foci at 24 hours following 10Gy was significantly higher in 53BP1-/- cells when compared to 53BP1+/+ cells (p<0.05). Inclusion of DNA-PKi in the media of irradiated cells eliminated this difference. This data supports the findings of our in vitro assay for a role of 53BP1 in NHEJ.

4.3.5 53BP1 is not solely epistatic with ATM or DNA-PK in determining cellular radiosensitivity

We have shown previously both ATM and DNA-PKcs can phosphorylate 53BP1 in response to DNA damage (Harding and Bristow, 2012). Our data in Figure 4-1 suggested that a similar radiosensitivity existed for cells deficient for either 53BP1 or ATM function. To determine if 53BP1 is epistatic with ATM or DNA-PKcs, we examined IR-induced clonogenic cell killing in 53BP1-/- cells following either ATM or DNA-PK inhibition (Figure 4-5). We observed a significant sensitization of 53BP1-null cells with ATM and DNA-PKcs inhibition with SER values of 1.321±0.055 and 2.079±0.170, respectively (see Figure 4-5A and Table 4.1). This
increased sensitization was significant at even 2Gy (Figure 4-5B). Surprisingly, despite our data in Figure 4-1 and previous literature suggesting that ATM is epistatic with 53BP1, we observed that 53BP1-null cells were further sensitized to IR following ATM inhibition. This suggests that in addition to survival pathways that depend on 53BP1, there are ATM and DNA-PK survival pathways independent of 53BP1 status.
FIGURE 4-4: 53BP1 loss confers increases residual γH2AX in 53BP1-/- MEFs. A) 53BP1+/+ (left panel) and 53BP1 -/- (right panel) were fixed at 30 minutes and 24 hours after irradiation with 2 and 10Gy, respectively. Cells were pulsed 1 hour before fixation with EdU and were stained with γH2AX, DAPI and EdU as indicated. Control treatment with DNA-PK inhibitor (DNA-PKi) was done 1h before irradiation and maintained until fixation. Non-irradiated cells are shown for comparison. The arrow denotes an S-phase cell eliminated from further analyses. B) Three independent experiments performed as in A) were quantified for the number of γH2AX foci per nucleus and the baseline (NIR value) was subtracted. The non-normalized NIR value is shown for comparison. Asterisk indicate p<0.05 by Mann-Whitney t-test.
FIGURE 4-5: Inhibition of ATM or DNA-PKcs enhances radiosensitivity of 53BP1-null MEFs. A) Clonogenic survival curves of 53BP1-/- cells treated with DMSO, ATMi or DNA-PKi shown as circles, squares and triangles, respectively. B) Surviving fraction at 2Gy of 53BP1-/- cells treated as in A). The asterisk denotes significant difference from wild-type DMSO control at p<0.001 as measured by Kruskall-Wallis 1-Way ANOVA.. All error bars represent SEM of at least 3 independent experiments.
4.4 DISCUSSION

53BP1 is a tumour suppressor gene and 53BP1 expression is often deficient in human tumours (Ward et al., 2003; Bartkova et al., 2005; Morales et al., 2006; Nuciforo et al., 2007). Here we present the novel findings that 53BP1 and its phosphorylation contribute to NHEJ repair of DSBs. This led to increased radiosensitivity when 53BP1 was lost and this could be further augmented by ATM or DNA-PKcs inhibition. Together this suggests that although 53BP1 contributes to a subset of NHEJ and IR-survival, it is not a sole determinant of ATM and DNA-PKcs-mediated radioprotection. Previous reports had suggested that 53BP1 confers radioresistance in part through promotion of NHEJ (Iwabuchi et al., 2003; Adams and Carpenter, 2006). ATM DSB-signaling leads to the processing of DNA ends to make them compatible for rejoining and also relaxes chromatin to permit repair of heterochromatin-associated DSBs and these activities are in part mediated by 53BP1 (Jeggo and Löbrich, 2005; Goodarzi et al., 2010; Noon et al., 2010). Our studies are the first to determine how 53BP1 and either ATM or DNA-PKcs in combination impact survival following ionizing radiation.

53BP1 can suppress aberrant rearrangements in V(D)J recombination by tethering distal immunoglobulin coding regions required for antibody diversity generation (Difilippantonio et al., 2008). Additionally, *in vitro* data has shown that the region of 53BP1 required for localization into IR-induce foci stimulates ligase IV/XRCC4 mediated end-joining (Iwabuchi et al., 2003). Using an *in vitro* repair assay we show that 53BP1-/- MEFs display reduced NHEJ efficiency. To extend this analysis we used antibodies to specifically block particular phosphoforms of 53BP1. We found that these phosphospecific antibodies reduced NHEJ in human cellular extracts suggesting that 53BP1 phosphorylation fulfills a functional role in NHEJ. This finding is
supported by previous evidence that loss of the N-terminal 53BP1 phosphorylation domain results in increased residual γH2AX foci indicative of DSBs (Ward et al., 2006).

To determine how 53BP1 and DNA-PKcs kinase combine to resolve DSBs in cells we used the residual γH2AX foci approach. We found that indeed, 53BP1-/- MEFs have increased residual γH2AX. Inclusion of DNA-PKcs inhibitor increased the number of residual γH2AX foci but also eliminated the difference between 53BP1 wild-type and knockout cells. This suggests that there are two DNA-PKcs dependent NHEJ pathways, one involving 53BP1 and the other independent of 53BP1 function. This possibility is diagrammed in Figure 4-6. It is clear that 53BP1 is phosphorylated by DNA-PKcs, but it is unknown whether 53BP1 contributes to further autophosphorylation of DNA-PKcs or DNA-PKcs activity on other targets. Since, aside from DNA-PKcs autophosphorylation, 53BP1 is the only DNA-PKcs target identified this will have to be examined using kinase assays specific to DNA-PKcs, which to our knowledge do not yet exist. This will be complicated by the similarity of target sequences for DNA-PKcs and the related ATM and ATR kinases (Durocher and Jackson, 2001). Additional functional biochemical and cellular studies are required using 53BP1 phosphomutants on a null-53BP1 background to define the role of 53BP1 phosphorylation on DNA-PKcs activity.

