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Synthetic lethality in NSCLC cells: Interrogating the therapeutic potential of ATM deficiency

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Synthetic lethality in NSCLC cells: Interrogating the therapeutic potential of ATM deficiency

by

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Abstract

Previous work by the Bebb and Lees-Miller labs demonstrated poly(ADP)ribose polymerase inhibitors (PARPi) induced synthetic lethality in Ataxia telangiectasia mutated (ATM) deficient cells. My work extended these studies to non-small cell lung cancer (NSCLC). In vitro, ATM deficient NSCLC cell lines were investigated by assessing DNA damage-induced phosphorylated target proteins and ionizing radiation (IR) sensitivity using the clonogenic assay. Two cell lines, H23 and H1395, with reduced ATM expression and impaired ATM dependent signalling were identified. H23 exhibited the expected increased sensitivity to radiation and PARPi. H1395 on the other hand displayed IR-induced ATM dependent phosphorylation and behaved like its ATM competent counterparts by being resistant to the investigative agents. These results suggest that while ATM deficiency may predict sensitivity to radiation and a novel combination of cisplatin and PARPi additional unidentified factors modulate this phenotype. These observations require validation in further studies.

Acknowledgements

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Finally, I want to express my utmost gratitude to my loving family; my husband and children for their supports and understanding; my parents and siblings for their incessant prayers and supports, I am really grateful to have you all around me.

To all cancer survivors

including my courageous dad

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
ALK	Anaplastic Lymphocytic Kinase
a-NHEJ	Alternate Non-Homologous End Joining
ATM	Ataxia telangiectasia mutated
A-T	Ataxia telangiectasia
ATR	ATM and Rad 3 related
BRCA1	Breast and Ovarian Cancer Susceptibility Protein 1
BRCA2	Breast and Ovarian Cancer Susceptibility Protein 2
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
c-NHEJ	Classical Non-Homologous End Joining
DDR	DNA damage response
DNA-PKcs	DNA dependent protein kinase catalytic subunit
DSBs	DNA double stand breaks
DBD	DNA binding domain
LIG III	DNA ligase III
LIG IV	DNA ligase IV
EGFR	Epidermal growth factor receptor
EXO1	Exonuclease 1
FAT	FRAP, ATM, and TRRAP domain
FATC	FAT-C terminal domain

HJ	Holliday junction
HR	Homologous recombination
HEAT	Huntingtin, Elongation factor 3, alpha subunit of PP2A and TOR1
IR	Ionizing radiation
KAP-1	KRAB-associated protein 1
LZ	Leucine zipper
MCL	Mantle cell lymphoma
MDC1	Mediator of damage checkpoint 1
MRN	MRE11, RAD50 and NBS1
NAD+	Nicotinamide adenine dinucleotide
NSCLC	Non-small cell lung cancer
NHEJ	Non-homologous end joining
NLS	Nuclear localization signal
PI3K	Phosphatidylinositol -3-kinase
PIKK	Phosphatidylinositol -3-kinase – like protein kinase
PRD	PIKK Regulatory Domain
PAR	Polymer of ADP-ribose
PARP	Poly (ADP) ribose polymerase
PARPi	PARP inhibitors
PNKP	Polynucleotide kinase/phosphatase
PUMA	p53 up-regulated modulator of apoptosis
53BP1	p53 binding protein 1

ROS	Reactive oxygen species
RPA	Replication protein A
RNF8	Ring finger protein 8
RNF168	Ring finger protein 168
SMC1	Structural maintenance of chromosome 1
TNM	Tumor Node Metastasis
TDP1	Tyrosyl-DNA phosphodiesterase
WRN	Werner syndrome helicase/exonuclease
XRCC1	X-ray cross-complementing gene 1
XRCC4	X-ray cross-complementing gene 4
XLF	XRCC4-like factor

Chapter One: Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide (WHO, 2012). Among Canadians, it is responsible for 26.7% of all deaths from cancer in both men and women (Statistics Canada, 2011). Lung cancer is classified as small cell lung cancer and non-small cell lung cancer (NSCLC), the latter accounting for about 85% of cases (Lu & Zhang, 2011). Based on the tumor node metastasis (TNM) staging system, NSCLC is categorised into stages 1-4, which have prognostic significance (Bergman et al., 2012).

The overall five-year survival rate for NSCLC has remained unchanged at less than15% due to several factors. Firstly, curative treatment with surgical resection is limited to early stage diseases (i.e. stage 1 & 2 NSCLC), which only represent 25-30% of NSCLC (Bergman et al., 2012). A high recurrence rate of about 35-55% following surgery further complicates the issue (Groome et al., 2007). Secondly, current treatment of the bulk of NSCLC (i.e. advanced NSCLC) uses cytotoxic, platin-based regimens, which are associated with low response rate and significant morbidity (Schiller et al., 2002). Since 80% of NSCLC patients are smokers (either past or current) the presence of co-morbidities precludes many from being candidates for cytotoxic treatment (Lu & Zhang, 2011). Lastly, although epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) inhibitors which specifically target NSCLC tumors that exhibit addiction to these pathways respectively, have shown promising results in selected patients, they are not curative and are associated with treatment failure that limit their use (Massarelli et al., 2013; Gerber & Minna, 2010). Clearly, the need for a novel therapy as well as predictive marker of treatment response in NSCLC cannot be overemphasized. One potential biomarker that has not been thoroughly investigated in NSCLC is Ataxia telangiectasia mutated (ATM).

The ATM is a nuclear protein that plays a central role in the cellular response to DNA double stand breaks (DSBs), termed the DNA damage response (DDR) (Canman & Lim, 1998). In mammalian cells, the DDR consists of co-ordinated multi-facet cellular processes some of which act as barriers to tumorigenesis, including DSB repair, cell cycle checkpoints and apoptosis (Jackson & Bartek, 2009; Bartkova et al., 2005; Halazonetis et al., 2008). Germ-line mutations affecting the ATM gene results in a cancer-predisposing human disorder A-T (Ataxia telangiectasia), (Gatti et al., 1988; Savitsky et al., 1995). Several aspects of DDR are impaired in A-T cells including abnormalities in DSB repair and cell cycle checkpoints after DNA damage, (Fig. 1.1); (Meyn, 1995; Shiloh, 1995).

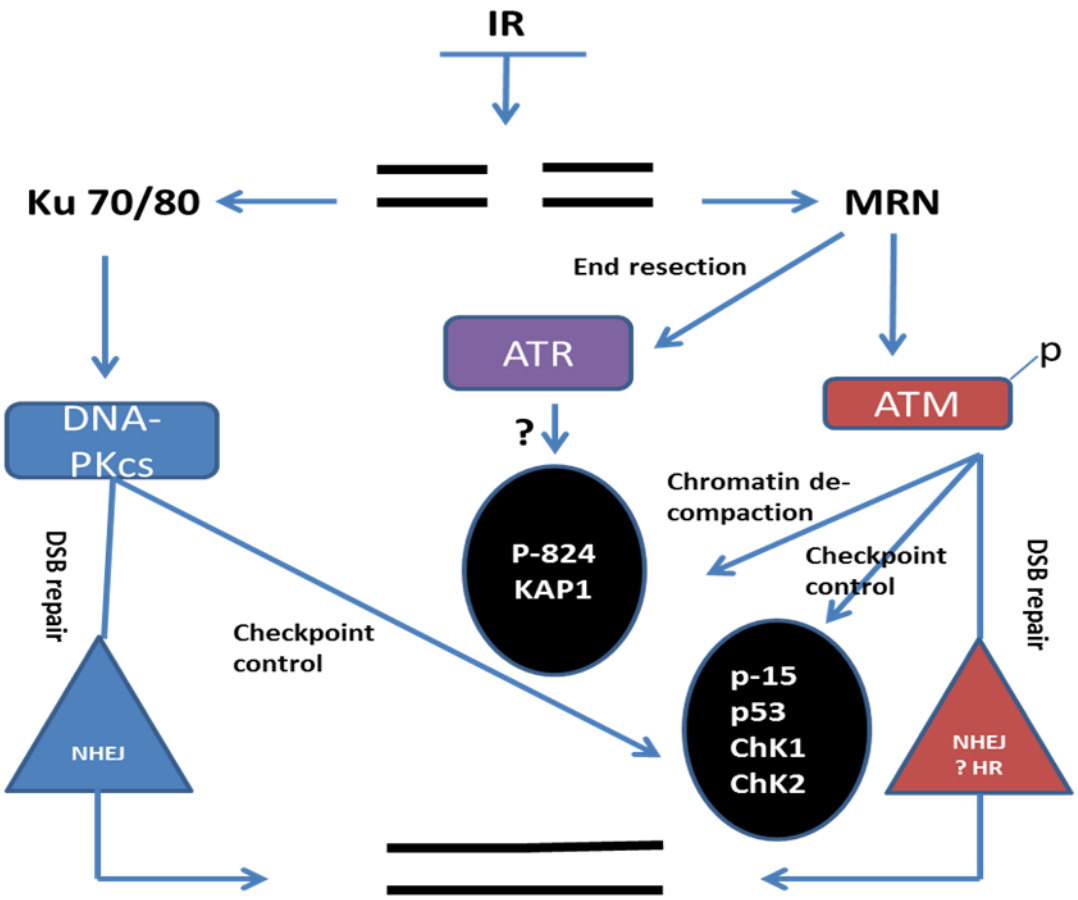


Figure 1.1: Major repair pathways for DNA double strand breaks (DSBs)
Ionizing radiation (IR) induced DSBs for instance, can be sensed by the protein complex MRN (MRE 11, RAD50 and NBS1) or Ku 70/80 heterodimer. MRN complex recruits inactive ATM to the damage site where it undergoes auto-phosphorylation (e.g. at serine 1981) to become activated. ATM dependent signaling regulates checkpoint controls, changes in chromatin architecture as well as DSB repair (NHEJ: non-homologous end joining and/or HR: homologous recombination) pathways via phosphorylation of several downstream targets including p53 and KAP1. Notably, DNA-PKcs (a primary regulator of NHEJ pathway) and ATR (mainly activated by DNA end resection and involved in replication-related DBSs but also implicated in IR induced DBSs) share some common downstream targets (e.g. p53, Chk1 and KAP1) with ATM. **(?)** indicates controversial areas of DNA damage response.

Genomic stresses that activate the DDR, including DNA damage, are common in malignant cells (Bartkova et al., 2005). Evidence for activation of the damage response is often shown by the presence of activated DDR markers such as phosphorylation of serine 1981 of ATM (p-S1981 ATM), serine 15 of p53 (p-S15 p53) and serine 139 of histone variant-H2AX (ɣ-H2AX) in cancerous lesions (Bartkova et al., 2005; Ismail et al., 2011). Due to the pressure posed on cancer cells by the DDR to arrest the cell cycle or drive towards apoptosis, it is not uncommon for these cells to dysregulate the DDR by selectively eliminating major DDR components for survival (Bartkova et al., 2005; Ismail et al., 2011). Inactivation of ATM due to gene mutations, epigenetic silencing and altered protein expression is found in many cancers including NSCLC (Rossi & Gaidano, 2012; Kang et al., 2008; Safar et al., 2005). Some of these ATM deficient tumors are associated with higher tumor grade and poorer survival outcomes (Kang et al., 2008).

Although, the inactivation of tumor suppressors such as ATM is a survival mechanism for cancer cells, often an opportunity is created for designing drugs that specifically target malignant cells with such inherent deficiency. An emerging approach uses the concept of synthetic lethality, in which case inactivation of either of 2 genes allows cell viability whereas the simultaneous inactivation of both genes results in cell death. Using this approach, poly(ADP-ribose) polymerase (PARP) inhibitors have been shown to selectively kill tumor cells deficient in homologous recombination (HR) DNA repair components (Bryant et al., 2005; Farmer et al., 2005; Weston et al., 2010; Williamson et al., 2010). A well-described example is tumors deficient in breast and ovarian cancer susceptibility protein 1 or 2 (BRCA1/2) (Bryant et al., 2005; Farmer et al., 2005).

In this study, the objective is to examine the role of ATM in the therapeutic response of NSCLC cells to targeted (PARP inhibitor) and genotoxic agents, (chemotherapeutic drugs and

radiation therapy). The potential clinical implications include the possibility of individualizing NSCLC treatment using ATM directed precision therapy.

1.1 Role of ATM dependent signaling

The ATM gene, found on the long arm of chromosome 11 (Gatti et al., 1998; Savitsky et al., 1995), encodes an approximately 350kDa protein, ATM (Gately et al., 1998; reviewed in McKinnon, 2012). ATM is ubiquitously expressed by different cell types and it belongs to the phosphatidylinositol 3-kinase (PI3K) – like protein kinases (PIKKs), (Savitsky et al., 1995). PIKKs are multi-branched family of large proteins that share a carboxyl-terminal PI3K signature which is conserved in all eukaryotes (Shiloh & Kastan, 2001; McKinnon, 2012). Members of the PIKK family such as ATM, ATM and Rad 3 related (ATR), and DNA dependent protein kinase catalytic subunit (DNA-PKcs), have overlapping protein targets and possess protein kinase activity directed at serine or threonine residues immediately followed by a glutamine (Kim et al., 1999). Although, ATM, ATR and DNA-PKcs have distinct biological roles, they all sense and trigger response to DNA damage and ultimately function to maintain the integrity of the genome (Falck et al., 2005; Shiloh & Kastan, 2001).

As a nuclear protein (Brown et al., 1997), ATM is activated by a specific form of DNA damage; DSBs, (Banin et al., 1998). DSBs may result from an exogenous genotoxic insult such as ionizing radiation (IR), radiomimetic chemicals as well as several chemotherapeutic drugs used in the treatment of cancers (Jackson & Bartek, 2009; Espinosa et al., 2003). Notably, IR induces DSBs both directly and indirectly (characteristically clustered oxidative damage) whereas many chemotherapeutic drugs including the platins and topoisomerase poisons, form DSBs indirectly e.g. cisplatin primarily induce DNA intra- and inter-strand crosslinks (ICLs) and then, ICL associated DSBs (Bishop et al., 1998). DSBs can also be induced during normal

cellular processes e.g. in the event of oxidative respiration that generate toxic reactive oxygen species (ROS); during germ cell's meiotic recombination and the immune system's antigen receptor gene rearrangement events (i.e. V (D)J and class switch recombination), (Valko et al., 2006; Richardson et al., 2004; Bassing & Alt, 2004; Shiloh & Ziv, 2012) . Of the different types of DNA damage the cells can incur, DSB poses the greatest threat. In mammalian cells, unrepaired or incorrectly repaired DSB is a frequent source of chromosomal rearrangements with carcinogenic potential (e.g. chromosomal translocation) and a trigger of cellular apoptosis, (Richardson & Jasin, 2000; Lips & Kaina, 2001; discussed in Khanna & Jackson, 2001). Elaborate mechanisms for resolving such stress from DNA damage have evolved, collectively referred to as the DDR. The DDR represents the genome maintenance machinery consisting of multiple integrated cellular events that detect DNA damage, signal its presence and promote its repair (Jackson & Bartek, 2009). Different DDR mechanisms are invoked depending on the type of DNA damage: ATM-dependent signaling is one of the well-studied responses to DSBs (Harper & Elledge, 2007).

1.1.1 Involvement of ATM in DSB repair

ATM is a large molecular weight protein with distinct domains (Shiloh & Kastan, 2001) (Figure 1.2). However, the only domain with a defined regulatory function is its kinase domain that is found on the carboxyl terminal region of ATM. As described in other PIKK e.g. ATR, the non-kinase portions of ATM is dominated by N-terminal HEAT (Huntingtin, Elongation factor 3, alpha subunit of PP2A and TOR1) repeat units; a unit of HEAT repeat consisting of paired interacting anti-parallel helices joined by a flexible intra-unit loop, (Perry & Kleckner, 2003). The N-terminus also serves as an important surface for interaction between ATM and other proteins e.g. p53 and the tyrosine kinase c-Abl, (Chen et al., 2003; Shafman et al., 1997). Upon

induction of DSBs, ATM is recruited to the site of DNA damage through a direct interaction with the NBS1 component of the MRN (MRE11, RAD50 & NBS1) protein complex (You et al., 2005). The MRN complex acts as an initial sensor of DSBs and is important for the activation of ATM as well as its retention at this site (Lee & Paull, 2005). Following its recruitment, ATM, which exists as an inactive dimer in unperturbed cells, undergoes auto-phosphorylation and dissociates to form the active monomeric protein, (Figure 1.1); (Bakkenist & Kastan, 2003; You et al., 2005).

Post-translation modifications of ATM such as phosphorylation (including auto-phosphorylation) and acetylation are essential for its kinase activity, (Figure 1.2). Although, the role of auto-phosphorylation in ATM activation is debatable, phosphorylated –S1981 (p-S1981) is often used as a marker of the active protein. Activated ATM has several targets which it phosphorylates at the vicinity of the DSB; its substrate phosphorylation being enhanced by a mediator protein MDC1 (mediator of damage checkpoint 1), as well as ATM - MRN complex interactions (Stucki et al., 2005; Lukas et al., 2004; Lee & Paull, 2005).

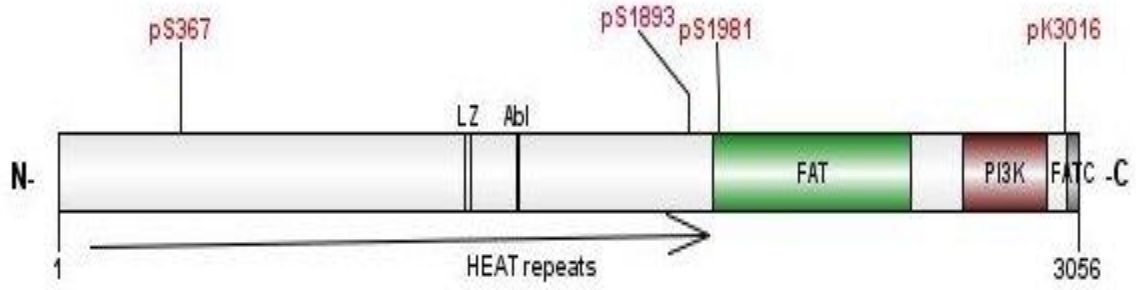


Figure 1.2: Schematic representation of basic domains and post-translational modifications of ATM protein
ATM is a 3056 amino acids long protein. The region containing signatures of its catalytic subunit of phosphatidylinositol 3-phosphate kinases (PI3K), FAT (a domain found in FRAP, ATM, and TRRAP proteins) and FATC (a carboxyl-terminal domain common to the PIK-related kinases) domains are shown in the C-terminal region (- C). In the N-terminal region (N-) are found the HEAT repeat units, (which occur in unique patch work and make up about 63% of the non-kinase portions of ATM, (Perry & Kleckner, 2003) and protein interaction motifs such as the leucine zipper (LZ) and Abl interacting domain (Abl). Also shown are the well characterised post-translational modifications such as phosphorylation (p) at serine (S) amino residues and acetylation (Ac) at lysine (K) residue that contribute to ATM kinase activation (Bakkenist & Kastan, 2003; Kozlov et al., 2011). Note that, ATM was detected in NSCLC cells used in this work with a rabbit monoclonal primary antibody (Epitomics) developed against residues surrounding the serine 1981 of ATM.