The first functional studies of 53BP1 suggested defects in cell cycle checkpoints, in particular the ATM-dependent early G2/M checkpoint measured by H3P early after DNA damage but this was not examined in the context of IR-survival. Similar to early work we have found that this is a true defect at 2Gy but not at 10Gy (Ditullio et al., 2002; Fernandez-Capetillo et al., 2002; Wang et al., 2002). Interestingly, this checkpoint is considered to be dose-independent when 53BP1 is intact (Xu et al., 2002). The reason for this dose-dependence when 53BP1 is absent is
FIGURE 4-6: Model for 53BP1 involvement in NHEJ. When a DNA double strand break is induced by ionizing radiation DNA-PKcs is rapidly activated where it contributes to NHEJ through two possible pathways. One, independently of 53BP1 and another that depends on 53BP1. Whether this is solely forward signaling of DNA-PKcs to 53BP1, possibly via phosphorylation, or whether this feedback to DNA-PKcs activity on other downstream targets is unknown.
a matter of debate, but the current dogma is that higher levels of damage enact multiple overlapping pathways that compensate for 53BP1 loss (Ditullio et al., 2002; Adams and Carpenter, 2006). We do not observe a G1/S checkpoint in either of these cell lines and this may be as a consequence of the immortalization process. Importantly, defects in this G1/S checkpoint such as loss of p53 or p21 are not associated with increased radiosensitivity (Slichenmyer et al., 1993; Wouters et al., 1997; Brown and Wouters, 1999). We do, however, observe a G2-accumulation in these cells. The lack of a G2/M checkpoint, but maintenance of G2-accumulation is similar to functionally null BRCA1 cells complemented with a BRCA1 mutated at Serine-1423 (Xu et al., 2002). These cells have normal radiosensitivity indicating that this particular phenotype does not impact on survival from IR. Combined with our clonogenic survival data this suggests that the role of 53BP1 in radiation survival is due primarily to a defect in DNA repair and not to the associated checkpoint defects.

We find that although 53BP1-/- MEFs are more radiosensitive than wild-type controls, ATM and DNA-PKcs can significantly radiosensitize each of these MEF cell lines. This indicates that although ATM and DNA-PKcs phosphorylate 53BP1 and although 53BP1 can contribute to ATM-mediated signaling, these kinases have effects that do not rely entirely on 53BP1. Together our data suggest that 53BP1 is an important determinant of cellular radiosensitivity that may have important therapeutic implications for 53BP1-deficient tumours. This is especially important in fractionated radiotherapy when small survival differences are exacerbated into large differences in cell killing that may be exploited as an improved therapeutic ratio. Additionally, we find that inhibition of DDR kinases ATM and DNA-PKcs significantly radiosensitize even 53BP1-/- cells suggesting that regardless of 53BP1 status these may be efficacious in clinical scenarios if protection of normal tissues can be achieved to maintain a favourable therapeutic
We also find that there are 53BP1 dependent and independent pathways for NHEJ indicating that tumours deficient in 53BP1 may be more susceptible to DNA damaging agents. Further studies in human cancer cell models and in xenograft tissues will be of great importance in evaluating the potential of these interventions in the context of microenvironmental factors and heterogeneous genetic backgrounds that are present in human cancer cohorts.

4.5 MATERIALS AND METHODS

4.5.1 Cell Culture and Treatments

The 53BP1+/+ and -/- MEF cell lines were obtained from P. Jeggo and cultured in Dulbecco’s Modified Eagles Medium (DMEM) media containing 10% fetal calf serum and 1X glutamine (Bioshop). HCT116 cells were a gift from B. Vogelstein and were cultured in McCoy’s 5A media containing 10% fetal calf serum. All cells were cultured in a humidified atmosphere containing 5% CO₂ in the presence of antibiotics and were verified to by mycoplasma free. ATM and DNA-PKcs inhibitors (Tocris Biosciences) were used at concentrations of 10µM and 4µM, respectively. Irradiations (Cs-137) were performed in a GammaCell 40 (Nordion) under oxic conditions at a dose rate of ~1Gy/minute.

4.5.2 Clonogenic Survival Assay

Clonogenic survival was assayed by standard procedures. Briefly, cells were seeded at known concentrations in triplicate into 6cm plates. Cells treated with ATM and DNA-PKcs inhibitors were seeded into the drug and allowed to incubate for 1-2h before irradiation. Following 24h of repair media was changed to that containing no inhibitors to limit excessive toxicity and colonies
were allowed to form from 5-10 days. Colonies were fixed and stained in a 50% ethanol solution containing 1% methylene blue and rinsed 3X in water.

In each case colonies of 50 or more cells were counted and surviving fraction was calculated by dividing the number of colonies in the treated group to that of the untreated group for at least 3 independent experiments. The geometric mean of these surviving fractions was plotted accordingly and SEM was calculated using GraphPad software (Prism). One-way ANOVA with a Bonferoni correction was used to determine statistical significance (p<0.05) between groups.

### 4.5.3 In vitro non-homologous end-joining assay

Non-homologous end-joining was assayed in vitro essentially according to Diggle et al (Diggle et al., 2003). Briefly, 10-20 15cm dishes (>10^8 cells) of subconfluent HCT116, 53BP1+/+ or 53BP1/- cells were harvested by trypsinization and washed 3X in cold PBS (−Mg –Ca). Cells were washed gently 1X in cold hypotonic lysis buffer (10mM Tris-HCl pH 8.0, 1mM EDTA and 1mM DTT) containing protease inhibitors (1mM benzamidine, 1µg/mL antipain, 2µg/mL aprotinin, 1µg/mL Leupeptin and 1mM phenylmethanesulfonylfluoride) and resuspended at a concentration of ~1x10^8 cells/mL in hypotonic lysis buffer as above. Cells were incubated for 20 minutes on ice and homogenized with ~30 strokes in a Dounce homogenizer with the tight pestle. Viability was verified to be <5% by trypan blue exclusion and cells were incubated a further 20 minutes on ice. One half volume of high salt buffer (50mM Tris-HCl pH7.5, 1M KCl, 1mM EDTA, 1mM DTT and protease inhibitors) was added and mixed gently before incubation on ice for 20 minutes. Cellular and chromatin debris was removed by conservative pipetting after ultracentrifugation at 4°C in a TLA-55 rotor at 55 000rpm for 3 hours. The supernatant was
dialysed (10K Snakeskin tubing; Thermo) overnight with one change against E-buffer (20mM Tris-HCl pH8.0, 0.1M Potassium acetate, 20% Glycerol, 0.5mM EDTA, 1mM DTT and protease inhibitors) at 4°C. Precipitated protein was eliminated from the resulting extract by centrifugation in a bench-top centrifuge and protein concentration was determined using A280 (NanoDrop) and only lysates with A260/280 ratios of less than 1.5 were used. Aliquotted lysates were frozen in LN\textsubscript{2} and stored at -80°C.