The first proteins to be phosphorylated by ATM in DDR include the histone variant H2AX, which is phosphorylated at S139 by ATM to form γ-H2AX (Rogakou et al., 1998). γ-H2AX interacts directly with MDC1, which in turn interacts with NBS1 to promote the retention of activated ATM around the DSB (Melander et al., 2008). Additionally, recruitment of MDC1 leads to the recruitment of two ubiquitin ligases, RNF8 (ring finger protein 8) and RNF168 which target H2A histone for ubiquitylation (Bekker-Jensen et al., 2010). Evidence suggests that, ubiquitylation events result in changes in the higher-order chromatin structure which exposes

specific binding sites for 53BP1 (p53 binding protein 1); another important mediator protein (Huyen et al., 2004). 53BP1 also attracts the MRN complex to the DSB site; further amplifying ATM-dependent DNA damage signaling (Lee et al., 2010). This orchestrated assembly of damage response proteins including MDC1, 53BP1 and p-S1981 ATM, which extends several kilobases from the break site is thought to concentrate repair factors at DSB to expedite the DNA repair process (Lobrich et al., 2010) (Figure 1.3). Notably, ATM is dispensable for initiating the assembly process of damage response proteins at DSB site since DNA-PKcs, a related protein, can redundantly phosphorylate histone H2AX; an early step in the process (Rogakou et al., 1998; Stiff et al., 2004).

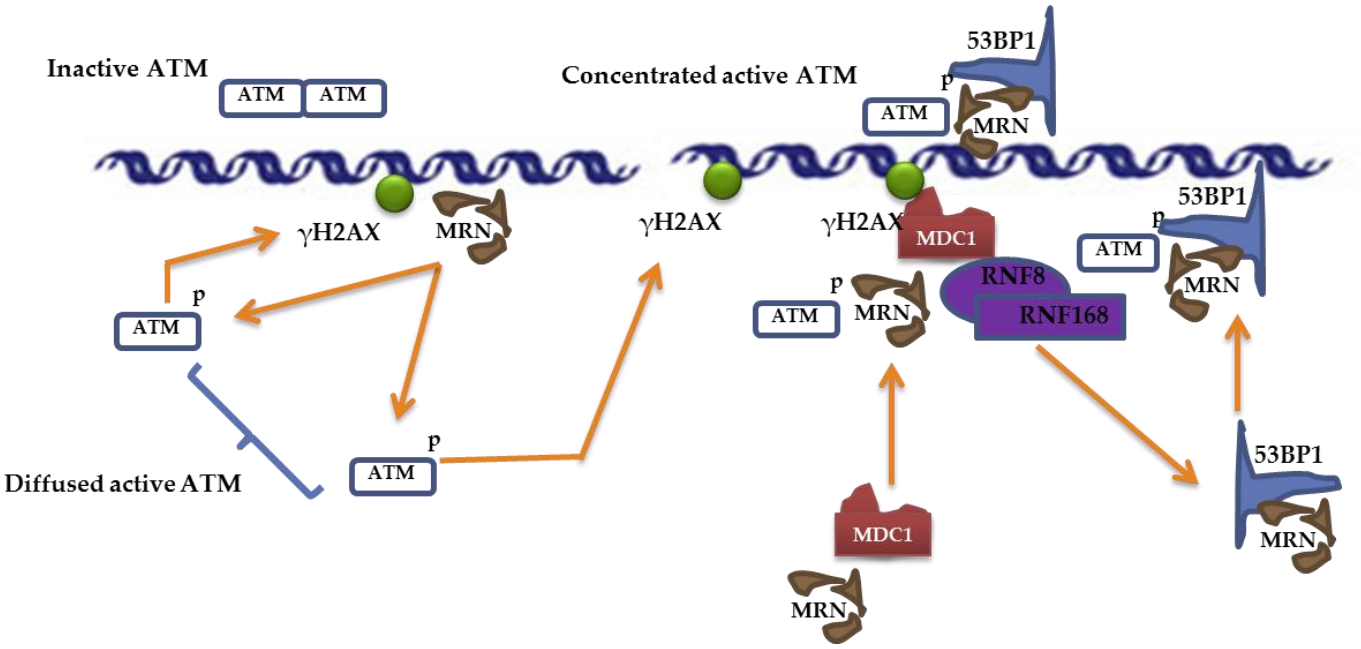


Figure 1.3: Orchestrated assembly of damage response proteins at the site of DSBs
Inactive dimeric ATM protein is recruited to DSB sites (by the MRN complex) where it undergoes auto-phosphorylation to form the active protein (pATM). Activated ATM phosphorylates H2AX histone variant around the vicinity of DSBs and the resulting γH2AX which is formed recruits MDC1 which in turn interacts with MRN complex to concentrate active ATM at DSB sites. In addition, MDC1 interacts with ubiquitin ligase RNF8 which partners with another ubiquitin ligase RNF168. The resulting poly-ubiquitylation of H2A histone by the ligases recruits 53BP1 to DSBs. 53BP1 also interacts with MRN complex to further concentrate ATM some kilobases around DSB sites.

In addition to its central role in DNA damage signaling, ATM is also implicated in major DSB repair pathways (Riballo et al., 2004), (Figure 1.4). Non-homologous end joining (NHEJ) is a predominant DSB repair pathway in mammalian cells that operates throughout the cell cycle; although, it is an error-prone DSB repair mechanism (reviewed in Mahaney et al., 2009; Wang & Lees-Miller, 2013). A-T cells have high levels of spontaneous unrepaired DSBs in the form of chromosome breaks, an indication of pre-replication DNA lesions. This observation suggests a NHEJ repair defect in ATM deficient cells (Zha et al., 2008). ATM deficiency also compromises V(D)J and class switch recombination, two events that utilise NHEJ for completion in immune cells (Rooney et al., 2004; Bredemeyer et al., 2006; Reina-San-Martin et al., 2004). Homologous recombination (HR) is another major DSB repair pathway which is most active in late S-phase and G2-phase of cell cycle when a homologous template is available for error free DNA repair (Moynahan & Jasin 2010; Yamamoto et al., 2012). However, the exact contribution of ATM in HR-mediated DNA repair is unclear. Although, several ATM substrates e.g. NBS1, CtIP, H2AX and BRCA1 may regulate HR (You et al., 2009; Xie et al., 2004; Shiloh, 2003); HR defects in the form of spontaneous chromatid breaks was observed only in cells treated with specific ATM inhibitors or in cells carrying homozygous kinase-dead ATM mutant whereas A-T cells show no such defect (Yamamoto et al., 2012; Daniel et al., 2012). Chromatid breaks generally represent post-replication DNA lesions (a time point when HR is most active) thereby suggestive of HR deficiency. In contrast, recent evidence suggests that ATM is dispensable for HR in mouse embryonic stem cells (Rass et al., 2013).

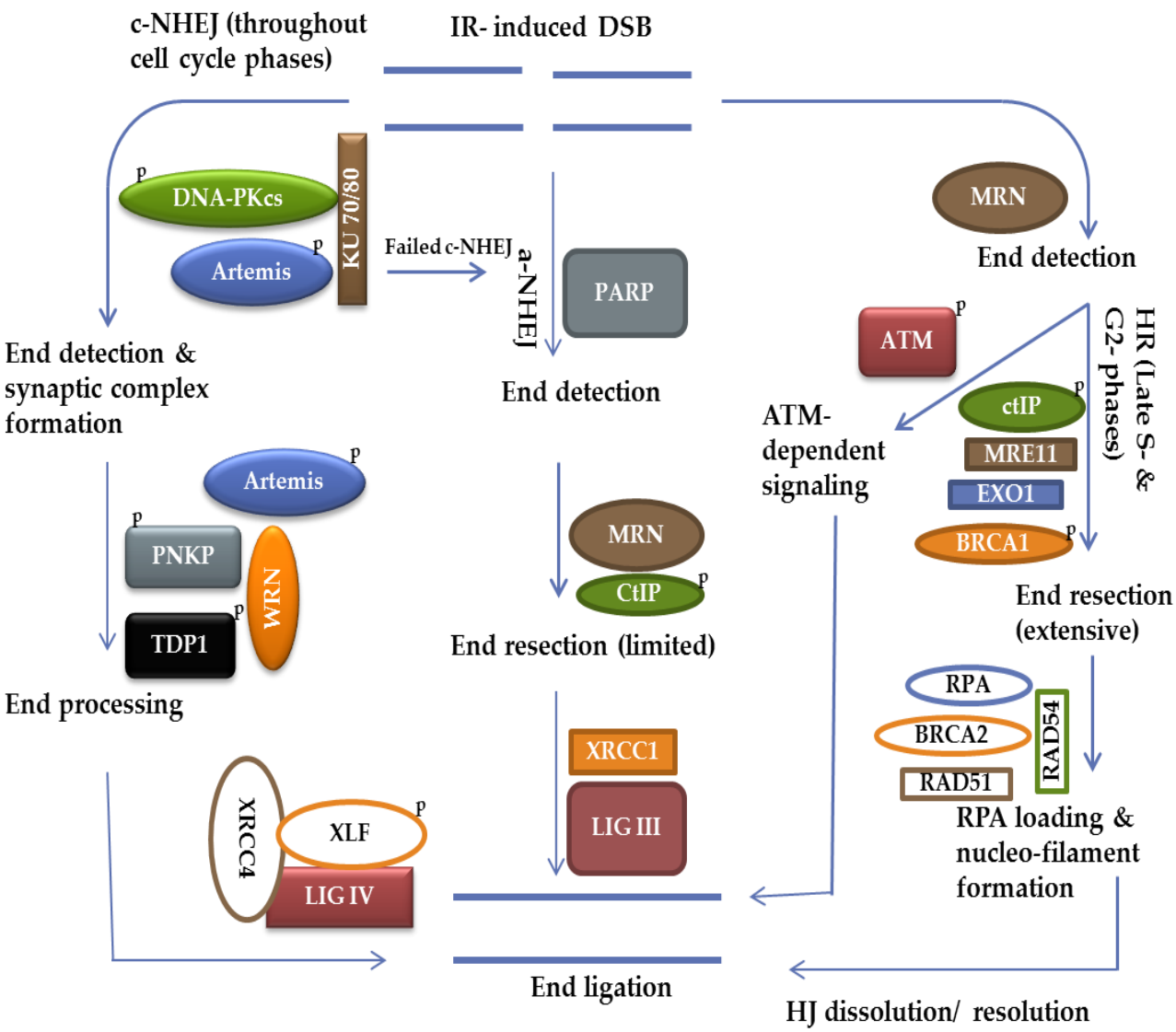

c-NHEJ (throughout cell cycle phases)
IR- induced DSB
DNA-PKcs
Artemis
KU 70/80
Failed c-NHEJ
a-NHEJ
PARP
End detection & synaptic complex formation
End detection
MRN
End detection
ATM
ctIP
MRE11
EXO1
BRCA1
HR (Late S- & G2- phases)
ATM-dependent signaling
Artemis
PNKP
WRN
TDP1
End processing
MRN
CtIP
End resection (limited)
End resection (extensive)
RPA
BRCA2
RAD51
RAD54
RPA loading & nucleo-filament formation
XRCC1
LIG III
XRCC4
XLF
LIG IV
End ligation
HJ dissolution/ resolution

Figure 1.4: Pathways for repairing DNA double strand breaks (DSBs)
DSBs can be repaired by 2 major pathways, NHEJ is triggered if DNA ends are detected by KU 70/80 heterodimer and is operational throughout the cell cycle phases. DNA-PKcs (in a complex with Artemis) is recruited by Ku and its auto-phosphorylation events regulate access of end processing proteins including PNKP (polynucleotide kinase/phosphatase), TDP1 (tyrosyl-DNA phosphodiesterase 1) and WRN (Werner syndrome helicase/exonuclease) at DNA break ends. DNA Ligation is then carried out by DNA ligase IV which exists in a complex with XRCC4 (x-ray cross-complementing gene 4) and XLF (XRCC4-like factor). Conversely, detection of DSBs by the MRN complex facilitates HR which is active only at late S- or early G2-phase. Limited DNA end resection, performed by MRE11 (of the MRN complex) with CtIP and then an extensive resection, done by EXO1 (exonuclease 1) with BLM helicase (not shown) are critical events in initiating the HR pathway. BRCA1 promotes DNA end resection and the resulting DNA overhang which is formed is rapidly coated by RPA (replication protein A). RAD51 can then replace RPA to form nucleo-filaments which invade homologous DNA segments resulting in D-loop formation (not shown), the process is facilitated by BRCA2 and /or RAD51 paralogs. Intermediate structures such as the holiday junctions (HJ) are formed which are either resolved or dissolved to complete the DNA repair process. In the absence of classical NHEJ (c-NHEJ), a less well-understood pathway, alternate NHEJ (a-NHEJ) is initiated when PARP binds the DNA breaks. In a-NHEJ, limited end resection by MRN and CtIP and ultimately DNA re-ligation by ligase III along with XRCC1 occur. (**p)** indicates ATM-dependent phosphorylation.

1.1.2 ATM involvement in other aspects of the DDR

Following DNA damage, cells respond by activating a wide range of physiological events. Because damage such as DSBs can induce cell death, the primary response is directed at repairing such damage as part of cellular survival mechanisms. In addition to activating the repair pathway, a temporary arrest of cell cycle progression also occurs via events that trigger cell cycle checkpoints (Shiloh & Kastan, 2001). This arrest of the cell cycle can occur before DNA replication (G1/S phase), during DNA replication (intra-S phase) or prior to cell division (G2/M phase) to provide time for DNA repair (Lukas et al., 2004). One of the readily observed consequences of ATM activation, following IR-induced DSB is activation of the tumor suppressor protein, p53 (Figures 1.1 and 1.5). ATM directly phosphorylates p53 at S15 leading to its stabilisation and accumulation within cells (Banin et al., 1998; Khanna *et al.*, 1998). The phosphorylated p53 (i.e. active p53) is a major player in the induction of G1/S checkpoint and to

some extent maintains the G2/M checkpoint (Agarwal et. al., 1995). Mediators of intra-S checkpoints include SMC1 (structural maintenance of chromosome 1), BRCA1, Chk2 (checkpoint kinase 2), RPA (replication protein A) and NBS1 proteins while Chk1 (checkpoint kinase 1), Chk2 and BRCA1 proteins are critical for the G2/M checkpoint. These mediators are activated in an ATM dependent manner when the damage is due to DSBs (Girard et al., 2002; Yazdi et al., 2002; Kurz & Lees-Miller, 2004; Deckbar et al., 2010; Shibata et al., 2010).

Although, the initial cellular response is aimed at survival, the cell can initiate a programmed cell death (apoptosis) in response to specific damage or when damage is incompatible with continuation of cellular life (Zhang et al., 2011). ATM mediated activation of p53 can induce the transcription of PUMA (p53 up-regulated modulator of apoptosis), (Figure 1.5) and BAX; proteins that promote apoptosis (Budhram-Mahadeo et al., 2006; Riley et al., 2008). Lastly, ATM activation following DSBs, probably impacts on chromatin architecture. KAP-1 (KRAB-associated protein 1), a co-repressor protein abundant at regions of compacted chromatin (heterochromatin), is directly phosphorylated by ATM at serine 824 (pKAP-1) after DSB induction (Ziv et al., 2006). Phosphorylation of KAP-1 promotes DSB repair at heterochromatic regions and enhances survival post-IR. Although, the underlying mechanism is not entirely clear, evidence suggests that pKAP-1 initiates important changes in heterochromatin required for the progression of DSB repair. At least, a transient relaxation of nucleosome compaction is observed following an ATM dependent KAP-1 phosphorylation (Goodarzi et al., 2010).

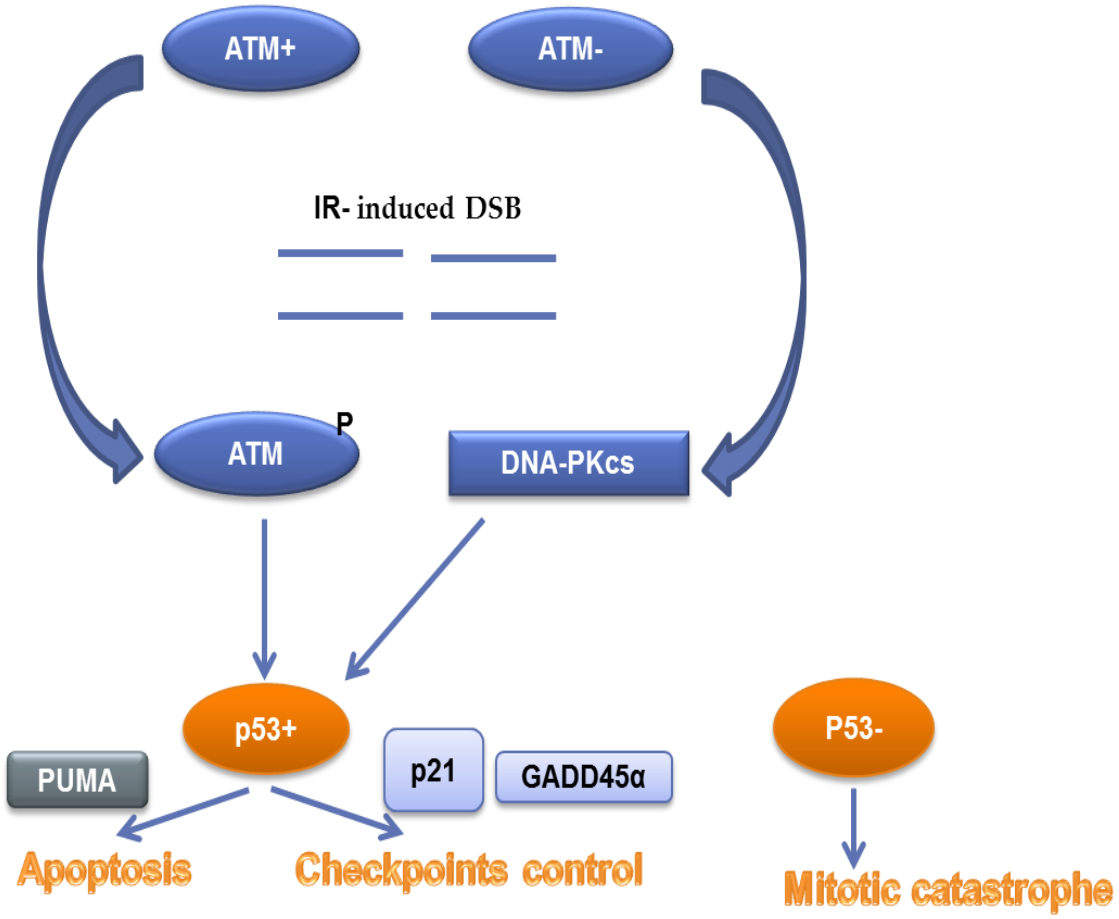


Figure 1.5: ATM-dependent p53 activation
Based on the cell type and extent of damage, IR-induced ATM-dependent p53 activation in ATM wild type cells can initiate arrest of cell cycle (checkpoint controls) or Apoptosis via p53-dependent control of target genes that influence cell cycle arrest (e.g. p21, GADD45α) or Apoptosis (e.g. PUMA). In the absence of ATM, p53 activation can redundantly occur through other PIKKs such DNA-PKcs. Failure of p53 activation e.g. in p53-mutant cells can result in death processes other than apoptosis e.g. mitotic catastrophe.

1.2 The poly(ADP-ribose) polymerases (PARP)

The PARP proteins are a large family of enzymes consisting of about 16 members including PARP-1 to 16; all possessing unique PARP signatures (Schreiber et al., 2006). The classical PARP proteins such as PARP-1, PARP-2, PARP-4 and PARP-5a & b (Tankyrases), typically catalyse the synthesis of a negatively charged polymer of ADP-ribose (PAR) from nicotinamide adenine dinucleotide (NAD+) and its addition on to protein targets (Mangerich & Burckle, 2011; Loseva et al., 2010; Kleine et al., 2008; Kickhoefer et al, 1999). The addition of PAR to proteins i.e. poly (ADP) ribosylation is a unique post-translation modification important in cellular functions such as DNA repair, transcription, mitotic spindle formation, telomere cohesion, intracellular trafficking and energy metabolism. Functionally, PARP-1, PARP-2 and more recently PARP3 are all implicated in DNA repair pathways (Schreiber et al., 2006; Rulten et al., 2011; Boehler et al., 2011; Fenton et al., 2013; Langelier et al., 2014).

PARP-1, the most extensively studied PARP protein, is highly conserved and its catalytic activity is increased several fold in response to DNA strand breaks (Schreiber et al., 2006). Most cellular PAR formation induced by genotoxic stresses e.g. IR, is attributable to PARP-1 activity (Tramontano et al., 2007; Mangerich & Burckle, 2011). In addition to PARylation of target proteins such as the DNA histones H1 & H2B, PARP-1 also forms PAR on itself (auto-modification). Upon induction of DNA strand breaks, PAR synthesis by PARP-1 can promote cellular survival from such damage by recruiting SSB repair (SSBR) factors e.g. XRCC1 (x-ray cross complementing 1) to SSB sites; influencing cellular DSB repair pathway choice (when the damage is a DSB) or alternatively facilitate cell death signals (Schreiber et al., 2006; Hochegger et al., 2006; Wang et al., 2006; Campalans et al., 2013).

PARP-2 in part, exhibits redundant functions with PARP-1 as evidenced by an overlapping phenotype between PARP-1 and PARP-2 knockout mice (Ménissier de Murcia et al, 2003). Similar to PARP-1, its catalytic activity is also stimulated by DNA strand breaks. Unlike PARP-1 however, PARP-2 is thought to play a minor role in SSB repair (Fisher et al., 2007). Nonetheless, PARP-2 seems to function independent of PARP-1 in gene transcription and maintenance of the genome (Yelamo et al., 2008). While PARP-1 or PARP-2 knockdown is tolerable, double knockout of both proteins in mice is embryonically lethal, suggesting that either of the proteins can compensate for each other's loss (Ménissier de Murcia et al, 2003).

1.2.1 PARP-1 in DSB repair and signaling

In addition to its catalytic domain, the PARP-1 protein possesses a DNA binding domain (DBD) containing two zinc fingers, one of which is essential for PARP-1 activation by DSBs (Ikejima et al., 1990; Bouchard et al., 2003). PAR is rapidly formed at the site of newly generated DSBs and is thought to facilitate proper dynamic assembly of DSB sensors such as MRE11 and other DSB repair proteins (Haince et al., 2008). An early DSB induced signaling cascade initiated by ATM can be regulated by PARP-1 activity. Haince et al., 2007 demonstrated physical interaction between ATM and PARP-1 via PAR molecules. PAR enhances ATM kinase activity (Goodarzi & Lees-Miller, 2004) however; the effect of PAR disruption (using small molecule inhibitor of PARP) on the activation of ATM dependent pathway is unclear. While there is a report that ATM dependent phosphorylation of downstream targets e.g. p53 is unaffected by PARP inhibition (Goodarzi & Lees-Miller, 2004) other evidence demonstrates reduced or delayed ATM substrate activation with PARP inhibitor after DSB induction (Haince et al., 2007). A recent study demonstrated the importance of PAR in MRN complex-mediated early activation of ATM via PAR interaction with NBS1 and its (NBS1) initial rapid relocation

to the DNA damage sites (Li et al., 2013). This finding suggests that PAR regulatory role on ATM is probably more of an early rather than a later event. Nevertheless, PARP deficient cells are selectively sensitive to ATM inhibitor (Bryant & Helleday, 2006). In fact, PARP-1/ ATM double knockout is embryonically lethal in mice (Menisser-de Murcia et al. 2001).

1.2.2 PARP inhibitors as anti-cancer treatment potentiation

Development of pharmacological inhibitors of PARP has made a major contribution to the initial recognition of PARP-1 as a DNA repair protein (Helleday 2011). In this regard, therapeutic effects of PARP inhibitors (PARPi) on cancer cells have only been associated with DNA damage and DNA repair mechanisms which implicate the DNA dependent PARPs as major mediators of response to these agents. Competitive PARPi are nicotinamide analogs that compete with NAD+ for PARP enzyme binding. PARP-1 is viewed as the main cellular target of PARPi because of its more prominent roles in DNA repair mechanisms than other DNA dependent PARPs (i.e. PARP-2 & 3); (Montoni et al., 2013). Because chemotherapeutic agents and radiation therapy, the mainstay of cancer treatment often induce cellular DNA damage that can activate PARP-1 to promote survival, PARPi presents a promising agent to increase the efficacy of these conventional cancer therapies. Moreover, the active role of PARP-1 in carcinogenesis provides additional rationale for using PARPi in cancer treatment (Zhang et al., 2005; Miwa & Masutani, 2007). For example, overexpression of PARP-1 which is found in different cancer types (Tomoda et al., 1991; Staibano et al., 2005; Lee et al., 2013, Michels et al. 2013b) is associated with resistance to cancer treatment (such as chemotherapy) including that of NSCLC (Lee et al., 2013; Michels et al. 2013b).

Several pre-clinical studies consistently demonstrate sensitisation effects of PARPi with DNA alkylating agents e.g. temozolamide (Masutani et al., 2000; Tentori et al., 2003; Donawho et al.,

2007) and topoisomerase 1 poisons e.g. camptothecin or its derivatives (Bowman et al., 2001; Miknyoczki et al., 2003). A similar effect is seen with combined PARPi and radiation treatment (Donawho et al., 2007; Dungey et al.; 2008). In other cases, there exist conflicting results of the potentiation effect of PARPi with DNA damaging chemotherapy such as the platin e.g. cisplatin (Michels et al., 2013a; Miknyoczki et al., 2003; Curtin et al., 2004). Moreover, growing evidence indicates synergistic cytotoxic effects of PARPi with targeted agents aimed at the EGFR such as cetuximab (a monoclonal EGFR neutralizing antibody) and lapatinib (EGFR small molecule inhibitor); (Nowsheen et al., 2011; Nowsheen et al., 2012).

1.2.3 Synthetic lethality with PARP inhibition

The application of synthetic lethality in cancer therapeutics offers the hope of targeting tumors with specific alterations that can be exploited using drugs but otherwise appear undruggabble (e.g. tumors with inactivating mutation of a tumor suppressor gene). Based on synthetic lethal approach, PARPi as a monotherapy for cancer treatment is used to target tumors with alterations in DDR proteins. This is best demonstrated with BRCA1/2 deficiency which confers impairments in HR DNA repair in the affected tumor (Farmer et al., 2005; Bryant et al. 2005). Treatment of BRCA 1 or 2 deficient tumors with PARPi results in cell death in vitro, inhibition of tumor growth in vivo (Farmer et al., 2005; Bryant et al. 2005; Rottenberg et al., 2008; Hay et al., 2009) and remarkable clinical responses in several early phase clinical trials (Fong et al., 2009; Tutt et al., 2010; Fong et al 2010; Audeh et al., 2010; Ledermann et al., 2012).

Though, a promising approach, the use of PARPi in cancer treatment is not without setbacks. Some tumors with known BRCA mutations respond poorly to PARPi treatment (Fong et al., 2009), hence a consistent predictive marker of response to PARPi treatment is yet to be identified; although evidence suggests pre-existing high levels of PAR in cancer cells as a strong

predictive marker; (Gottipatti et al., 2010). Most importantly, the mechanism of action by which the currently available PARPi mediate cytotoxicity in BRCA deficient tumors remained to be clearly defined (Helleday 2011).

Synthetic lethal interactions between PARP inhibitor and other DNA repair proteins as well as microRNAs have also been proposed (Murai et al., 2013, Neijenhuis et al., 2013). Protein examples include those involved in HR pathway such as MRE11, NBS1, RAD51and ATM (McCabe et al., 2006; Vilar et al., 2011; Patel et al., 2012); post-replication repair pathway e.g. RAD18 and the Fanconi anaemia proteins (Murai et al., 2013). Recent findings explaining PARPi mediated cytotoxicity in DNA repair deficient cells support several mechanisms (Murai et al., 2013). First, PARP inhibition disrupts SSB repair and induces formation of PARP-DNA complex both of which can impede DNA replication and result in stalling and collapse of replication fork. Second, collapsed forks can in turn generate one ended DSB which are classical HR substrates. PARP-DNA complex induced one ended DSB on the other hand, may require HR and/ or additional repair pathways as well as checkpoint control; the latter being more implicated in the sensitivity of ATM deficient cells to PARPi (Figure 1.6).

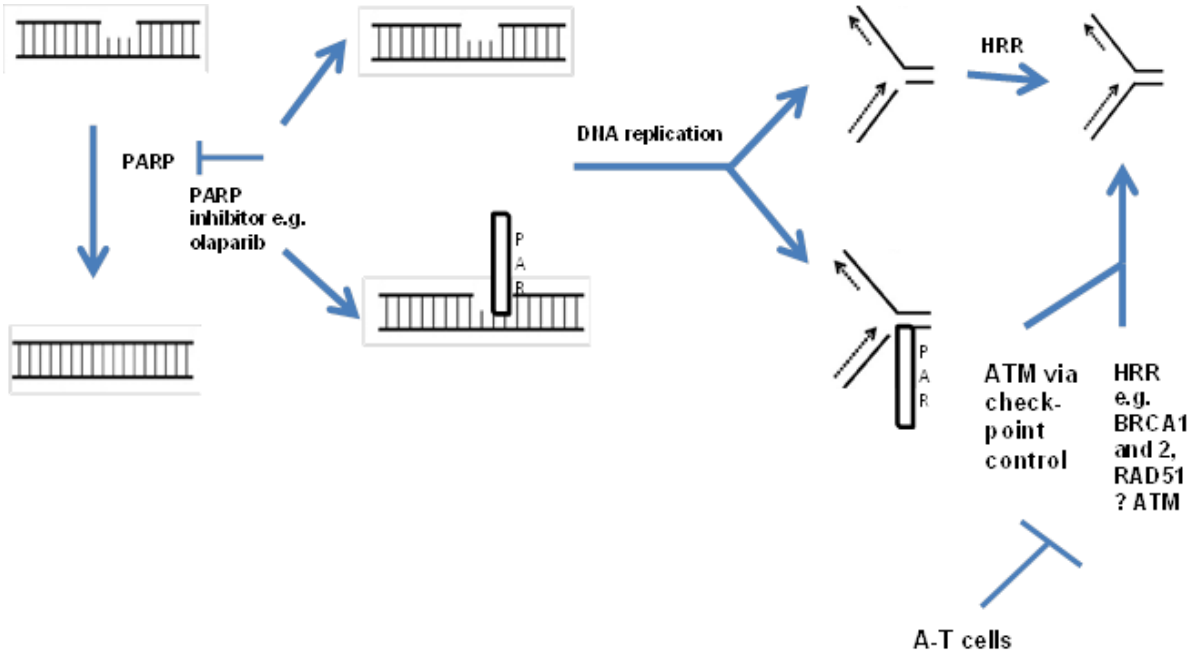


Figure 1.6: Model mechanisms of PARP inhibitor induced cell death in ATM deficient cells PARP inhibition impairs SSB repair pathway and /or results in PARP trapping within SSB intermediates. DNA replication fork encountering such damage stalls and collapses to form more complex damage e.g. one ended DSBs that may require HRR and /or additional repair pathways as well as activate cell cycle checkpoints for cellular survival. ATM deficiency as a result of mutations either reduces cellular DNA repair capability and /or impairs the checkpoint control therefore resulting in cell death.

1.3 ATM and its role in cancer

The understanding of ATM's cellular functions has grown from an extensive research on A-T, a human disorder resulting from ATM mutations. Over 80% of the mutations are truncating forms which result in an unstable protein that is undetectable in A-T cells (Lavin et al., 2004). Some major hallmarks of A-T include predisposition to childhood hematological malignancies e.g. lymphomas and leukemia and exquisite sensitivity to radiation treatment. The phenotype of A-T cells is characterised by defects in several aspects of DDR and significant level of genomic instability in the form of chromosome breaks and abnormal chromosome structures which can foster malignant transformation of cells (Gatti et al., 1988).

About 10% of A-T patients succumb to cancer related death. Generally, lymphoid malignancies both of B and T cell origins as well as spectrum of leukemias affect A-T patients more than other cancer types especially at their young age. Epithelial tumors (e.g. breast cancer), brain and other cancer types are also seen especially in older patients (Reiman et al., 2011; Shiloh & Kastan, 2001). ATM inactivation via mechanisms like mutation, epigenetic silencing of the gene or altered protein expression has also been documented in sporadic cancers, both of hematological origin (e.g. lymphomas) and solid tumors (e.g. NSCLC), (Gronbaek et al., 2002; Rossi & Gaidano, 2012; Safar et al., 2005; Buzhanov et al., 2010; Mazumder Indra et al., 2011; Kang et al., 2008; Biankin et al., 2012). Several of the ATM deficient tumors are associated with higher tumor grade and poorer clinical outcomes including NSCLC (Klimowicz et al., unpublished data; Tommiska et al., 2008; Mazumder Indra et al., 2011; Kang et al., 2008).

The impact of ATM deficiency on cancer management is twofold according to whether the deficiency is germ line based or limited to the malignant clone. On the one hand, a relationship between functional ATM polymorphism and development of severe toxicities to radiation therapy for cancers including NSCLC has been described in individuals who are relatively healthy prior to developing cancers (Zhang et al., 2010; Yang et al., 2011; Xiong et al., 2012 & Raabe et al., 2012). On the other, lymphoid malignancies e.g. mantle cell lymphoma (MCL) are radio-sensitive tumors and their radio-sensitivity are partly ascribed to the high rate of inactivating somatic ATM mutations commonly found in this type of tumors (Filippi et al., 2006). This raises the spectre of ATM directed radiation treatment for cancers. Conversely, use of a small molecule inhibitor of ATM sensitised several cancer cells to IR in vitro and in vivo (Collis et al., 2003; Rainey et al., 2008; Kuroda et al., 2012, Batey et al., 2013)

Perhaps, a more promising treatment strategy extends the PARPi synthetic lethality approach to ATM deficient tumors. Pharmacological inhibition of PARP-1 is differentially cytotoxic in vitro and in vivo in ATM deficient mantle cell lymphoma (MCL) cells, (Weston et al., 2010; Williamson et al., 2010). Williamson et al. (2012) further demonstrated an enhanced PARPi cytotoxicity in ATM and p53 double knockdown MCL cells. Unlike in hematopoietic tumors, the exploitation of ATM deficient solid tumors (including NSCLC) with PARPi has not been thoroughly investigated.

1.3.1 ATM deficiency in NSCLC

The more commonly diagnosed type of lung cancer is NSCLC, the aetiology of which has not been fully explained by tobacco smoking. Paradoxically, NSCLC in non- smokers has a greater proportion of DNA copy number alterations than found in smokers, however, the means by which these alterations occur is yet to be understood (Thu et al., 2012). Recently, changes in DNA repair genes including *ATM* have been implicated in lung carcinogenesis (Lo et al., 2010; Shen et al., 2012). ATM ranked as the 7th most mutated gene in NSCLC (Ding et al., 2008) and reduced level of the protein (ATM) found in about 20% of resected NSCLC samples is associated with poor overall survival (Klimowicz et al., unpublished data). Conversely, resistance to chemotherapeutic drugs attributable to evasion of apoptosis has been reported in NSCLC cells characterised by ATM loss (Riabinska et al., 2013; 2013; Lundholm et al., 2013).