A 3.2kb fragment of plasmid DNA (pPCR1+3 cut with BstXI; gift of A. Kiltie) was isolated using gel extraction (Qiagen) and quantified using NanoDrop. A reaction of 20-80ng of DNA was incubated at 37°C with 40ug of cell free extract (above) and 4µL of HEPES end-joining buffer (50mM HEPES pH 8.0, 40mM Potassium acetate, 0.5mM Magnesium acetate, 1mM ATP, 1mM DTT and 0.1mg/mL BSA) diluted to 20µL final volume. Following indicated times 2µL DNase-free RNase was added for 10 minutes followed by 4 µL Deproteinization solution (10mg/mL proteinase K, 2.5% SDS, 50mM EDTA, 100mM Tris-HCl pH 7.5 in water) for 10 minutes. DNA was isolated by phenol/chloroform/isoamyl alcohol and separated by electrophoresis in a 0.7% agarose 1X TBE gel at 50V for 3h. The gels were dried at 60°C for 30 minutes under vacuum and stained in SYBR green I (1X in TBE, Invitrogen) overnight (Kiltie and Ryan, 1997). Experiments were performed at least twice.

As indicated some assays omitted ATP or Mg. In indicated cases ATM or DNA-PKcs inhibitors (500nM) or antibodies were added 1h prior to addition of the plasmid fragment. Antibodies were: 53BP1\textsuperscript{Total} (Cell Signaling #4937), 53BP1\textsuperscript{Ser25/29} (Cell Signaling #2674), 53BP1\textsuperscript{Ser1778} (Cell Signaling #2975), IgG (Cell Signaling #2729), Ku70 (Santa Cruz #SC5309) and XRCC4 (Abcam #Ab145). These antibodies were spiked into reactions at the highest possible
concentrations given the concentration of the cell extract used and a final reaction volume of 20µL. Where indicated rejoined fragments were re-cut with BstXI according to standard protocol (NEB) and fragments were isolated by chloroform extraction.

4.5.4 Western Blotting

To assay protein activation in the NHEJ assay reactions were scaled up to include 100µg of protein and plasmid concentrations that equaled the above descriptions. Here, the deproteinization step was eliminated and 1X NuPage loading buffer containing 10mM DTT (Invitrogen) was added and incubated at 70°C for 10 minutes. Proteins were separated on 4-8% Tris-acetate gels and transferred to nitrocellulose as described (Harding and Bristow, 2012). Primary antibodies were: 53BP1<sup>Total</sup> (Cell Signaling #4937), 53BP1<sup>Ser25</sup> (Novus NB100-1803 Lot 1A), 53BP1<sup>Ser1778</sup> (Cell Signaling #2975), DNA-PKcs<sup>Thr2056</sup> (Gift of D. Chen), DNA-PKcs<sup>Total</sup> (Serotec #AHP318), ATM<sup>Ser1981</sup> (Epitomics #2152) and Ku70 (Santa Cruz #SC5309). Blots were imaging using the Odyssey system (Licor) as described (Harding et al., 2011; Chapters 2 and 3).

4.5.5 Residual γH2AX analysis

Immunofluorescence was performed as described previously using EdU incorporation 1h before fixation and staining as described (Harding et al., 2011). Primary antibody was γH2AX (Upstate/Millipore JBW301 #05-636, 1/800 dilution). Secondary detection was with: Alexa Fluor 488 Donkey Anti-Mouse (A21202; 1:500). For inhibitor treatments these were added 1h prior to irradiation and maintained until fixation. Cells were imaged as described previously except imaging was done using a 40X objective (0.95NA) yielding pixel dimensions of 0.4µm x 0.4µm with z-stack distances of 0.4 µm (Harding et al., 2011). Images were deconvolved using
25 iterations of 3D-blind deconvolution with Autoquant software (Media Cybernetics). At least 50 non-S-phase nuclei (excluded by EdU positivity) were counted for each of 3 independent experiments. Images were prepared for publication using ImageJ (NIH) and quantified using Image Pro Plus (Media Cybernetics). Data was analysed using GraphPad software (Prism) and standard error of the mean is plotted. Statistical significance was calculated using Mann-Whitney t-Test and p<0.05 as the cutoff.

4.5.6 Flow Cytometry

Cells were irradiated with indicated doses at ~80% confluence, trypsinized and fixed at indicated times using ice cold 70% ethanol. Cells were stored at -20°C until stained.

BrdU/PI

At 1 hour before fixation BrdU (Sigma) was added to the media at a concentration of 10µM. Cells were washed 2X in 1% BSA/PBS solution. Cells were resuspended in 1mL 2N HCl/0.2mg/mL Pepsin solution and incubated at room temperature for 30 minutes. To this solution 3mL of 0.1M Sodium tetraborate pH 8.2 was added followed by 2X PBS/BSA washes. Approximately 10⁶ cells were resuspended in 50µL, PBS/BSA/0.5% Tween-20 with 20µL BrdU-FITC conjugate antibody (BD Biosciences) and incubated for 1hr at room temperature. Cells were washed 2X in PBS/BSA/Tween-20 and resuspended in PBS/BSA/Tween containing 0.5mg/mL RNase A and 50µg/mL Propidium iodide (Sigma). Cells were analysed on a FACS Calibur instrument and analysed using FlowJo software.
**Phospho-H3 (Ser10)**

Cells were fixed as above and stained with phospho-H3 (Ser10) antibody from Cell Signaling (#9706) for 1hr at room temperature. Cells were washed 1X in PBS/BSA/Tween-20, resuspended in RNase A/Propidium iodide solution and analysed on a FACS Calibur and analysed using FlowJo software. Gates were chosen to select for diploid cell phases and may include some tetraploid G1-phase cells as a component of the G2 population.
4.6 Supplementary Figures

SUPPLEMENTARY FIGURE 4-1: Using SF2 calculated in Figure 1 extrapolated surviving fractions for multiple fractions of 2Gy were calculated from 5-40 fractions simulating daily clinical fractionation schedules. These extrapolations assume equal killing at each fraction, no repair between fractions and no other tumoural changes (i.e. reoxygenation, redistribution etc.). The 53BP1+/+ ATMi and 53BP1-/- DMSO curves are superimposable.
4.7 REFERENCES


CHAPTER 5
SUMMARY AND FUTURE DIRECTIONS
5.1 THESIS SUMMARY

In many cancers, increasing tumour stage and aggression relates to increasing levels of hypoxia and genetic instability (Bristow and Hill, 2008). This instability is characterized by gene mutation, allelic loss and/or gain and epigenetic changes relating to oncogene and tumour suppressor gene function. Genetic instability can further be modified by the tumour microenvironment (e.g. in hypoxic cells) leading to complex and dynamic genetic changes during tumour progression (Bristow and Hill, 2008). The interrogation of specific DNA damage responses using biomarkers that reflect the cellular responses to exogenous and endogenous DNA damage in oxic and hypoxic cells may allow a better understanding of tumour responses to radiotherapy and chemotherapy (Chan and Bristow, 2010). In this thesis I have examined how 53BP1 and its phosphorylation contribute to the DDR in oxic and hypoxic cells.

In Chapter 1 I reviewed the literature on the 53BP1 protein, its contribution to DSB sensing, repair and cell cycle checkpoints. I hypothesized that 53BP1 phosphorylation is downstream of the MRN-ATM-RNF168 signaling axis following replication stress and ionizing radiation and that 53BP1 contributes to NHEJ and subsequently clonogenic survival from IR.

In Chapter 2, I conducted experiments using a panel of phosphospecific antibodies to explore 53BP1 response in cells exposed to oxic or anoxic gassing conditions. I observed that 53BP1 is phosphorylated at Serine-25 specifically in anoxic S-phase cells that have arrested during DNA replication. This lead to replication stress activating ATM; this kinase was only partially responsible for 53BP1 phosphorylation in these conditions. This suggested that ATM might not be the only kinase responsible for 53BP1 phosphorylation under other conditions.
To further work addressing 53BP1 phosphorylation following exogenous DNA damage, in **Chapter 3**, I conducted experiments to interrogate ATM and DNA-PKcs kinase activity and phosphorylation of 53BP1 in cells treated with ionizing radiation. Importantly, using isogenic cell lines, I observed that defects in the MRN-ATM-RNF168 signaling pathway did not eliminate 53BP1 phosphorylation. Furthermore, other studies using DDR-defective cells showed that RNF168 was required for 53BP1 localization to DSBs, but was dispensable for phosphorylation. My results for the first time uncoupled 53BP1 phosphorylation, from DSB localization, and supported parallel pathways for 53BP1 biology during the DDR. I also summarized the data from Chapters 2 and 3 to show how staining for different 53BP1 phosphoforms *in situ* may give unique information regarding different cancer phenotypes or response to cancer treatment.

Finally, based on a potential role for DNA-PKcs kinase activity and 53BP1 phosphorylation, I studied whether 53BP1, and its phosphorylation by either ATM or DNA-PKcs, contributed to NHEJ and radiation cell survival. These studies were presented in **Chapter 4**. Surprisingly, I observed that 53BP1-null cells can be further sensitized by ATM or DNA-PKcs inhibition. This suggested that the loss of 53BP1 is not a limiting factor for use of such small molecules to increase killing of cells. It also suggests that 53BP1 is not entirely epistatic to ATM in a serial DDR pathway and supports my biochemical and cellular data in Chapter 3. Additionally, using *in vitro* and cellular assays for NHEJ, I observed that 53BP1 and its phosphoforms contribute to the final level of DSB repair. These novel data suggest that there are both 53BP1-dependent and 53BP1-independent pathways for NHEJ. The key findings from Chapters 2-4 are summarized diagrammatically in **Figure 5-1**.
The understanding of how 53BP1 mediates cell killing in response to cancer therapies in experimental tumour models will be of key importance given the reported loss of 53BP1 in a subset of human cancers (reviewed in Chapter 1). The novel data shown in Chapters 2-4 lead to a number of outstanding questions that can be addressed by future studies and are outlined in Figure 5-2. I have shown that 53BP1 and its phosphorylation contribute to NHEJ, but did not study the role of 53BP1 in HR. It is also unclear how 53BP1 contributes to survival in tumour cells rather than normal cells either in vitro or in vivo following radiotherapy and/or chemotherapy. Such studies would be of interest using agents in addition to IR that initiate the DDR to enact cell kill (e.g. DSB-inducing agents such as bleomycin or topoisomerase I or II inhibitors). It may be of further interest to also study agents that specifically target DNA replication during S-phase, where I observed that 53BP1 has distinct responses to replication-associated damage.

Despite extensive studies on multiple tumour sites for DDR components, including 53BP1, it is unclear how the DDR response varies in epithelial versus mesenchymal tissues. For example, prostate cancer epithelium may activate the ATM and DNA-PK pathway differently than prostate stromal cells or surrounding seminal vesicle tissue and androgen-mediated pathways of DSB induction may underlie TMPRSS2:ERG fusions (Mani et al., 2009; Haffner et al., 2010; Zhang et al., 2011). Many DDR proteins respond in prostate cancer, targeting of defective DDR responses in prostate cancer using inhibitors of ATM, DNA-PKcs or PARP1, could lead to novel therapies (Shaheen et al., 2011; Fraser et al., 2012). I propose that in the future these issues be the focus of 53BP1 studies and detail below some preliminary data and suggestions for future study.
Ionizing radiation induces DNA-PKcs and, via MRE11, ATM kinase activities. Together these kinases phosphorylate 53BP1 upstream of chromatin recruitment and RNF168-mediated ubiquitylation leads to accumulation of 53BP1 at the DSBs as IRIF. Hypoxia also activates ATM, and likely ATR, which phosphorylates 53BP1 but does not lead to 53BP1 foci formation. Replication stress activates ATR and also ATM (see Chapter 1) both of which may contribute to 53BP1 phosphorylations and chromatin recruitment. Functionally 53BP1 and its phosphorylation contribute to NHEJ and IR-survival and 53BP1 also has a dose-dependent role in the G2/M checkpoint.
Does hypoxia-induced ATM-activation signal to 53BP1-phosphorylation?

Do upstream signaling pathways control localization of phosphorylated 53BP1 to IRIF?

Does 53BP1 and phosphorylation contribute to NHEJ and survival from IR?

Does 53BP1 loss in human tumour models sensitize cells to the rapteutics?

Do upstream signaling pathways control localization of phosphorylated 53BP1 to IRIF?

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

Summary and Future Directions

Does 53BP1 loss contribute to HR or sensitivity to novel therapeutics?

Does 53BP1 loss in human tumour models sensitize cells to the rapteutics?

Is 53BP1 a predictive or prognostic factor in prostate cancers?