Interestingly, studies have shown that ATM deficient NSCLC cells become susceptible to genotoxic agents when repair proteins such as DNA-PKcs are inactivated using specific small molecule inhibitors (Jiang et al., 2009; Riabinska et al., 2013). This observation suggests an over-reliance on other repair proteins for survival in the event of ATM loss. Although, it is not yet described in association with ATM, the observation of an overly expressed PARP in NSCLC

(Lee et al., 2013; Michels et al. 2013b), warrants the exploitation of PARP inhibition in NSCLC cells.

Given that NSCLC is one of the most genetically unstable malignancies, (Pikor et al., 2013), and report of frequent ATM mutations in NSCLC, (Ding et al., 2008), one could predict the occurrence of ATM deficiency in a significant proportion of NSCLC and that those cases may be candidates for exploitation of synthetic lethality using pharmacological PARP inhibition. This project therefore, aims to examine in vitro, the impact that ATM disruption has on NSCLC cells including their response to PARP inhibition.

1.4 Hypotheses

Based upon the observation that ATM protein expression is lost in about 20% of resected NSCLC specimens (Klimowicz et al., unpublished data), we hypothesise that some NSCLC cell lines will be ATM deficient. Given that, A-T cells are extremely radiosensitive and the consistent reports of increased radiation sensitivity in ATM inactivated cells (Collis et al., 2003; Rainey et al., 2008; Kuroda et al., 2012, Batey et al., 2013), we predict that ATM deficiency in NSCLC cells will confer an A-T phenotype of increased radiation sensitivity. Lastly, based on previous work performed in our lab revealing PARP inhibition lethality in ATM deficient MCL (Williamson et al., 2010; 2012) and gastric cancer cells (Kubota et al 2014), we hypothesize that through a synthetic lethality mechanism; PARP inhibition will be cytotoxic to ATM deficient NSCLC cells and will sensitize ATM deficient NSCLC cells to DNA damaging chemotherapeutic agents.

1.4.1 Specific aims:

1. Using western bloting, a panel of NSCLC cell lines will be screened for ATM protein loss and assessed for consequences of ATM loss on signaling functions such as IR-inducible ATM dependent phosphorylation of S1981 ATM, S15 p53 and S824 KAP1.
2. With the clonogenic assay, sensitivity to IR will be measured to assess the consequences of ATM protein loss on the phenotype of NSCLC cells.
3. To examine the role of ATM in PARP inhibitor induced lethality and sensitivity to cytotoxic agents using the clonogenic assay as routinely used in the Bebb & Lees Miller laboratories.

If ATM deficiency in NSCLC cells confers increased radiation sensitivity and PARP-induced synthetic lethality, clinical trials will have to be designed to inform us on how the findings are clinically applicable.

Chapter Two: Materials and Methods

2.1 Human cell lines and culture

NSCLC cell lines (NCI-H23, NCI-H226, NCI-H522, NCI-H460, NCI-H1395, NCI-H1793 and HCC 4006) derived from male and female NSCLC patients (see Table A in the Appendices) were obtained from American Type Culture Collection (ATCC). NCI-H1793 was cultured in RPMI-1640 medium (Gibco) containing 5% (v/v) fetal bovine serum (FBS), (Gibco) and ITS (0.005 mg/ml Insulin, 0.01 mg/ml Transferrin & 30 nM Sodium selenite-final concentration) supplements, while the remaining cell lines were cultured in antibiotic-free RPMI-1640 medium supplemented with 10% (v/v) FBS. The lymphoblastic cell line referred to as BT is derived from normal (non-A-T) control patient and was confirmed to be ATM proficient, L3 is a lymphoblastic cell line from an A-T patient and is well characterized as an ATM deficient cell line. Both cell lines i.e. BT and L3 (originally from Drs. Martin Lavin (Queensland Institute for Medical research, Australia) and Yossi Shiloh (Tel Aviv University, Israel) were obtained from Dr. Lees-Miller's lab and cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS. Cell lines were cultured with antibiotic-free media under strict aseptic conditions and maintained at 5% CO_2 and 37°C.

2.2 Drugs and small molecule inhibitors

DNA alkylating drug, cisplatin (3.3 mM), (Hospira Montreal QC) already reconstituted in sterile water was obtained from the pharmacy in the Tom Baker Cancer Centre and stored at room temperature away from light. Cisplatin was further diluted to concentrations 0.01, 0.1, 0.5 and 1 µM using RPMI-1640 medium just prior to cell treatment which lasted for 24 hours. Small molecule inhibitors of ATM (KU55933-Kudos Pharmaceuticals, Cambridge, UK) and PARP (AZD2281-AstraZeneca, London, UK) dissolved in dimethyl sulfoxide (DMSO) as 50 mM stock

and DNA-PKcs inhibitor (NU7441- Kudos Pharmaceuticals, Cambridge, UK) dissolved in DMSO as 10 mM stock were obtained from Lees-Miller's lab and stored at -80°C. ATM and DNA-PKcs inhibitors were diluted in DMSO to 5 and 10 μM respectively prior to cell treatment and used to treat cells for 1 hour. PARPi was also diluted in DMSO to either a dose range of 0.1, 0.3, 1 and 3 μM (when used alone) for continuous cell treatment or a single dose of 0.3 μM (when combined with Cisplatin) for 24 hours treatment.

2.3 Ionizing radiation

Cell lines in culture media inside 6cm³ dishes were irradiated using a Gamma cell 1000 Cesium-137 (^{137}Cs) source, (MDS Nordion, Ottawa, Canada). The Gamma cell 1000 irradiator is a self-contained radiation source with a built-in double-walled source capsule (stainless steel inner and outer capsules) containing radioactive ^{137}Cs, (Appendix figure B). It has a sample chamber with magnetic based turnable which securely holds a metal canister containing cells in culture dishes. With the aid of a touch screen control, radiation can be delivered onto cells, the radiation dose depending on the pre-set time of exposure. For instance, by setting the exposure time at 11, 21 and 42 seconds, it delivers about 0.5, 1, and 2 Gy respectively onto cells; corresponding to a dose rate of 2.9 Gy/min.

2.4 Whole cell extraction

Logarithmically growing cells were harvested and washed twice in cold Phosphate Buffer Saline (PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM $NaHPO_4$, 1.8 mM $KHPO_4$) at pH 7.5. The cells were centrifuged at 1363 x g for 5 minutes at 4°C and cell pellets were suspended in NET-N lysis buffer (150 mM NaCl, 0.2 mM EDTA, 50 mM Tris-HCl, at pH 7.5) containing 1% NP-40 (v/v), protease inhibitors (0.2 mM PMSF, 0.1 μg/mL pepstatin, 0.1 μg/mL aprotinin and 0.1 μg/mL leupeptin) and a phosphatase inhibitor (0.1 μM microcystin-LR). Cells in lysis buffer

were then incubated on ice for 10 minutes and sonicated twice at 2 second pulses and 10% amplitude (Fisher Scientific, model 500). Cells were centrifuged again at 9279 x g for 10 minutes at 4°C and whole cell extracts were generated from the supernatants. Protein concentrations were determined using the detergent compatible Lowry assay (BioRad) with Bovine Serum Albumin (BSA) as standard.

2.5 Western blotting

Fifty micrograms (50µg) of protein extracts mixed with 4X Laemeli sample buffer (glycerol, 1 M Tris pH 6.8, β-Mecaptoethanol, Sodium Dodecyl Sulfate-SDS, Bromophenol Blue) were resolved on either 8% or 10% acrylamide gel depending on the target protein size, using SDS running buffer (50 mM Tris, 384 mM glycine, 0.1% SDS, pH 8.3). Proteins were then transferred to nitrocellulose in electroblot buffer (48 mM Tris-HCl, 39 mM glycine, 20% (v/v) methanol) at 100 V for 1 hour. For large size target proteins (high molecular weight proteins), 0.035% SDS was added to electroblot buffer for the protein transfer. Immunoblots were blocked in 25% skim milk in Tris-Buffered Saline with Tween-20 (TTBS: 20 mM Tris-HCl pH 7.6, 500 mM NaCl, 0.1 % (v/v) Tween-20) for 0.5 hour and the protein of interest probed for with target specific primary antibodies at dilutions as indicated (Table C in the Appendix).

2.6 Clonogenic survival assay

Cells were washed in 1X PBS, trypsinized and re-suspended in 5ml of fresh media plus serum. The cells were then seeded in 60 mm^3 sterile culture dishes in triplicate at varying cell densities, between 1 X 10^2 to 3 X 10^3 cells/ml (depending on the cell line) and were allowed to adhere to the culture dishes overnight at 37ºC, 5% CO_2. Cells were either left untreated or treated with IR, PARP inhibitor or cisplatin at a dose range as indicated for each of the treatment agent. Cell treatment with chemotherapeutic drugs lasted for 24 - 48 hours, after which the drug

containing media were aspirated. Cells were then washed twice in 1X PBS and fresh media plus serum were added to the cells. Eight days after cell incubation, each of the control dishes (untreated cells) was examined under the microscope for visible colonies (a colony is ≥ 50 cells cluster). At 10-14 days, media were aspirated from cells and the surviving colonies were fixed with 1ml/dish clonogenic fixing solution containing 89% (v/v) of water, 3% (v/v) acetic acid and 8% (v/v) methanol for 2 minutes. The fixed cells were stained with 1ml/dish crystal violet staining solution (0.5% (w/v) crystal violet powder added to10% (v/v) formalin in Phosphate buffer saline: PBS) for 5 minutes and washed 3 times with distilled water. The numbers of purple colonies per dish were counted with the Colcount system (Oxford Optronix Ltd, Oxford, UK). Plating efficiency (PE) for each cell line was determined by dividing the number of colonies observed with the total number of cells seeded. Surviving fractions (SF) were calculated as follows: SF = (number of cells observed/number of cells seeded) X (PE/ 100), and normalized to control untreated cells. Cell survival data were plotted as logarithm of the surviving fractions (on the y-axis) versus dose (on the x-axis). Error bars indicate SEM of 3 independent experiments.

2.7 Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-PCR) analysis

Logarithmically growing cells in media plus serum were either exposed to 2Gy radiation or left untreated. After 1, 2 and 3 hours, cells were harvested for RNA extraction using a RNeasy Mini Kit, (QIAGEN) and the RNA extracted were quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltman, MA, USA) according to manufacturer's protocols. To generate copy DNA (cDNA) for RT-PCR, 1µg of total RNA from each sample was reverse-transcribed using a qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA) for first-strand synthesis as specified by the manufacturer. One microliter (1 µl) of cDNA product was used as the template in a 20 µl PCR reaction containing 10 µl of PerfeCTa SYBR

Green FastMix (Quanta Biosciences), 0.5 µl of each gene-specific primer (250 nM final concentration; sequences in Table D), and 8 µl of nuclease-free water according to the manufacturer's protocol. Real-time PCR was performed using an ABI 7500 Fast System (Applied Biosystems, Life Technologies, New York USA) using the following amplification parameters: 95°C for 2 min, followed by 40 cycles of 95°C/10s, 60°C/30s. Baseline and threshold cycle number (Ct) were determined automatically. Water was used as a no-template control for the PCR reaction. β-glucoronidase (GUSB) was selected as the most stable reference gene for the cell lines, and relative expression of p21, GADD45α (GADD45 alpha), and PUMA (p53 up-regulated modulator of apoptosis) in untreated and IR-treated conditions was calculated using the comparative (ΔΔCt) method with the untreated cells as the reference samples.

2.8 Statistical analysis

Statistical analyses were performed using Microsoft Excel. Data were derived from the average of 3 experimental replicates. Each data point in the clonogenic survival assay was an average of 3 technical replicates. Statistically significant differences in clonogenic survival between cells were determined using two-tailed Student's t-test and p-values less than 0.05 were considered significant.

Chapter Three: Results

3.1 Pre-existing loss of ATM protein expression and function in NSCLC cell lines

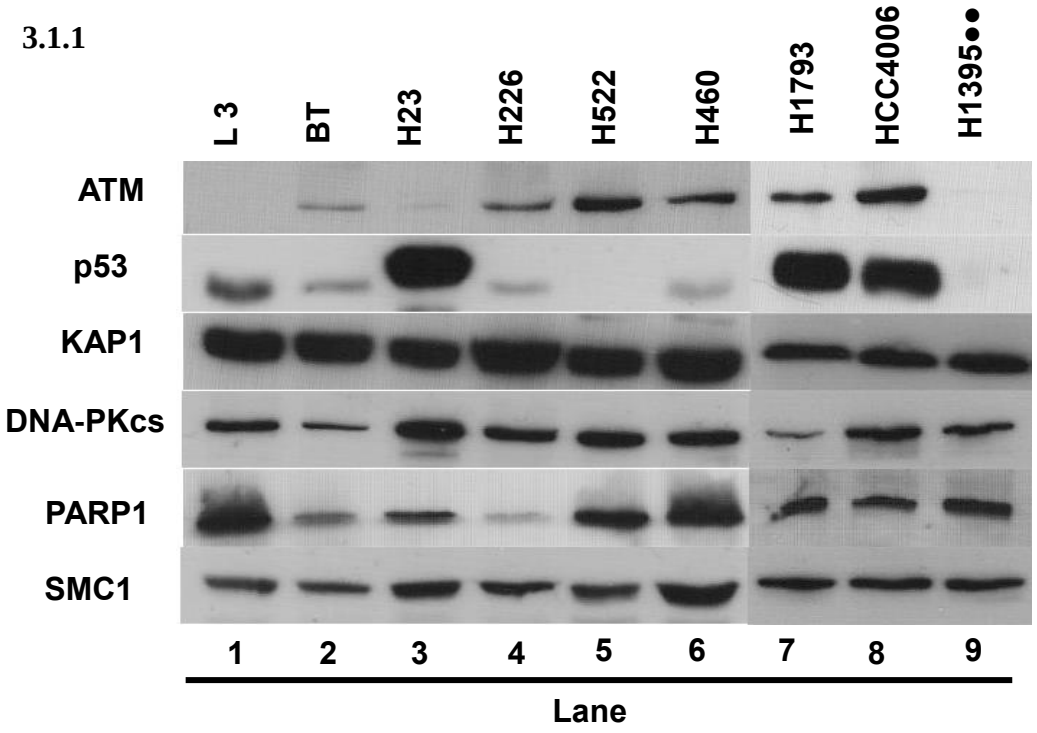
The primary focus of this project is on ATM in NSCLC. As such, I initially characterised a panel of NSCLC cell lines for ATM status using lymphoblastoid cells derived from control (BT cells) and A-T patients (L3 cells) as positive and negative controls respectively. Seven cell lines (H23, H226, H522, H460, HCC4006, H1793 and H1395) were examined. ATM mutations were previously reported in H23 (missense mutation resulting in glutamine substitution for proline at position 1919) and H1395 (missense mutation resulting in threonine substitution for alanine at position 2666), (CCLE, http://www.broadinstitute.org/ccle). In addition, p53 mutations have been described in H23 (TP53 missense mutation), H522 (TP53 frame-shift deletion), and H1793 (TP53 missense mutations), (O'Connor et al., 1997; http://www.atcc.org/~/media/PDFs/Culture%20Guides/Cell_Lines_by_Gene_Mutation.ashx), (see Table A in Appendix A).

Here, I found reduced ATM levels in H23 and H1395 cells by western blot (Figures 3.1.1 and 3.1.2 A, Appendix figure E1). In the case of H1395, ATM appeared undetectable with short exposures (30 seconds to 1 minute) (Figures 3.1.1, 3.1.2 A and 3.3 A; Appendix figures E1 and E3 A) whereas slight ATM bands were visible with long exposures (3 - 5 min.), (Figures 3.2 A and E3 B, C in the Appendix). The amount of ATM in H23 cells was 11% relative to BT (positive control cells), with quantification using the ImageJ (Figure 3.1.2 A). Analysis of the difference between ATM levels in BT cells and either H23 or H1395 demonstrated statistical significance with Student's t-test (Figure 3.1.2 A).

By examining the levels of other DNA damage responsive proteins such as PARP-1, DNA-PKcs, KAP1 and p53 in the cell lines, I found undetectable p53 protein in H1395 and H522.

While this observation may be consistent with the p53 gene deletion status of H522 (www.atcc.org), it is an intriguing finding in H1395 whose p53 gene status was previously classified as non-mutant (Catalogue of somatic mutations in cancers (COSMIC) database, http://cancer.sanger.ac.uk/cosmic/sample/overview?id=684681). In contrast, p53 levels in H23 and H1793 were high in comparison to normal lymphoblastoid cell, BT (Figures 3.1.1 and 3.1.2 B). High p53 level was also seen in HCC4006 cells, although, to my knowledge, there was no report of p53 mutation in this cell line (HCC4006). Surprisingly, a high level of PARP-1 was detected in the ATM negative control cell line, L3, whereas NSCLC cells with reduced or undetectable ATM have about the same levels of PARP-1 when compared with BT cells (Figures 3.1.1 and 3.1.2 C). High levels of PARP-1 were also detected in H55 and H460 cells. Relative to BT cells, DNA-PKcs level seems greater in most of the NSCLC cell lines (Figures 3.1.2 D, E, and E2 E). Variation in KAP1 levels among the NSCLC cell lines appeared negligible (Figure 3.1.1; see Appendix figure E1).

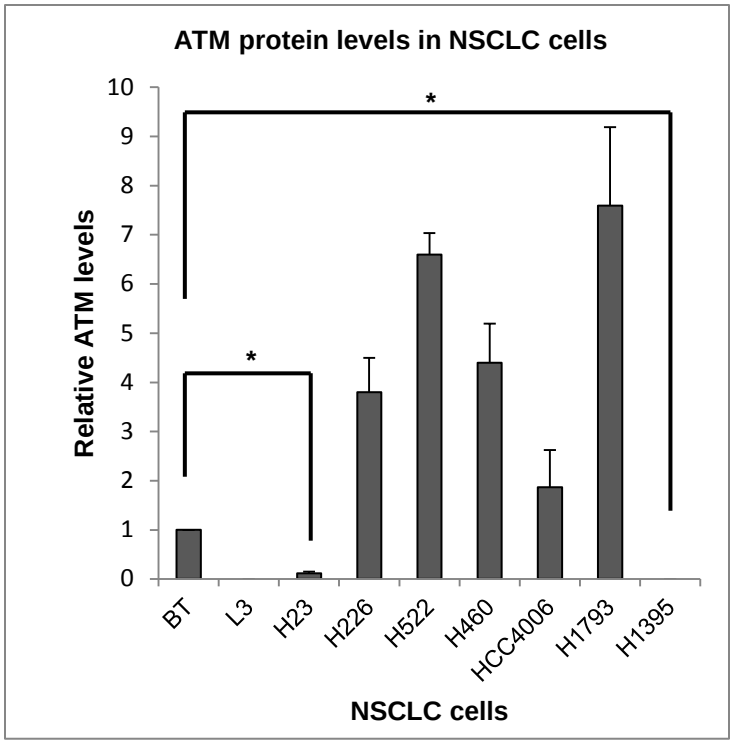
3.1.1



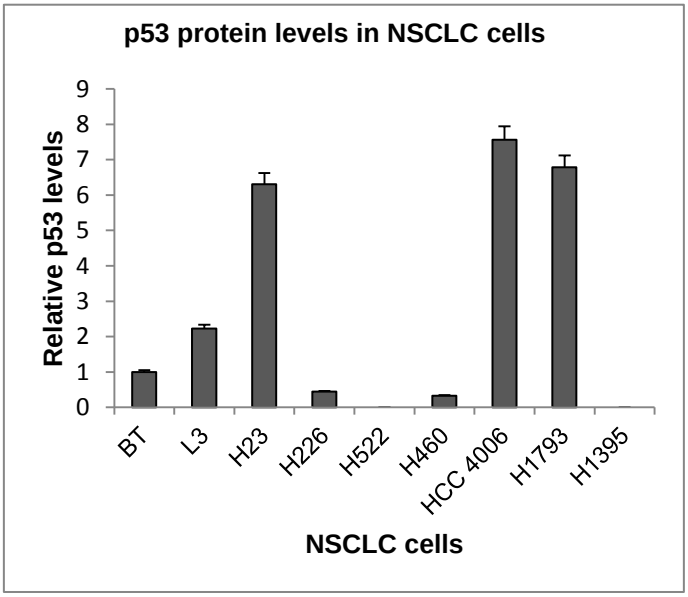
●●Short exposure and undetectable ATM in H1395 compare to figures 3.2 A and E3 B, C

3.1.2

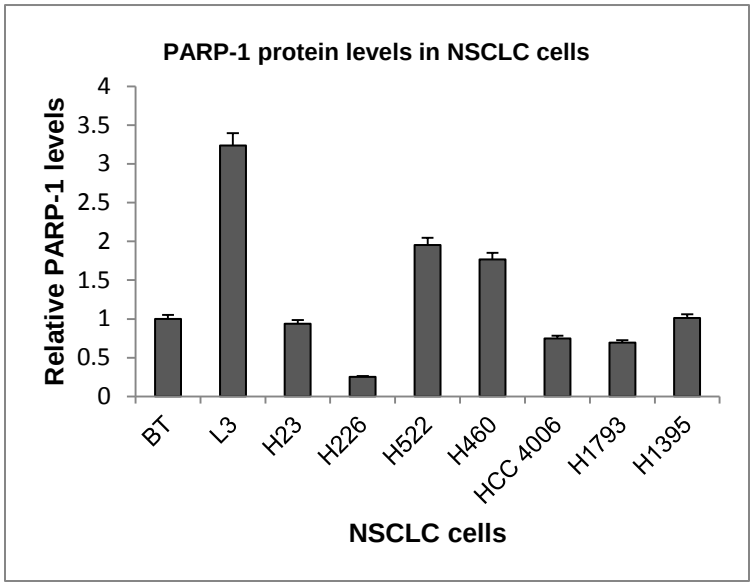
A


ATM protein levels in NSCLC cells
Relative ATM levels
10
9
8
7
6
5
4
3
2
1
0
*
*
BT
L3
H23
H226
H522
H460
HCC4006
H1793
H1395
NSCLC cells

B


p53 protein levels in NSCLC cells
Relative p53 levels
9
8
7
6
5
4
3
2
1
0
BT
L3
H23
H226
H522
H460
HCC 4006
H1793
H1395
NSCLC cells

C


PARP-1 protein levels in NSCLC cells
Relative PARP-1 levels
4
3.5
3
2.5
2
1.5
1
0.5
0
BT
L3
H23
H226
H522
H460
HCC 4006
H1793
H1395
NSCLC cells

D

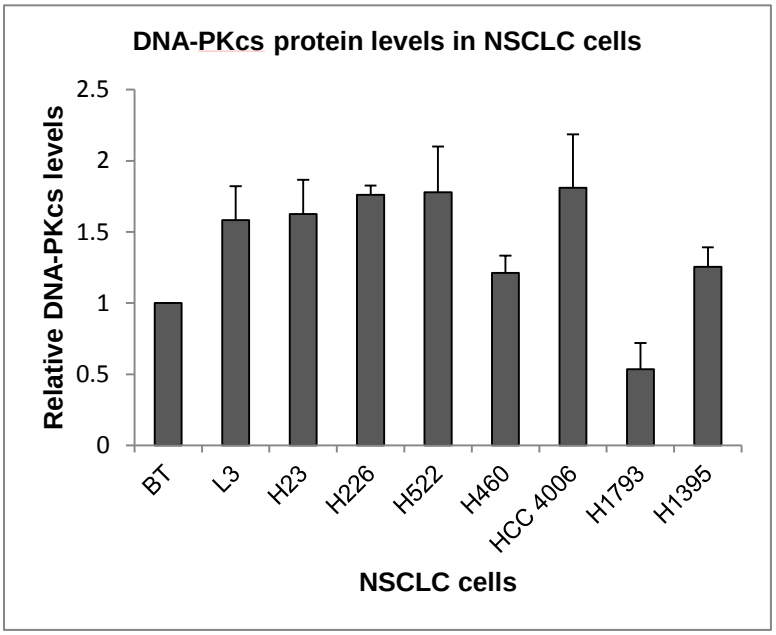

DNA-PKcs protein levels in NSCLC cells
Relative DNA-PKcs levels
2.5
2
1.5
1
0.5
0
BT
L3
H23
H226
H522
H460
HCC 4006
H1793
H1395
NSCLC cells

Figure 3.1: Analysis of DNA damage responsive proteins in NSCLC cell lines
3.1.1. DNA damage responsive proteins including ATM in NSCLC cells: Western blot of 50 µg whole cell extracts (WCE) from the cells (H23, H226, H522, H460, H1395, H1793 and HCC4006) resolved on a low bis-acrylamide SDS- PAGE gel (7.9% : 0.1% v/v acrylamide/ bis-acrylamide was used to make an 8% gel). L3 (ATM deficient) and BT (ATM proficient) are negative and positive control cells for ATM protein respectively. SMC1 (structural maintenance of chromosome 1) protein serves as the loading control. Note that the film exposure for lanes 7 – 8 was not exactly the same as for lanes 1 – 7 because they are 2 separate gels. There is loss of ATM protein in both H23 (lane 3) and H1395 (lane 9).
3.1.2. Quantification of the relative levels of ATM, p53 PARP-1 and DNA-PKcs proteins in NSCLC cells: ATM, p53, PARP-1 and DNA-PKcs proteins in the cells (H23, H226, H522, H460, H1395, H1793 & HCC4006) were quantified using an image processing program, ImageJ (developed by the National Institutes of Health) after normalisation to the loading (SMC1) and positive (BT) controls. Error bars represent standard error of mean (SEM) from 3 independent experiments. Student's t- test was used for data analysis. The p-value = 0.009 for the mean ATM level in H23 compared to the mean ATM in BT. The p-value = 0.01 for the mean ATM level in H1395 compared to the mean ATM in BT. p-values < 0.05 were considered statistically significant and are represented by asterisks, n = 3.

3.2 Relationship between ATM levels and ATM signaling in NSCLC cell lines

A lack of ATM would be expected to generate an A-T phenotype denoted by defective ATM signaling and checkpoint controls. Following IR treatment of the cells, I examined IR-inducible ATM dependent phosphorylation sites including p-S1981 ATM, the well characterized marker of ATM activation, as well as p-S15 p53 and p-S824 KAP1 which are largely but not exclusively ATM dependent phosphorylation sites (Shiloh & Kastan 2001, Hu et al., 2012). Phosphorylation of these sites (i.e. p-S1981 ATM, p-S15 p53 & p-S824 KAP1) varied between cell lines (Figure 3.2; Appendix figure E2). ATM phosphorylation i.e. p-S1981 was not detected in either H23 and very faint p-S1981 bands seem apparent in H1395 cells with either long film exposure or increased concentration of anti-ATM antibody (Epitomics), (Figures 3.2 A; E3 B; E5 A). This is suggestive of defective ATM activation and consistent with overall reduction in ATM levels in these cells by western blot. However, KAP1 was phosphorylated in H1395, but not in either H23 cells or A-T patient's derived cell line i.e. L3 (Figure 3.2; Appendix figures E2 A and C). These data suggest the possibility of ATM functionality in H1395 cells. Because IR-induced DSBs can also activate DNA-PKcs in addition to ATM and since both proteins share similar downstream targets (e.g. p53 phosphorylation at serine 15), (Shiloh & Kastan, 2001); I also probed for DNA-PKcs and its IR inducible auto-phosphorylation site, p-S2056 and both were detected in all the NSCLC cells (Figure 3.2; Appendix figure E2 A), indicating that DNA-PKcs was equally activated in the cells post-radiation.

To determine if KAP1 phosphorylation was ATM dependent, 1 hour prior to IR treatment; the cells were pre-treated with a potent and selective ATM inhibitor (KU55933), which has been

shown to inhibit 50% of in-vitro ATM activity at 12.9 nM (Hickson et al., 2004). There was loss of KAP1 phosphorylation with ATM inhibition even in H1395 cells, this finding further in support of residual ATM activity in H1395, (Figure 3.3; Appendix figures E3 A and B). Conversely, pre-treatment of cells with ATM inhibitor had no effect on DNA-PKcs phosphorylation at serine 2056, (Figure 3.3; Appendix figure E3), confirming its selectivity for ATM as previously described (Hickson et al., 2004). At the same time, inhibition of DNA-PKcs with the small molecule inhibitor, NU7441 had no effect on KAP1 phosphorylation in the cells, ruling out the possibility of KAP1 phosphorylation by DNA-PKcs, (Figures 3.3; E3 A and B).

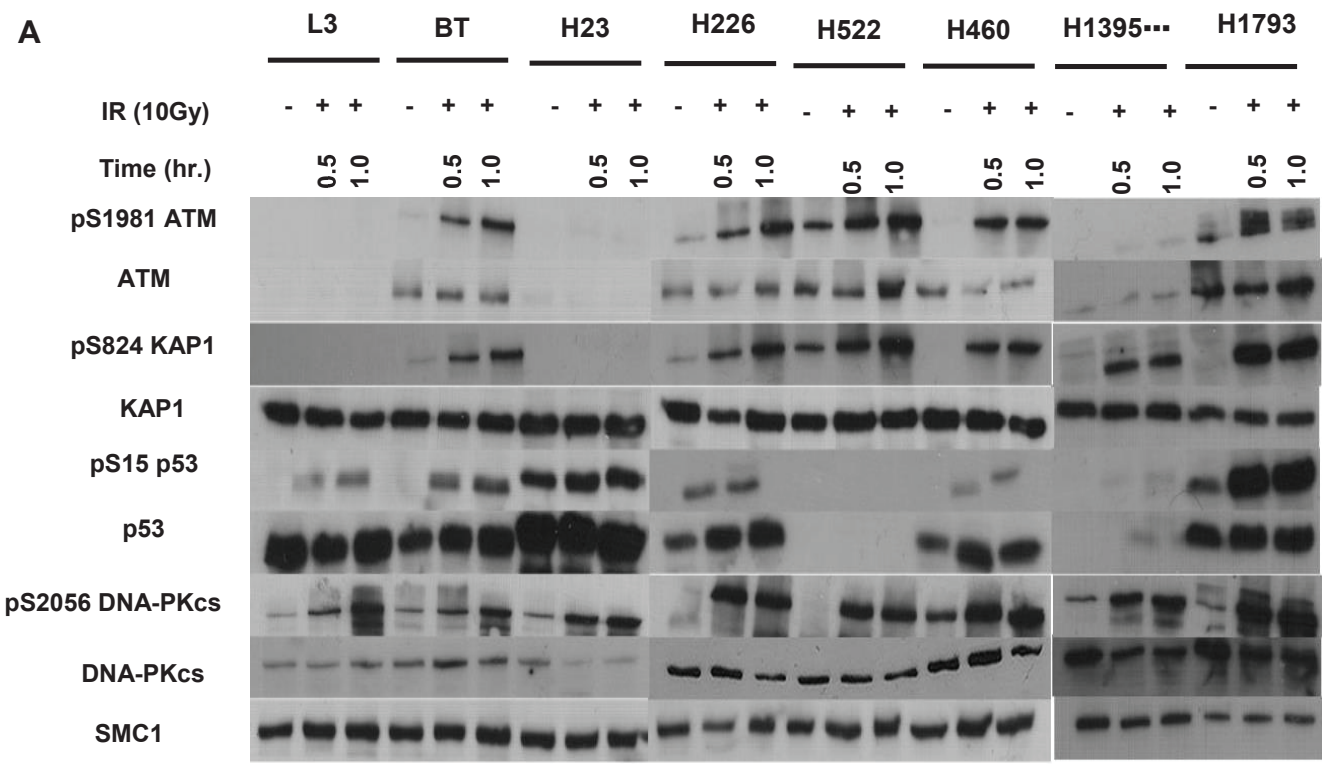
3.3 Relationship between ATM levels and radiation sensitivity in NSCLC cell lines

Loss of ATM function would also be expected to generate the radiation sensitivity phenotype characteristic of A-T cells. To further test for ATM functionality, the sensitivity of cells to radiation treatment was measured by assessing survival after 1, 2 and 4 Gy of irradiation. Similar to cells lacking ATM (e.g. A-T cells), H23 cells exhibiting ATM loss were extremely radio-sensitive and had the least number of cells surviving (close to 1%) after 4Gy of IR (Figure 3.4). In contrast, H1395 cells also with reduced ATM by western blot (Figures 3.2A; E3 B; E5 A), were not dramatically sensitive to IR. In fact, H1395 was among the most resistant cells to IR only second to H1793, an ATM proficient counterpart, (Figure 3.4).

From the findings above, I identified H23 cells as clearly an ATM deficient cell line which exhibits known hallmarks of A-T cells vis-à-vis radio-sensitivity and impaired ATM-dependent signaling. On the other hand, H1395 probably has some functional ATM and thus behaved as an ATM proficient cell line. Other cell lines i.e. H226, H460, H522 and H1793 were considered ATM proficient cells.

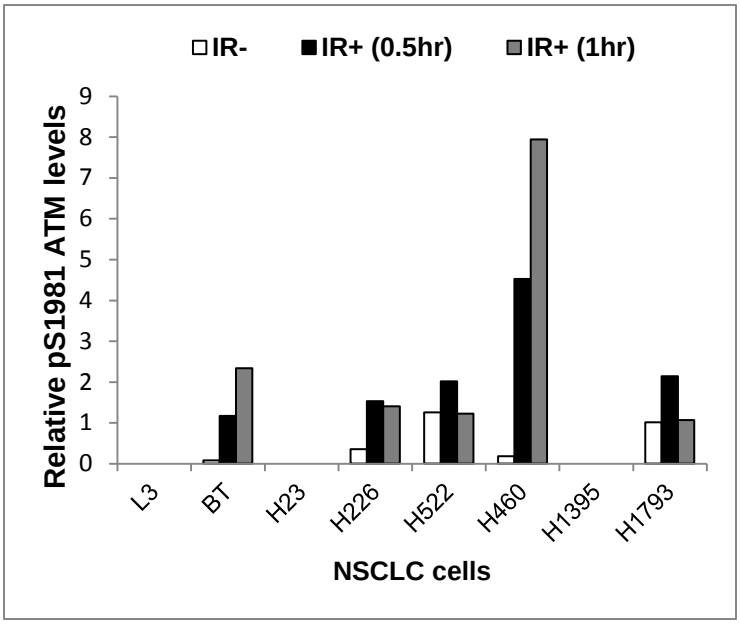
To confirm the ATM expression status of the NSCLC cells lines, the western blot was repeated by Petersen L.F in our lab using a second antibody (Millipore) generated to full-length ATM, but this antibody could not detect ATM in H1395 (Appendix figure E5).