FIGURE 5-2: Thesis summary figure.
5.2 FUTURE DIRECTIONS

5.2.1 53BP1 as a Determinant of in vivo Radiosensitivity

I have shown that a loss of 53BP1 leads to in vitro radiosensitivity in non-transformed MEF cells. I have also observed that complicating factors in tumours, for example increased S-phase fractions and anoxia, may impact on the biology of 53BP1 responses to DSBs. I suggest that functional studies using tumour models are fundamental to the understanding of how 53BP1 loss in tumours may be exploited clinically given these confounding factors. This requires the development of tumour models which are isogenic aside from 53BP1 expression.

Due to difficulties experienced during direct siRNA-knockdown of 53BP1 and other genes in populations of cells in vitro and the disappointing effects on radiosensitivity (see Appendix B), I have begun collaborations with Dr. Gaetano Zafarana in the Bristow laboratory. We are using a conditional 53BP1 shRNA system in clonal 22RV1 tumour cells lines as detailed in Figure 5-3. We have chosen to use the 22RV1 prostate cancer cell line because on comparative genomic hybridization it shows comparable genetic abnormalities to intermediate risk prostate cancer (see below) and it efficiently forms xenografts in immunocompromised mice. Into this cell line we have stably overexpressed the TET repressor and integrated shRNA sequences targeting 53BP1 and have selected clones. Under normal conditions in vitro the shRNA is not expressed but when induced by doxycycline the transcriptional repression is relieved and shRNA is produced to silence 53BP1 RNA and protein expression (Figure 5-3). This produces a much more homogeneous population of 53BP1 knockdown than siRNA approaches with the added benefit that the level of knockdown can be titrated by the doxycycline concentration (Appendix B, Figure 5-3 and Zafarana et al., 2009).
FIGURE 5-3: Development of an inducible shRNA system for 53BP1 knockdown. A) Schematic of the 22RV1 inducible shRNA system. 22RV1 cells were transfected by electroporation with a vector expressing the Tet Repressor (TetR) and a puromycin selection cassette. Isolated colonies were picked and screened for TetR expression and a single clone was transfected with a vector encoding one of two different 53BP1-shRNA sequences (A or B) under control of the H1 promoter into which a Tet operon (TetO) is inserted. This is predicted to insert as an array of 5-20 copies into the genome (G. Zafarana, unpublished observations). Clones were isolated and induced with 250ng/mL Doxycycline resulting in decreased 53BP1 mRNA as measured by TaqMan qPCR. Control expression of the 22RV1 (Par) and 22RV1-TetR (TetR) are shown as controls and all expression is relative to the respective clones without doxycycline. The two target sequences for 53BP1 were: (A) 5’AAGATACTGCCTCATCACAGT3’ corresponding to residues 610-630 and (B) 5’AAGAACGAGGAGACGGTAATA3’ corresponding to residues 340-360 of the 53BP1 mRNA (accession number NM_001141980). Continued on next page.
FIGURE 5-3 Continued: Development of an inducible shRNA system for 53BP1 knockdown. B) Immunofluorescent images of 53BP1 staining in a HCT116 cells treated with siRNA to 53BP1 or a negative control showing heterogeneous knockdown of 53BP1 despite apparent efficiency by western blotting. In comparison, 22RV1-TetR-sh53BP1 clone A21 shows homogeneous knockdown when induced with doxycycline (+Dox, 250ng/mL 48h) with equivalent appearance of 53BP1 knockdown by western blot.
Once established, I anticipate that these cell lines will form solid tumours in which I can modulate 53BP1 expression in tumour-bearing animals in the following studies. First, tumour cell radiosensitivity can be studied *in vivo* using tumour growth delay and *ex vivo* clonogenic survival assays at 53BP1-suppression levels of 50% and 100% to, theoretically, mimic single or dual copy-loss of 53BP1. These are both observed in human tumours (Ishkanian et al., 2009 and Chapter 1). This should give an indication of whether IR-induced cell death is 53BP1-dependent leading to increased growth delay and tumour cell clonogen radiosensitivity *in vivo*. Although growth delay experiments are relatively simple, they do not give an indication of whether a particular factor (i.e. 53BP1 status) will result in increased radiocurability of a tumour. For this, one requires an assay to determine the tumour control dose to give 50% cure (called TCD50 assays) (Baumann et al., 2008). Here, using only conditions observed to give increased growth delay, one should determine whether 53BP1-positive tumours require higher doses to give 50% cure. If that is the case, then one could be confident that 53BP1 status is a determinant of curability in this experimental model.

Throughout all of these studies it will be important to examine how 53BP1 responds to IR *in vivo* to gain mechanistic insight. For example, one can monitor cells in S-phase at the time of irradiation by injecting mice with EdU just prior to radiation or one can monitor hypoxia/anoxia by injection of EF5. Both EdU and EF5 can be detected on slices of tumour by immunohistochemistry and co-staining with 53BP1 and its phosphoforms using immunofluorescence would identify how 53BP1 localization is affected by these microenvironmental factors. Hypoxia does not necessarily exist in a static state so examining 53BP1 status in relation to currently or previously (cycling) hypoxic regions using two different
hypoxia markers (EF5 and Pimonidazole) administered sequentially would provide insights to this issue. I suggest 3 questions in particular that could be examined in this system:

1) Is 53BP1 phosphorylation active in anoxic regions of tumours and does this depend on S-phase?
2) Does IR-induced 53BP1 phosphorylation change across oxygen gradients as marked by EF5 intensity and cycling hypoxia?
3) Do areas of hypoxia or active proliferation show increased regression following IR when 53BP1 shRNA is induced?

Together this model system will further the functional and mechanistic understanding of how 53BP1 contributes to radiation survival in vivo and to what extent the microenvironment will influence these responses.

5.2.2 53BP1 in Homologous Recombination

In Chapter 1, I outlined previous studies that suggested a role for 53BP1 in the suppression of the HR pathway of DSB repair. Cells rendered HR-deficient by loss of BRCA1/2 incur increased chromosomal abnormalities when treated with PARPi (Bouwman et al., 2010; Bunting et al., 2010). This treatment results in excessive DSBs during S-phase that are rejoined into aberrant NHEJ rearrangements. When 53BP1 is also lost in these cells fewer chromosomal abnormalities are recorded and the cells lose their sensitivity to PARPi, presumably due to decreased NHEJ (Bouwman et al., 2010; Bunting et al., 2010). Interestingly, none of these studies explore how 53BP1 loss in isolation contributes to survival from agents that are repaired by HR. For example, are 53BP1/- cells less sensitive to PARPi as a consequence of increased reliance on HR rather
than the deleterious NHEJ? In Figure 5-4, I show that 53BP1-/- MEFs are in fact more sensitive to PARPi than are wild-type cells. One would also predict that if 53BP1 were purely HR-suppressive, then chemotherapeutics creating damage types that are repaired through HR would result in increased resistance to such drugs. Mitomycin C, repaired by a mechanism that requires HR factors, is equally efficient at killing 53BP1-/- and +/+ cells. A defect in HR is in keeping with a severe proliferation defect in these 53BP1-/- MEFs compared to wild-type controls (Figure 5-4). This indicates that 53BP1, although it may contribute to HR-suppression, can also under these experimental conditions contribute to survival from PARPi and from MMC.