3.2

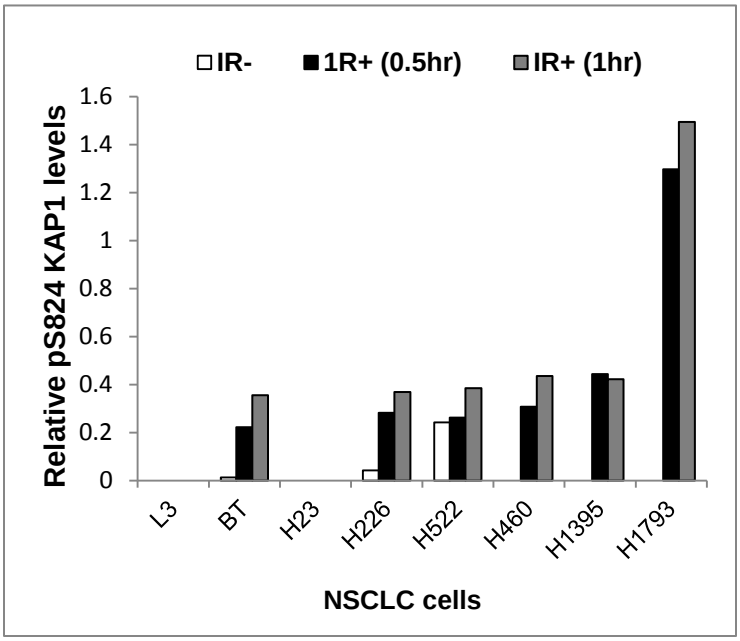


▪▪▪Long exposure and faint ATM bands in H1395 compare to figures 3.1.1, 3.1.2 A, 3.3 A and E1, E3 A

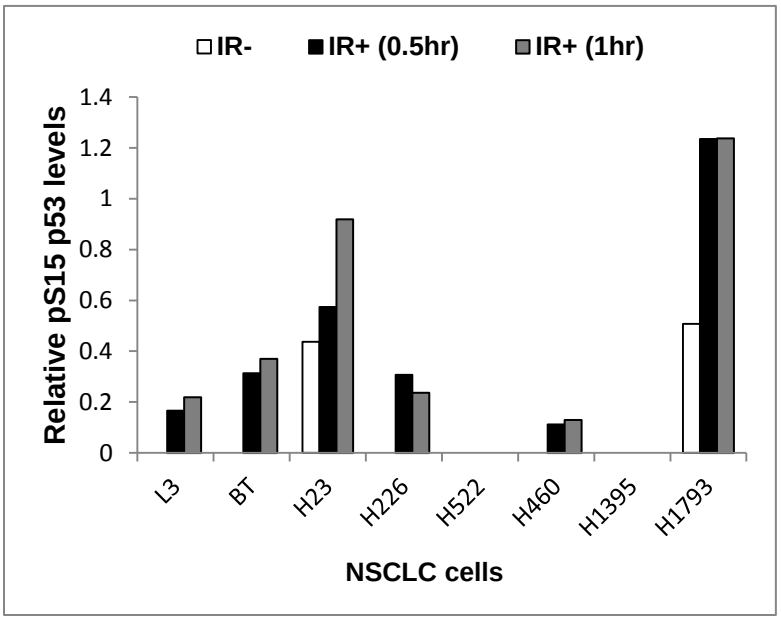
B


IR-
IR+ (0.5hr)
IR+ (1hr)
Relative pS1981 ATM levels
0
1
2
3
4
5
6
7
8
9
L3
BT
H23
H226
H522
H460
H1395
H1793
NSCLC cells

C


IR-
1R+ (0.5hr)
IR+ (1hr)
Relative pS824 KAP1 levels
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
L3
BT
H23
H226
H522
H460
H1395
H1793
NSCLC cells

D


IR-
IR+ (0.5hr)
IR+ (1hr)
Relative pS15 p53 levels
1.4
1.2
1
0.8
0.6
0.4
0.2
0
L3
BT
H23
H226
H522
H460
H1395
H1793
NSCLC cells

E

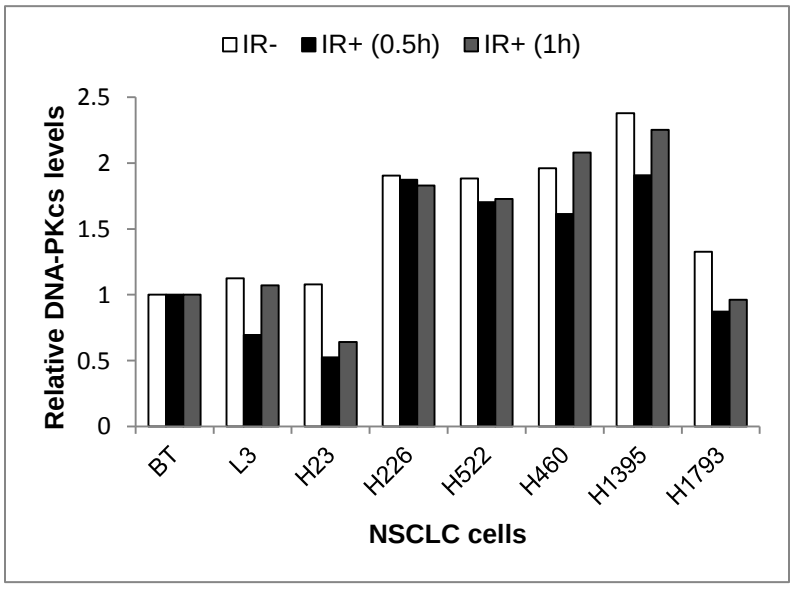
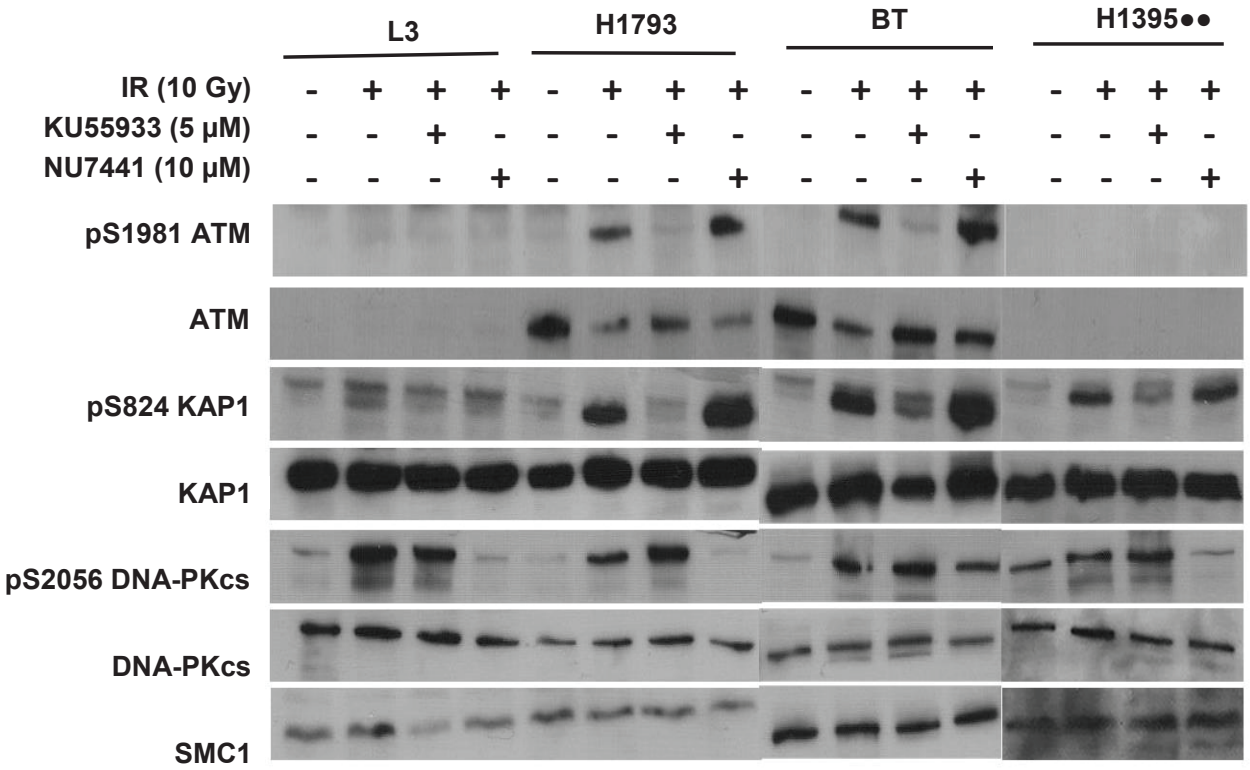

IR-
IR+ (0.5h)
IR+ (1h)
Relative DNA-PKcs levels
2.5
2
1.5
1
0.5
0
BT
L3
H23
H226
H522
H460
H1395
H1793
NSCLC cells

Figure 3.2: Differential phosphorylation of ATM downstream targets in NSCLC cell lines
Cells were either treated with 10 Gy of IR or left untreated. At 0.5 and 1 hour post-radiation, whole cell extraction was performed with detergent-containing NET-N lysis buffer plus protease and phosphatase inhibitors. 3.2A- western blot performed as in figure 3.1.1. Immunoblots were probed for ATM, KAP1, p53, DNA-PKcs proteins and their phosphorylated variants (p-S1981 ATM, p-S824 KAP1, p-S15 p53 and p-S2056 DNA-PKcs) with specific primary antibodies and phospho-specific antibodies as indicated. ATM-proficient BT cell line is shown as the positive control for ATM-dependent phosphorylation sites. SMC1 acts as the loading control. Relative levels of pS1981 ATM (3.2B), pS824 KAP1 (3.2C) and pS15 p53 (3.2D) were quantified using ImageJ after normalizing to ATM for pS1981 ATM, KAP1 for pS824 KAP1 and p53 for pS15 p53 respectively. For DNA-PKcs, the protein (DNA-PKcs) was normalized to SMC1 and expressed relative to BT cell as follows: DNA-PKcs in untreated cells (IR-) relative to DNA-PKcs in untreated BT cell i.e. IR- NSCLC cell/ IR- BT cell, DNA-PKcs in the treated cells (IR+ 0.5h or 1h)) relative to DNA-PKcs in treated BT cell i.e. IR+ (0.5h) NSCLC cell/ IR+ (0.5h) BT cell or IR+ (1h) NSCLC cell/ IR+ (1h) BT cell respectively.

3.3

A


L3
H1793
BT
H1395●●
IR (10 Gy) - + + + - + + + - + + + - + + +
KU55933 (5 μM) - - + - - - + - - - + - - - + -
NU7441 (10 μM) - - - + - - - + - - - + - - - +
pS1981 ATM
ATM
pS824 KAP1
KAP1
pS2056 DNA-PKcs
DNA-PKcs
SMC1

B

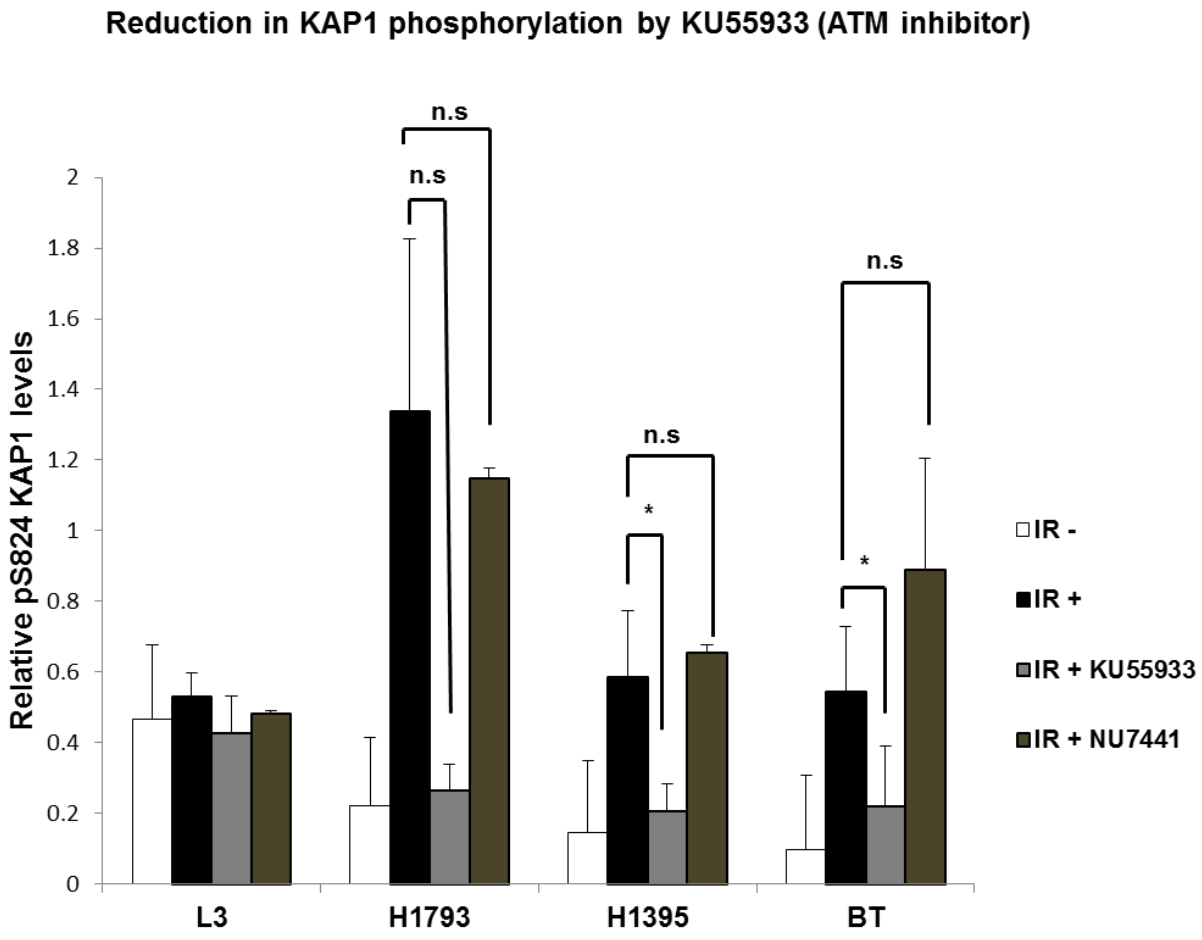

Reduction in KAP1 phosphorylation by KU55933 (ATM inhibitor)
Relative pS824 KAP1 levels
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
n.s
n.s
n.s
n.s
*
*
L3
H1793
H1395
BT
IR -
IR +
IR + KU55933
IR + NU7441

Figure 3.3: KAP1 phosphorylation at serine 824 is ATM dependent in H1395
3.3A. Cells were either pre-treated with 5 µM ATM inhibitor, (KU55933), 10 µM DNA-PKcs inhibitor (NU7441)1 hour prior to 10 Gy of IR or left untreated. WCE was harvested (as in figure 3.2A) after another 2 hours of cell incubation. Western blot was performed as in figure 3.1.1; specific primary antibodies and phospho-specific antibodies were used to detect target proteins and their phosphorylated forms as indicated. ATM-dependent phosphorylation sites, p-S1981 ATM and p-S824 KAP1 were lost with ATM inhibition but not with DNA-PKcs inhibitor in the cells including H1395. SMC1 serves as the loading control, BT and L3 cells are the positive and negative control cells for ATM dependent phosphorylation sites. Note that the film exposure for H1395 bands are not necessarily equivalent to others as it is a separate blot.
3.3B. Quantification of pS824 KAP1 levels from the average of 3 replicates using ImageJ and error bars represent SEM of the 3 replicates. Student's t- test was used for the results analysis. The p-values = 0.11 for H1793 IR+ versus IR+ KU55933; 0.025 for H1395 IR+ versus IR+ KU55933 and 0.013 for BT IR+ versus IR+ KU55933 respectively. p-values < 0.05 were considered statistically significant and are represented by asterisks while p-values > 0.05 were considered non-statistically significant and are represented by n.s; $n = 3$.

3.4

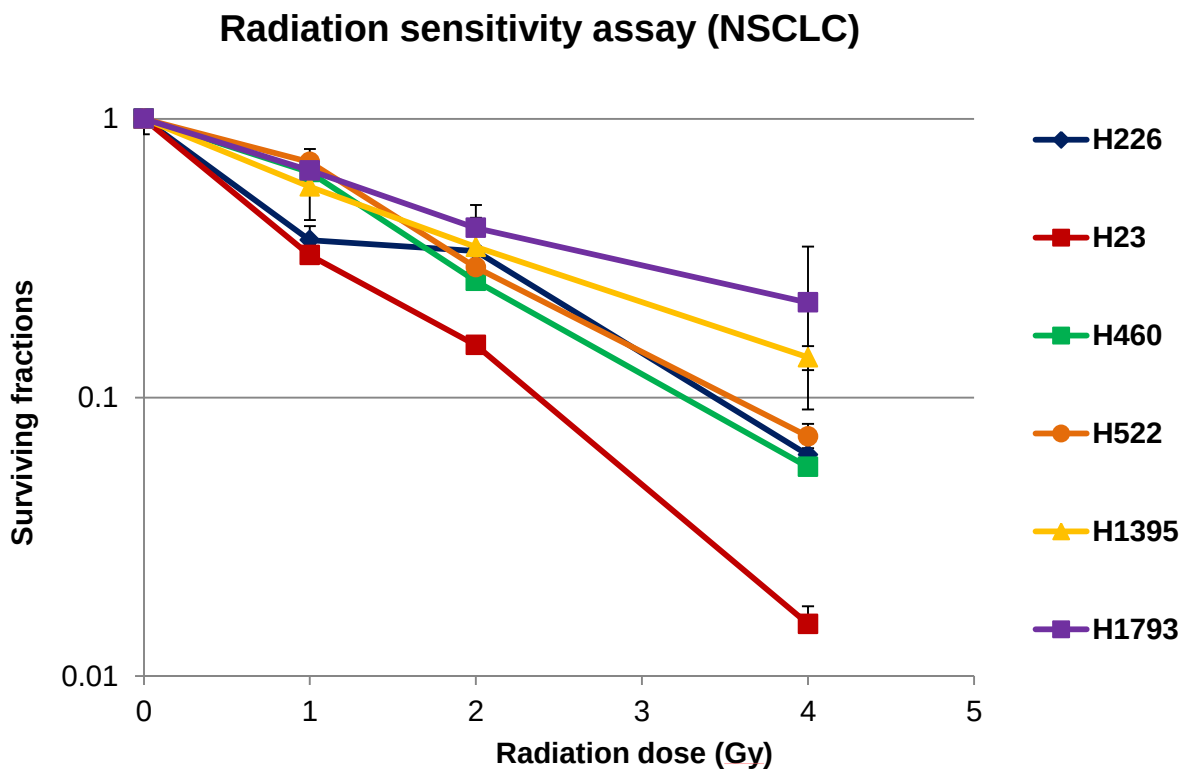

Radiation sensitivity assay (NSCLC)
Surviving fractions
1
0.1
0.01
0
1
2
3
4
5
Radiation dose (Gy)
H226
H23
H460
H522
H1395
H1793

Figure 3.4: ATM deficiency in H23 changes sensitivity to radiation treatment in clonogenic survival assays

NSCLC cells (H226, H23, H460, H522, H1395 & H1793) in 5ml of fresh media plus serum were plated in 3 separate 6cm plates at varying cell densities (1 X 10^2 - 3 X 10^3 cells/ml) depending on the cell line. The cells were allowed to adhere to plates and then treated with or without IR, doses as indicated. Surviving fractions (SF) were calculated as follows: SF = (number of cells observed/number of cells seeded) X (Plating Efficiency (PE)/ 100), and normalized to control untreated cells. PE for each cell line was determined by dividing the number of colonies observed (under microscope, 10 x magnifications) with the number of cells seeded. Error bars indicate SEM of 3 experiments with 3 replicates.

3.3 ATM deficient H23 cells are preferentially sensitive to PARP inhibition

Several reports, including work previously performed in our laboratory, indicate a synthetic lethal interaction between ATM and PARP (Williamson et al., 2010; Weston et al. 2010, Patel et al., 2011; Montoni et al., 2013; Kubota et al., 2014). The PARP inhibitor, olaparib (also known as AZD2281) which inhibits PARP-1 and PARP-2 is under clinical development for treating different malignancies including NSCLC (Fong et al., 2009; Tutt et al., 2010; Fong et al 2010; Audeh et al., 2010; Gelmon et al., 2011; Ledermann et al., 2012; Montoni et al., 2013). Here, I tested the hypothesis that ATM deficient cells exhibit selective sensitivity to olaparib compared to their ATM proficient counterparts. The cells were exposed continuously to increasing concentrations of olaparib or DMSO as a control. As shown in figure 3.5, olaparib (PARPi) induced greater dose-dependent reduction in the viability of H23 cells with no functional ATM, in clonogenic survival assays. Again, H1395 exhibited no dramatic response to olaparib in a similar manner as ATM competent cells (H226, H460, H522 and H1793).

Williamson et al. (2012) previously reported that disruption of p53 transcriptional activity resulted in increased sensitivity of ATM deficient MCL cells to olaparib. Because of the p53 mutant status of H23 (O'Connor et al., 1997) and my observation of a constitutively phosphorylated p53 in this cell line (H23) irrespective of IR-induced DNA damage, (Figures 3.2 A and D), I speculated that p53 transcriptional activity is dysregulated in H23 cells which may contribute to its sensitivity to olaparib. To determine whether p53 is functional in H23, I performed quantitative RT-PCR to examine the IR-induced expression of p53 responsive genes in the cell lines (H23, H460, H1793, H1395, H226 and H522). In keeping with impaired p53 activation, no changes in expression of the checkpoint control genes, p21 and growth arrest and

DNA damage-inducible 45 alpha (GADD45α) nor the pro-apoptotic gene, PUMA were detected in H23, H1793 and H522 cells, as measured by p21, GADD45α and PUMA transcript levels (Figure 3.6). Whereas in H460 and H226 cells, IR treatment largely increased p21 levels and a slight increase in PUMA levels up to 3 hours post-radiation. However, fold changes in GADD45α varied between the two cell lines (H460 and H226) with increasing levels only seen in H460 cells, (Figure 3.6). Taken together, the findings suggest that, p53 deficiency alone does not confer sensitivity to PARP inhibition, since ATM proficient cells with p53 disruption (i.e. H1793 and H522) were not largely affected by olaparib, (Figure 3.5). However, olaparib-induced cytotoxicity in H23 may be influenced by both ATM and p53 disruptions.

3.5

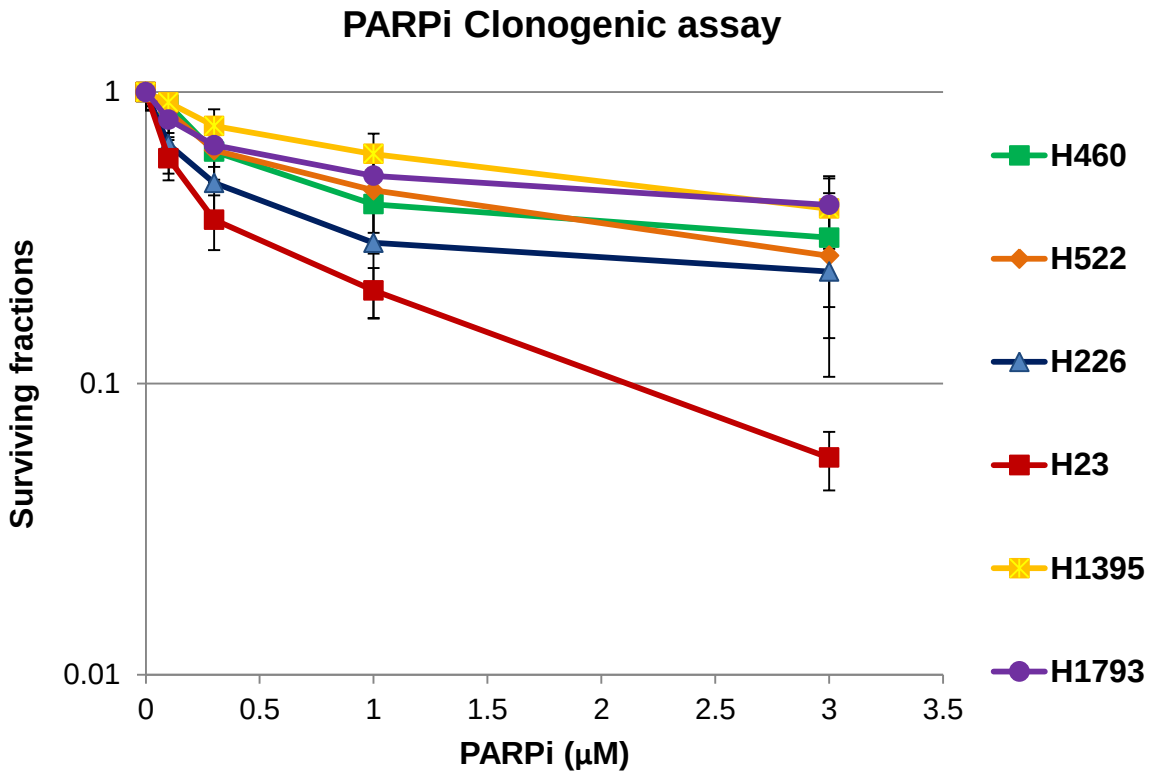
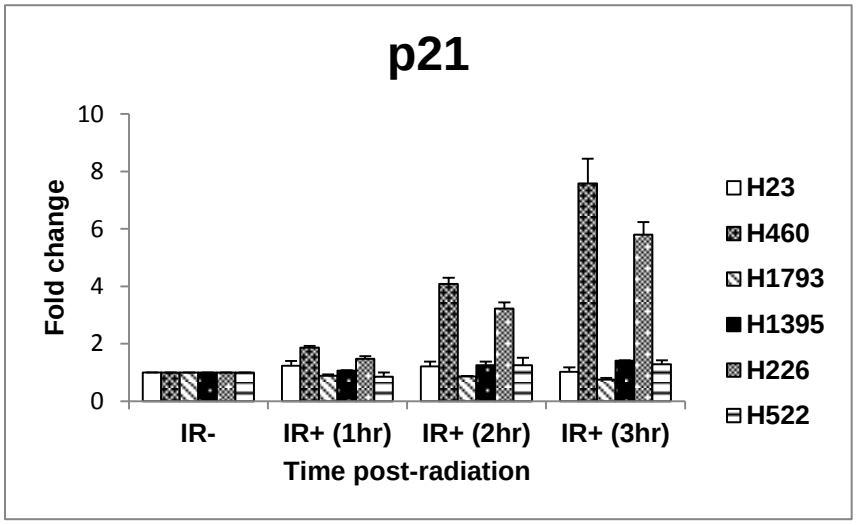

PARPi Clonogenic assay
Surviving fractions
1
0.1
0.01
0
0.5
1
1.5
2
2.5
3
3.5
PARPi (µM)
H460
H522
H226
H23
H1395
H1793

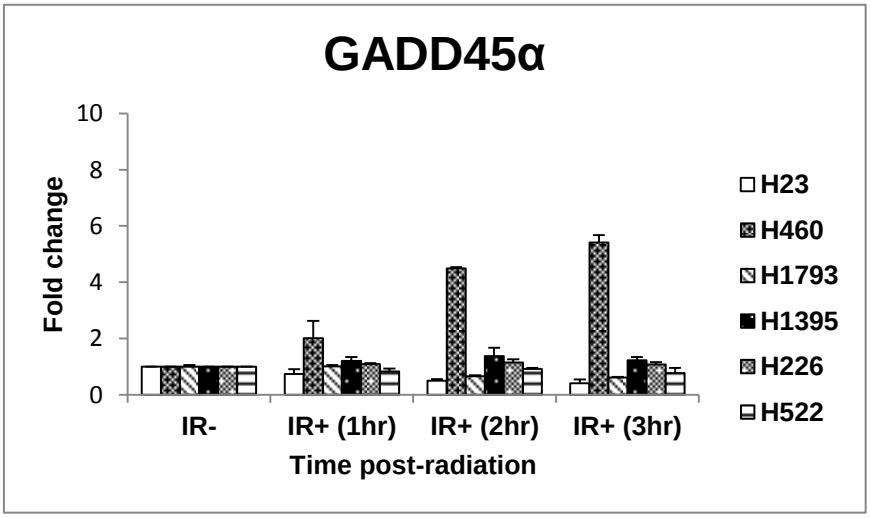
Figure 3.5: ATM deficiency in H23 cells changes sensitivity to targeted treatment
NSCLC cells in serum-containing media were continuously exposed to increasing doses of PARP inhibitor (AZD2281) as indicated, in clonogenic survival assays. After 10-14days of incubation, colonies formed were counted, the PE and SF were analysed as in figure 3.4. Error bars indicate SEM of 3 experiments with 3 replicates. PARPi denotes PARP inhibitor (olaparib).

3.6

A


p21
Fold change
10
8
6
4
2
0
IR-
IR+ (1hr)
IR+ (2hr)
IR+ (3hr)
Time post-radiation
H23
H460
H1793
H1395
H226
H522

B


GADD45α
Fold change
10
8
6
4
2
0
IR-
IR+ (1hr)
IR+ (2hr)
IR+ (3hr)
Time post-radiation
H23
H460
H1793
H1395
H226
H522

C

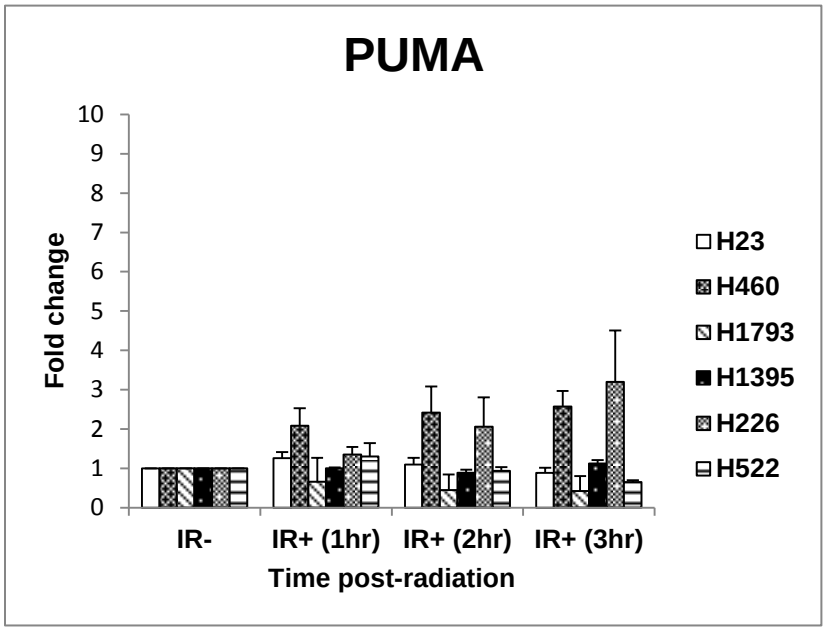

PUMA
Fold change
10
9
8
7
6
5
4
3
2
1
0
IR-
IR+ (1hr)
IR+ (2hr)
IR+ (3hr)
Time post-radiation
H23
H460
H1793
H1395
H226
H522

Figure 3.6: Impaired IR-inducible p53-target gene transcription in NSCLC cells by quantitative RT-PCR

Cells were treated with IR (2 Gy) as indicated, in medium plus serum or left untreated. Total RNA was isolated after 1, 2 and 3 hours of incubation. For each RNA sample, 1 μg of total RNA was used for first-strand synthesis and 1μl of cDNA product was used as the template in a 20 μl PCR reaction containing 10 μl of PerfeCTa SYBR Green FastMix (Quanta Biosciences), 0.5 μl of each gene-specific primer (250 nM final concentration) and 8 μl of nuclease-free water. β-glucoronidase (GUSB) was selected as the most stable reference gene for the cell lines and the untreated cells serve as reference samples. Relative expression levels of the genes of interest, p21 (A), GADD45α (B) and PUMA (C) in untreated and IR-treated conditions were calculated using the comparative (ΔΔCt) method. Error bars indicate SEM of 3 biological replicates

3.4 PARP inhibition increases Cisplatin toxicity in ATM deficient H23 cells

Cisplatin mediates cytotoxicity via the induction of DNA adducts i.e. intra-strand and inter-strand DNA crosslinks (Basu and Krishnamurthy, 2010). However, limited tumor response and development of resistance are major barriers to the use of cisplatin in solid tumors like NSCLC. Given that ATM-/- fibroblasts are sensitised to alkylating agent by PARP inhibition, (Loser et al., 2010), one could predict that the efficacy of cisplatin may be improved in ATM deficient NSCLC cells with the use of PARPi. To test the prediction, cell lines were initially exposed to increasing concentrations of cisplatin as a monotherapy for 24 hours after which their clonogenic survival was determined. Since there were no cell lines with just ATM deficiency, the cells were matched by taking into consideration their ATM and p53 status; ATM wild type and p53 mutant cells (H522 & H1793) versus cells mutant for both ATM and p53 (H23 & H1395), (Table A in the Appendix; ATCC-http://www.atcc.org/~/media/PDFs/Culture%20Guides/Cell_Lines_by_Gene_Mutation.ashx). Loss of clonogenic survival was greater in H23, (ATM deficient, p53 mutant) and H522, (ATM proficient, p53 mutant) than the other cells (H1793 and H1395); suggesting that ATM could not predict for response to cisplatin in NSCLC cells (Figure 3.7).

To determine whether pre-treatment of ATM-deficient NSCLC cell lines with olaparib will potentiate cisplatin-induced cytotoxicity, cells were either simultaneously exposed to increasing concentrations of cisplatin plus a fixed dose of olaparib or a dose range of cisplatin alone for 24 hours. Next, cells were allowed to form colonies in fresh media for 10-14days and the surviving fractions were determined. Combination of olaparib with cisplatin further decreased clonogenic survival in H23 cells (ATM deficient cell) but not in H1395 or ATM proficient cells (H1793 and

H522) and the decrease in cellular survival reached statistical significance as shown in Figure 3.8.

3.7

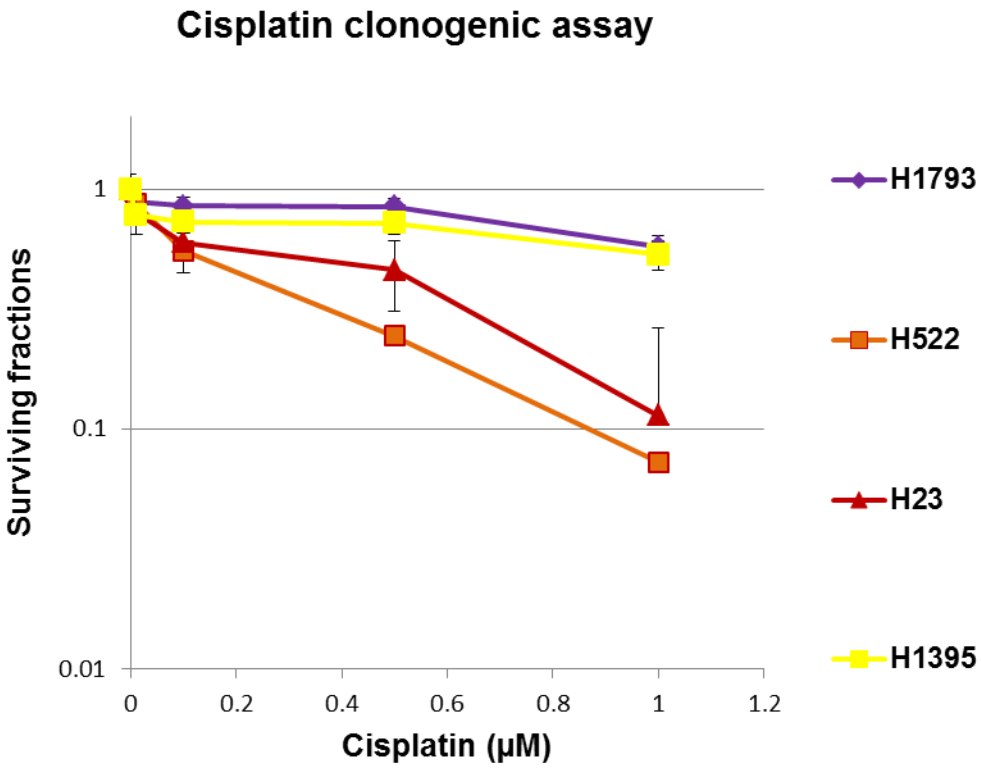
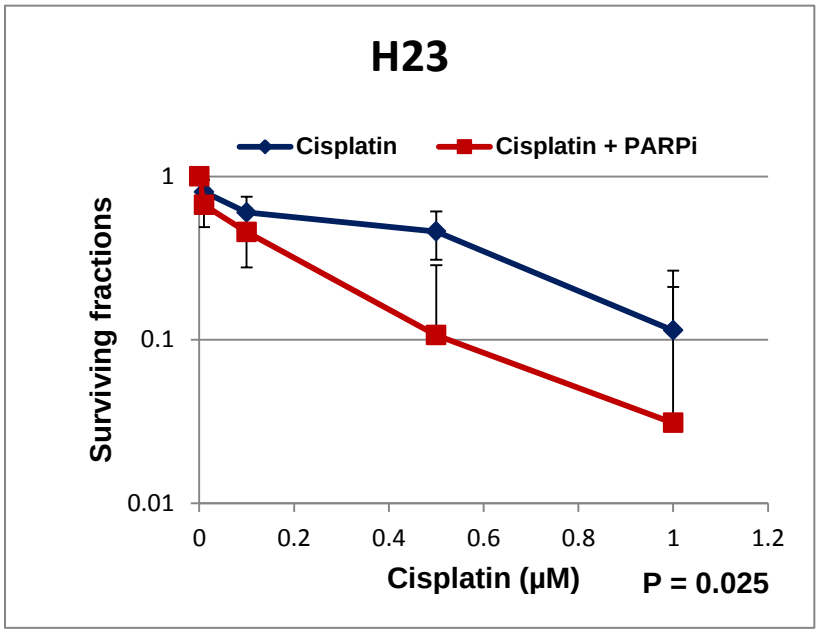