These observations present a number of possibilities including 53BP1 involvement in other repair pathways. For example, 53BP1 may be directly involved in the repair of single strand breaks or in base excision repair when PARP is inhibited. This would manifest as sensitivity to agents such as H2O2 or methyl methanesulfonate (MMS). This should be tested both in the MEF system and also in the 22RV1-Tet-sh53BP1 system described above. Since the response of 53BP1 is dependent on cell-cycle phase and oxygenation status the best way to integrate these into functional studies is to use the in vivo xenograft 22RV1-Tet-sh53BP1 model system. If 53BP1 were functionally important for tumour survival to these treatments then such tumours should respond to PARPi and to chemotherapies that induce damage predominantly repaired by HR including MMC and etoposide (Helleday et al., 2008). As in the case of radiation treatment, immunohistochemical analyses of these tissues may also be suggestive of underlying mechanisms.
FIGURE 5-4: 53BP1 loss causes cellular sensitivity to PARP inhibition. Sensitivity of MEFs to 2.5uM of ABT-888 (PARPi) and 1ug/mL of MMC (1 hour) was examined by clonogenic survival. PARP inhibitor was maintained through the duration of the assay whereas MMC was added for 1h at 16h post-seeding of the clonogenic assay. * denotes p<0.001 between treatment and respective untreated cell line. ** denotes p<0.01 of particular treatment between cell lines. Test was 1-way ANOVA with Bonferroni multiple comparisons correction.
5.2.3 53BP1 as Prognostic Factor in Prostate Cancer Therapy

Prostate cancer is the most common malignancy in men with more than 25,000 men being diagnosed annually and more than 4000 men dying of metastatic prostate cancer per year (Canadian Cancer Statistics 2011; www.cancer.ca). The current prognostic factors of pathologic Gleason score (GS), TNM staging and the level of prostate specific antigen (PSA; ng/ml) are used to place men with localized prostate cancer into low, intermediate and high risk of local and systemic spread. Some of the low risk patients are thought to have indolent disease that instead of treatment would be better served by active surveying for disease progression in which up to 2/3’rds of these men could be spared the morbidity of surgery or radiotherapy (Cooperberg et al., 2011). Identifying patients that require more radical treatments or no treatment at all is a major goal of current research in this area.

As described above no study has examined 53BP1 expression in prostate cancer. Our lab has collected pre-treatment biopsies from ~150 men diagnosed with intermediate risk prostate cancer and treated with modern image-guided radiotherapy (IGRT). This is a heterogeneous group of patients who, despite having similar clinical presentation of disease have ~20-40% chance of failing therapy. These pre-treatment biopsies have been placed on tissue microarrays for rapid analysis of putative biomarkers by immunohistochemistry that can be correlated with clinical outcome. In addition DNA from these patients has been subjected to array comparative genomic hybridization (aCGH) to determine copy loss in this cohort of patients. We have not detected any loss in the genomic region of 53BP1, but this does not include gene expression changes that may result in reduced 53BP1 protein. Currently DNA from these patients is being studied as part of the International Cancer Genome Consortium (ICGC) and being whole genome sequenced to
identify copy number variations and DNA mutations that may be present. Most importantly at least 5 years of clinical follow-up is associated with these data and can be used to correlate any observations with clinical outcome for validation in prospective studies.

I have shown that 53BP1 and its phosphoforms respond \textit{in situ} to IR-induced DSBs in 22RV1 human prostate cancer xenografts and using 53BP1 phosphospecific antibodies, I have validated their use in \textit{ex vivo} irradiated primary human prostate tissue (\textbf{Figure 5-5 and 5-6}). These antibodies are therefore suitable for use on the tissue microarrays that have been produced using specimens from patients in whom outcome is known following surgery or radiotherapy.

Therefore, 53BP1 protein status (positive, negative, focal, phosphorylated, etc) in these primary tissues, could be evaluated as novel prognostic factors.

I suggest that three specific questions be addressed using this material:

1) Is 53BP1 expression variable within and between patient biopsies and is decreased or punctate expression specific to cancer tissue?
2) Is 53BP1 phosphorylated in primary prostate specimens and how does this correlate with proliferation (i.e. Ki67) or hypoxia (i.e. GLUT1) markers in adjacent sections?
3) Do any of these phenotypes correlate with clinical outcome and are they new and independent prognostic factors that aid in personalized medicine protocols?

Current whole genome sequencing efforts (e.g. The Cancer Genome Atlas (TCGA) and ICGC) will document allelic loss, mutation and epigenetic modification of genes involved in the DDR
FIGURE 5-5: 53BP1 phosphorylation occurs in vivo in response to IR. 22RV1 tumours were induced in the hind flank of CD1 Nude mice. Non-irradiated (NIR) tumours or tumours from mice treated with 10Gy of whole-body radiation were resected 1h post-IR and subjected to immunohistochemistry using antibodies indicated above. Slides were scanned using an Aperio scanner; scale bar is 100µm in large images and 10µm for inlays.
FIGURE 5-6: 53BP1 phosphorylation occurs in ex vivo irradiated prostate cancer. Radical prostatectomies were obtained and placed into transport media, dissected and either mock-irradiated or irradiated with 2Gy and fixed in formalin 1 hour later and parafin embedded. Tissue sections were stained for markers as indicated. Zoomed features are from areas identified as cancer (Gleason Score 3). Slides were scanned using an Aperio scanner; scale bar is 100µm in large images and 10µm for inlays.
pathway, including 53BP1 and will be invaluable data to understand the role of 53BP1 in prostate cancer. Most importantly, these studies will determine whether 53BP1 status is a factor that contributes to success or failure of IGRT prostate cancer therapy. This will give an evaluation of the \textit{prognostic} value of 53BP1 for IGRT. In order to determine the value of 53BP1 as a \textit{predictive} factor, there would have to be a role for 53BP1 in determining outcome following radiotherapy, but not surgery (e.g. 53BP1’s role is treatment-specific). If it is true that, for example, 53BP1 loss is associated with poor response to IGRT then one may opt to use alternative therapies, such as radical prostatectomy or adjuvant chemotherapy, in novel clinical trials based on 53BP1 status \textit{a priori}.

\textbf{5.3 CONCLUSION}

In this thesis I have examined how 53BP1 phosphorylation occurs in response to endogenous replication stress, to hypoxic stress, and to ionizing radiation. I have examined the pathways that control phosphorylation of 53BP1 following IR and how upstream pathways combine with 53BP1 to control survival from ionizing radiation. These studies suggest that 53BP1 responses can no longer be considered as linear pathways but instead are subjected to crosstalk and influence multiple pathways in the DDR. I propose a number of extensions to this work using a novel cell line and currently available clinical data to further understand how 53BP1 contributes to the DDR \textit{in vivo} of xenograft and primary human tissue. It is hoped that these studies will form the basis for further studies of 53BP1 biology as it pertains to the DDR response on a mechanistic basis and how 53BP1 contributes to survival from IR and other cancer therapies on a functional level.
5.4 REFERENCES


APPENDIX 1
A DNA-DAMAGE INDUCED COMPLEX OF 53BP1, p53 and PHOSPHORYLATED ATM AT THE SITE OF DOUBLE STRAND BREAKS

This Appendix contains an excerpt from the published manuscript on which I was a joint co-first author. Reproduced with permission of the Radiation Research Society:

A1.1 ABSTRACT

Our lab had previously shown that phosphorylated p53 (p53^{Ser15}) localized to sites of IR-induced DSBs (Rashid et al., 2005). Subsequently, using exogenously expressed YFP-tagged p53 constructs in the p53-null cell line we found that YFP-p53^{WT} and YFP-p53^{Δ367-393} associated with ATM^{Ser1981} and 53BP1 in the nuclear, chromatin-bound fractions following DNA damage. However, YFP-p53^{Δ1-299} fusion proteins, which lack transcriptional trans-activation and the Ser15-residue bound to ATM^{Ser1981}, but not 53BP1. It was necessary to confirm these analyses using endogenously expressed proteins. Using sub-nuclear UV-microbeam and immunoprecipitation analyses of irradiated normal human fibroblasts (NHF) I confirmed a physical interaction between endogenous p53 and ATM or 53BP1. Based on these observations, we proposed a model whereby a pre-existing pool of p53 responds immediately to radiation-induced DNA damage using the C-terminus to spatially facilitate protein-protein interactions and the DDR at sites of DNA damage.

A1.2 RESULTS

A1.2.1 Co-localization and co-immunoprecipitation of endogenous 53BP1 and p53 with ATM kinase activity following DNA damage

Despite experimental data suggesting that ATM phosphorylates p53 and 53BP1 during the DDR, little evidence exists that these phosphorylations occur directly at sites of DNA damage. Using GM05757 contact-inhibited G0/G1 normal human fibroblasts (NHF), we determined whether p53, 53BP1 and ATM were all recruited to DSBs (induced with a sub-nuclear microbeam-induced) and whether these proteins could be pulled down as IR-induced complexes using biochemical immunoprecipitation. Unfortunately, all tested aliquots of ATM^{Ser1981} antibody were
not successful in giving clean ATM-specific nor reproducible cell staining after IR, and therefore 53BP1Ser25 was used as a surrogate target for ATM activation in staining studies. Figure A1-1A indicates that γ-H2AX was activated and 53BP1 was recruited within UV-microbeams within 30 minutes following lasing. Furthermore, both p53Total and p53Ser15 co-localized with 53BP1 and γ-H2AX based on 2D and 3D immunofluorescent images (Figure A1-1B). 53BP1 phosphorylation on Serine-25 was reported to be ATM dependent and indeed, this phosphorylation was inhibited using a small molecule inhibitor of ATM (KU55933; Figure A1-1C). We observed that p53Ser15 co-localized with 53BP1Ser25, indicating co-recruitment of phosphorylated p53-53BP1 complexes at DSBs in the presence of ATM kinase activity.

We next conducted immunoprecipitations for endogenous 53BP1-ATM Ser1981-p53 Ser15 complexes in irradiated whole-cell extracts of G0/G1 NHFs (Figure A1-1D). Western blots of equal input protein show induction of ATM Ser1981, p53 Ser15 and no change in 53BP1 levels. Immunoprecipitations confirmed when p53 Ser15 is induced by IR co-immunoprecipitation occurs with both ATM Ser1981 and 53BP1 concurrent with our UV-microbeam findings shown in Figure A1-1A. Taken together, these data suggest an endogenous complex can form between p53 Ser15-ATM Ser1981-53BP1 following induction of DSBs.
FIGURE A1-1: Interactions between endogenous p53, 53BP1 and ATM^{Ser1981} to DSBs in normal human fibroblasts. In A), UV microbeam-induced DSBs activates γH2AX and results in co-localization of 53BP1, p53Total and p53Ser15 within the lased track in G0/G1 contact-inhibited normal human fibroblasts (NHF s). Scale bar is 10µm; (B) 3D-renderings of co-localized regions observed in Figure A1-1A using Imaris software: (C) UV microbeams activate ATM activity within the lased tracks where ATM-dependent downstream phosphoforms, p53^{Ser15} and 53BP1^{Ser25} interact. When ATM kinase activity is inhibited following 1h pre-treatment with 10µM ATM inhibitor (Ku55933; ATMi), 53BP1^{Ser25} does not localize to γH2AX within the microbeam track. Scale bar is 10µm. Continued on next page.
FIGURE A1-1 Continued: Interactions between endogenous p53, 53BP1 and ATM<sup>Ser1981</sup> to DSBs in normal human fibroblasts. D) Co-immunoprecipitation of endogenous proteins from G0/G1-GM05757 NHFs before IR or 30 minutes and 2h following 10Gy. Input protein is indicated on the leftmost of the panel. Immunoprecipitation western blots are centre and right and the immunoprecipitating antibody is indicated above the respective gel lanes. Representative blot of 2-3 similar experiments are shown.
A1.3 MATERIALS AND METHODS

A1.3.1 Fluorescence Microscopy and UV-microbeam induced DSBs

For sub-nuclear DSB induction, cells were pre-incubated as sub-confluent GM05757 fibroblasts plated on 24mm ,1.5 coverslips in 10µM BrdU (Sigma-Aldrich) for 36h before transferring the coverslip to a Chemlde Chamber for lasing (Quorum Technologies). This chamber was placed into a Pathology Devices Live Cell stage-top incubator maintaining 37°C, 5% CO2 and 30% humidity affixed to an Olympus IX81 microscope operated with Metamorph Software (Olympus Canada). A 355nm PowerChip Nanolaser (Spectral Applied Research) was directed through the right side port, reflected with an 80/20 (reflectance/transmission) beamsplitter through the 100X 1.4NA UPLSAPO lens and output power was controlled using a Laser Merge Module (LMM5; Spectral Applied Research). Cells were monitored during lasing using low-level brightfield illumination and a Cascade 512B EM-CCD Camera (Roper Scientific). Focus was maintained manually as the stage (ASI, Applied Scientific Instruments) was moved at a constant velocity using µManager and ASI scripts. Laser power was measured using an Ophir Optronics Thermopile detector at the entrance to the side port and maintained at 4mW. Cells were fixed 30 minutes post-lase as previously described (26). Immunofluorescent images of 0.25µm widefield sections were captured using the above instrument and a 60XO 1.4NA UPLSAPO lens. All images were deconvolved using 25 iterations of 3D-blind deconvolution using Autoquant software (Imaris). Maximum intensity 3D-projections were created, standard intensity/contrast adjustments were made and images were cropped for publication using ImageJ software (NIH). 3D-renderings were created using Imaris (Bitplane).
A1.3.2 Immunoprecipitation and Western Blot Analyses

Protein for endogenous immunoprecipitation was collected from G0/G1 fibroblasts by scraping into NETN buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP40, 20U/mL Benzonase nuclease (EMD Chemicals), 1mM Benzamidine, 1µg/mL antipain, 5µg/mL Aprotinin, 1µg/mL Leupeptin, 1mM Phenylmethylsulfonyl fluoride, 2mM imidazole, 10mM NaF, 1.15mM Sodium molybdate, 4mM Sodium tartrate, 2mM Sodium pyrophosphate, 2mM β-glycerophosphate and 2mM Sodium orthovanadate), incubated with agitation at 4°C for 30 minutes and cleared by centrifugation. Protein was quantified using BCA reagent (Pierce) and 1mg was used per immunoprecipitation. Lysates were incubated with primary antibody (1µg of antibody per 1mg of protein lysate) overnight, and incubated with Protein G-beads for 1 hour, all kept at 4°C. Finally, beads were washed twice with Tris buffered saline+0.1% Tween 20 and prepared for electrophoresis and western blot analyses. Western detection was either using Licor or ECL (Pierce SuperSignal Pico).

Primary antibodies used in this study include: DO-1 (amino-terminus-specific p53; Calbiochem OP43), Ab421 (C-terminus-specific p53; Calbiochem OP03), Ab1801 (amino-terminus-specific p53; Novocastra NCL-P53-1801) from Novocastra; polyclonal and monoclonal phospho-specific p53Ser15 from Cell Signaling (9284 and 9286 respectively); monoclonal γ-H2AX from Millipore (05-636); monoclonal phospho-specific ATMSer1981 from Epitomics (2152); BP53-12 (pantropic p53; SC-263) from Santa Cruz; monoclonal β-tubulin from Sigma (T4026), monoclonal 53BP1 from Chemicon (MAB3802); polyclonal 53BP1 from Alexis (210-419-R050) and Cell signaling (2675); and rabbit and mouse IgG control antibodies from Jackson Immunoresearch Laboratories.
Secondary antibodies were 800CW or 680CW goat anti-mouse and anti-rabbit (LI-COR) or Trueblot HRP-conjugated (eBioscience).

A1.4 REFERENCES

APPENDIX 2
USE OF siRNA IS NOT FEASIBLE IN STUDIES OF CLONOGENIC SURVIVAL FOR 53BP1, ATM OR DNA-PKcs
A2.1 EXTENDED ABSTRACT

To study the contribution of 53BP1 to survival following IR in cancer cell lines with an “isogenic” system we used siRNAs targeting 53BP1. As shown in Figure A2-1B 53BP1 knockdown was very efficient using these methods, however no radiosensitivity was noted. This was of concern as multiple studies using MEF cell lines had shown 53BP1 loss was associated with radiosensitivity. I noted that the knockdown of 53BP1 across the population of siRNA treated cells was very heterogeneous when examined by immunofluorescence (see Chapter 5). To further this analysis I tested both the knockdown of ATM and DNA-PKcs using siRNA, both targets that should markedly radiosensitize the cells, and compared this to small molecule inhibition of these kinases in clonogenic survival. As shown in Figure A2-1B ATM and DNA-PKcs were efficiently knocked down however these gave little, if any radiosensitization. This was in contrast to small molecule inhibition of kinase function which markedly radiosensitized these cells. This suggested that the heterogeneity associated with transient siRNA knockdown in populations of cells rendered these unsuitable methods for examining radiosensitization by clonogenic survival. This is likely due to the small fraction of clonogenic cells that are transfected with sufficient siRNA to confer radiosensitivity and would make future analyses of epistatic interactions impossible using this system. To overcome these limitations one must develop a cell line with stable expression of a shRNA molecule in a clonal culture to eliminate these variables. We have begun the development of such a system as outlined in Chapter 5.
FIGURE A2-1: Use of siRNA is unsuitable for clonogenic survival assays with ATM, DNA-PKcs and 53BP1. A) Clonogenic survival of HCT116 cells treated with a non-targeting siRNA (CNTL) or siRNA directed towards 53BP1 shows no difference in survival to ionizing radiation (0-6Gy). Western blotting of cell lysates taken at the time of clonogenic seeding shows efficient 53BP1 knockdown. Standard error of the mean for 3 independent experiments is shown on the survival curves. Continued on next page.
FIGURE A2-1 Continued: Use of siRNA is unsuitable for clonogenic survival assays with ATM, DNA-PKcs and 53BP1. B) Clonogenic survival of h1299 cells treated with siRNA to ATM or DNA-PKcs as compared to treatment with small molecule ATM (KU55933) or DNA-PKcs (NU7441) inhibitors. Cognate westerns showing knockdown of ATM and DNA-PKcs or inhibitor of ATM and DNA-PKcs kinases as measured by phosphorylation of DNA-PKcs at Serine-2056 or CHK2 Threonine-68, respectively. No survival difference is noted with knockdown of either ATM or DNA-PKcs, however marked radiosensitization is noted with either small molecule inhibitor. Standard error of the mean for at least 2 independent experiments is shown on the survival curves.