Cisplatin clonogenic assay
Surviving fractions
1
0.1
0.01
0
0.2
0.4
0.6
0.8
1
1.2
Cisplatin (μM)
H1793
H522
H23
H1395

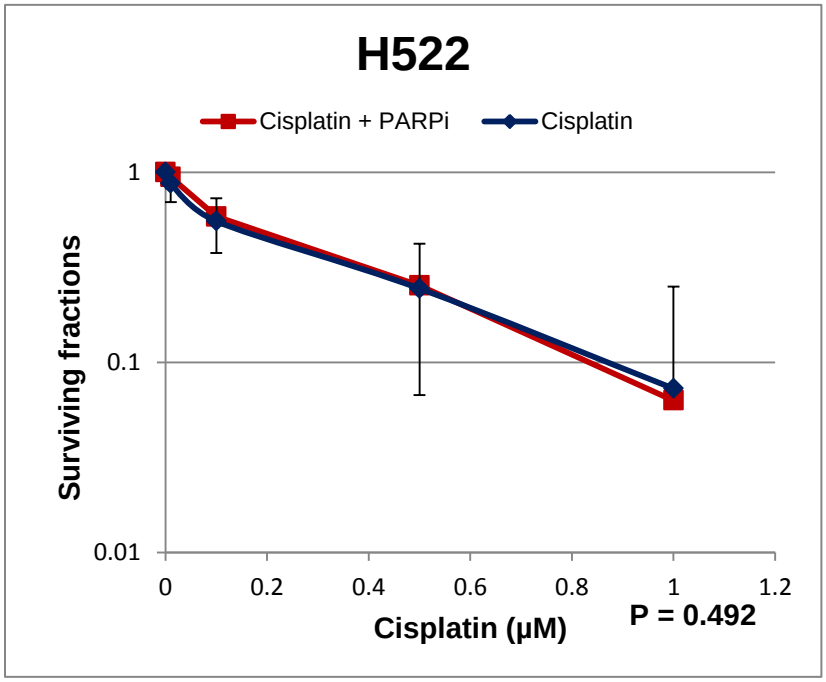
Figure 3.7: Cisplatin induces NSCLC cell death independently of ATM status
Cells growing in media plus serum were treated with concentration range of cisplatin as indicated (0.01, 0.1, 0.5 and 1μM). After 24 hours, the cisplatin containing media were removed; cells were washed in PBS and were replaced in fresh media to allow for colony formation. The surviving colonies were determined after 10 – 14 days and normalized to control untreated cells in clonogenic survival assay as described in figure 3.4. SEM was analysed from 3 independent experiments each with 3 replicates and was shown as error bars

3.8

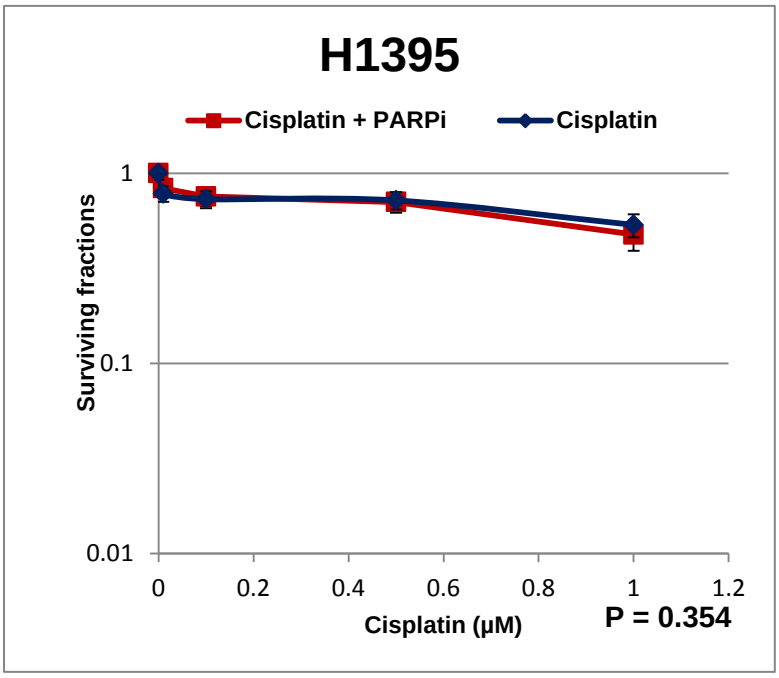
A


H23
Cisplatin
Cisplatin + PARPi
Surviving fractions
1
0.1
0.01
0
0.2
0.4
0.6
0.8
1
1.2
Cisplatin (μM)
P = 0.025

B


H522
Cisplatin + PARPi
Cisplatin
Surviving fractions
1
0.1
0.01
0
0.2
0.4
0.6
0.8
1
1.2
Cisplatin (μM)
P = 0.492

C


H1395
Cisplatin + PARPi
Cisplatin
Surviving fractions
1
0.1
0.01
0
0.2
0.4
0.6
0.8
1
1.2
Cisplatin (μM)
P = 0.354

D

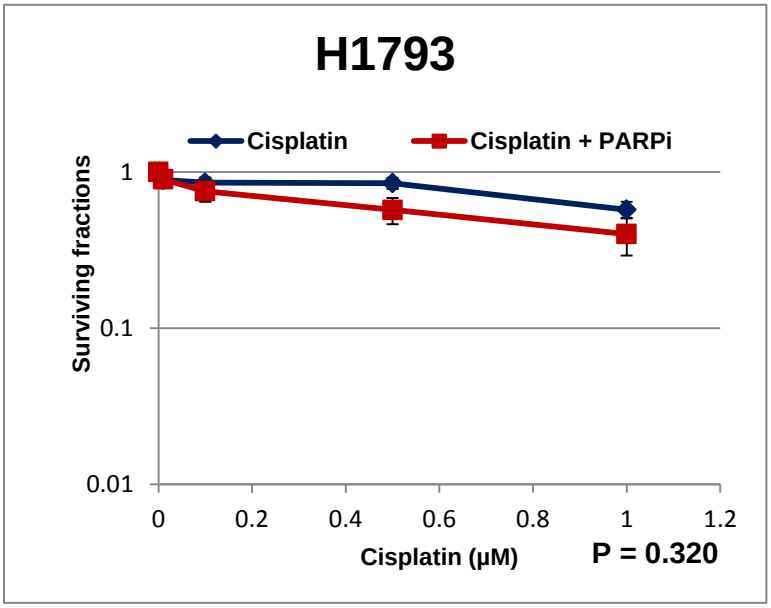

H1793
Cisplatin
Cisplatin + PARPi
Surviving fractions
1
0.1
0.01
0
0.2
0.4
0.6
0.8
1
1.2
Cisplatin (μM)
P = 0.320

Figure 3.8: PARP inhibition sensitizes ATM deficient H23 to Cisplatin treatment
NSCLC Cells (a) H1793, (b) H522, (c) H23, & (d) H1395 were exposed for 24 hours to an increasing dose of Cisplatin treatment (0.01, 0.1, 0.5 and1µM) along with either PARP inhibitor (0.3µM) or serum free RPMI-treated control in clonogenic survival assays. The surviving fractions were determined as in figure 3.4 and normalized to controls for each cell line. Results were analysed using Student's t-test and p values < 0.05 were considered statistically significant, $n = 3$. Error bars represent SEM of 3 experiments.

3.5 Summary and implications

This chapter describes several observations made in NSCLC cell lines while exploring the precise role of ATM pathway in the context of sensitivity of the cells to therapeutic agents. We found distinct cell lines (H23 and H1395) with abnormality of ATM levels and impaired ATM dependent signaling out of 7 NSCLC cells that we tested; implying the occurrence of ATM pathway disruptions in a significant proportion of NSCLC cells. In addition, p53 inactivation in several of our NSCLC cell lines (H1793, H1395, H522 and H23) was demonstrated. The fact that p53 is inactivated in those cells identified as having abnormal ATM (H23 and H1395) suggests that, there is the chance of ATM loss in p53-inactivated NSCLC cells. Surprisingly, we identified IR-induced ATM dependent phosphorylation site i.e. pS824 KAP1 in H1395 cells. On the other hand, ATM defective H23 cells exhibit the well-known A-T phenotype of increased radiation sensitivity and were largely sensitive to PARP inhibition as monotherapy as well as in combination with cisplatin treatment which is consistent with our expectations. However, it is difficult to attribute the characteristics observed in H23 to ATM because in contrast to H23, H1395 was resistant to our investigative agents including IR, olaparib, and cisplatin and behaved in a similar way as ATM competent counterparts. Likely reasons as to why H1395 cells do not behave as expected are discussed in the next chapter with suggestions for future directions.

Chapter Four: Discussion

Mammalian cells are under constant challenge from various sources of DNA damage including therapeutic cytotoxic agents used for cancer treatment. Largely, NSCLC patients at some point will require these cytotoxic agents which spare not the surrounding important tissues the impact of DNA damage. Thus inherent deficiencies in malignant cells are potential ways through which these cells can be specifically targeted while sparing surrounding normal cells. Defects in the ATM pathway in anti-cancer drug development have been exploited via PARP inhibition with success in preclinical studies of hematological cancers in our laboratory, (Williamson et al., 2010; 2012; Kubota et al., 2014). Whether this promising approach is applicable in solid tumors like NSCLC is yet to be thoroughly investigated. In the present work, a panel of NSCLC cell lines were examined for ATM status, with the primary goal of determining the therapeutic potential of ATM deficiency in these cells. In vitro, ATM deficiency in NSCLC cells were investigated by assessing phosphorylated target proteins induced by IR-related DNA damage and by assessing sensitivity to IR. NSCLC cells identified as ATM deficient were tested for sensitivity to targeted and cytotoxic DNA damaging agents. Unexpectedly, we found that not all ATM deficient NSCLC cell lines are radio-sensitive, which is contrary to the general belief of increased radiation sensitivity in ATM-deficient cells (Shiloh & Kastan 2001; Rainey et al., 2008; Kuroda et al., 2012). The startling observation that one ATM deficient cell was PARPi sensitive while another was insensitive further add a layer of complexity.

4.1 ATM deficiency and variation in cellular phenotype

As shown in chapter 3, both H23 and H1395 cells exhibit an abnormal IR-induced S1981 ATM phosphorylation (Figure 3.2), creating an impression of some defects in ATM pathway activation in both cells given that, pS1981 ATM is often used as a marker of ATM activation. It is however intriguing to detect ATM dependent KAP1 phosphorylation in H1395 cells post-radiation (Figure 3.3). Based on this observation, one could assume the presence of residual ATM activity in H1395 cells. On one hand, some ATM seems apparent in H1395 with longer film exposure (see figure E3 C in the appendix). On the other hand, additional probing for ATM by Petersen L.F in whole cell lysate harvested from H1395 using a different antibody generated to full-length ATM failed to detect the protein (i.e. ATM), (Appendix figure E5). Given the findings which suggest minute quantity of ATM as sufficient to normally activate cellular DDR in response to DNA damage (Stewart et al., 2001; Stankovic et al., 1998), further evaluation of ATM status in H1395 will be required. In our hands, methods that refine protein detection and analysis of well characterized damage-induced ATM dependent cellular responses should be performed to clearly delineate the presence or absence of ATM kinase activity in H1395 cells. Western blots on nuclear extracts from H1395 or immunoprecipitation which can reliably detect very low levels of ATM as previously shown in other cells (Stewart et al., 2001) can be performed in addition to characterizing other aspects of ATM functions. It is not uncommon to find loss of ATM protein expression as being interpreted as ATM inactivation (Tommiska et al., 2008; Mazumder Indra et al., 2011).When one consider the fact that, cells may harbor functional ATM which can probably be missed using antibody detection methods (e.g. western blot, immunohistochemistry), it calls for caution in the interpretation of mere undetectable ATM

protein especially in clinical tumor specimens as evidence of loss of functional ATM. Perhaps, this scenario might explain controversies surrounding the prognostic implications of ATM deficiency in cancers. Some groups correlated ATM loss with poorer clinical outcomes (Kang et al., 2008; Beggs et al., 2012; Klimowicz et al., unpublished data) in tumors such as gastric, colorectal and lung cancers, while others report either no association with or favorable prognosis in ATM deficient head and neck carcinoma and leukemia respectively, (Lim et al., 2012; Haidar et al., 2000). Close review of some of these studies e.g. in a meta-analysis, might inform the development of a universally acceptable, more robust technique to specifically detect ATM functionality in malignant cells.

In addition to differential phosphorylation of damage inducible target protein between H23 and H1395 cells, response of the cells to targeted and DNA damaging agents also differs (Figures 3.4, 3.5, 3.7 and 3.8). As mentioned in chapter 1, increased sensitivity to radiation attributable to DSB repair defects, is a well-known characteristic of A-T cells (Riballo et al., 2004; Shiloh & Kastan 2001). Previous reports also confirm radiation sensitivity in the context of inactivating ATM mutations or using specific ATM inhibitors in malignant cells, (Collis et al., 2003; Filippi et al., 2006; Rainey et al., 2008; Kuroda et al., 2012, Batey et al., 2013). Thus, at maximal dose of 4Gy IR, we envisaged many of the IR induced DNA damage to be DSBs which should be lethal especially in ATM defective cells (Riballo et al., 2004). It was both intriguing and unexpected that H1395 was radiation resistant (Figure 3.4). Goodarzi et al. (2011) described IR-induced pS824 KAP1 as an enabling factor for DSB repair, thus, detecting pS824 KAP1 in H1395 seems consistent with an efficient DSB repair and might in part explain the lack of radiation sensitivity in this cell. Although multifactorial, increased DNA repair efficiency is

implicated in cisplatin resistance just as DSB repair integrity has been proposed as having impact on the sensitizing effect of PARPi (Basu and Krishnamurthy, 2010; Loser et al., 2010). Whether this is the case in H1395 cells in which we demonstrated PARPi and cisplatin resistance (as single agents or in combination treatment) remain unclear. Therefore, it will be worthwhile comparing for instance, DSB repair efficiency between H1395 and H23 cells in further studies. In general, whether ATM modulates response to cytotoxic treatments or PARP inhibition will require further investigations. ATM knockdown and or knock-in approach in an isogenic line would be ideal to study the precise role of ATM in the cells, considering the existence of between-cells genetic variations. The *ATM* gene can be restored back into H23 cells (i.e. cells having ATM loss) or alternatively, shRNA knock-down of *ATM* could be performed in one of the ATM proficient NSCLC cells (as previously done in our lab (Williamson et al., 2010). The ATM presence or absence will then be confirmed by western blot and the genetically altered cells can be tested for sensitivity to targeted and cytotoxic agents. Assuming the *ATM* knock-down cells show similar response as what we saw in H23, radio-sensitivity and PARPi-induced cytotoxicity in NSCLC cells can be reliably attributed to ATM. However, if the cells (*ATM* knock-down cells) exhibit responses different from H23, this opens up exploration of other genetic traits that possibly confer favorable therapeutic response in H23 cells.

4.2 Possible consequences of ATM loss in NSCLC

Based upon our observations (chapter 3), H23 cells clearly harbor some deficiencies in the ATM pathway and display phenotypes consistent with A-T cells and ATM deficient tumors (Figures 3.4 and 3.5). Impaired p53 signaling was demonstrated in H23 cells in addition to ATM deficiency (Figure 3.6). However, it seems unlikely that p53 functional defects alone will

account for the cytotoxicity seen with PARPi both as monotherapy and as combined treatments in H23 if one considers that, ATM proficient-p53 disrupted cells (H522 and H1793) were largely unaffected by the same agents (Figures 3.2, 3.5 and 3.6). This supports the finding that PARPi induces cell death independently of the p53 status (Nguyen et al., 2011).

Characteristically, A-T cells exhibit increased genomic instability often described as a potential mechanism for new mutations and therefore an enabling state for carcinogenesis (Hanahan and Winberg 2011). Although not the ideal for measuring genome instability states (Pikor et al., 2013), preliminary data derived from ATM gene sequencing performed in our lab (Petersen et al. unpublished, see Appendix E, Table E1) identified more base substitutions in H23 cells. Additionally, previous karyotyping of cancer cell lines ranked H23 in the top list of cells with chromosomal instability in the context of chromosome number (Roschke et al., 2003). Although, one report describes NSCLC as one of the most genetically unstable malignancies with high copy number alterations that are indicative of chromosomal instability, (Pikor et al., 2013), to my knowledge however, no correlation between ATM and genomic instability in NSCLC tumors has been described, thus studies to elucidate ATM status in the context of genetic heterogeneity will be informative. A great resource for instance to perform such analyses is the Cancer Cell Line Encyclopaedia (CCLE, http://www.broadinstitute.org/ccle) that has detailed genetic characterization of a large panel of human cancer cell lines. Moreover, when viewed from the point that A-T is cancer predisposing and of emerging evidences implicating ATM alteration in lung carcinogenesis (Yang et al., 2007; Lo et al., 2010; Shen et al., 2012), it raises further question as to whether ATM loss is a cause or consequence of malignant transformation in H23 cells. Examination of early-stage NSCLC specimens for ATM status may

predict the point in the carcinogenic process at which ATM is lost. Given that, NSCLC in non-smokers has (surprisingly) a greater proportion of altered genome than in smokers (Thu et al., 2012), further analysis is warranted to differentiate ATM functional status in the two cohorts (NSCLC in smokers versus in non-smokers), to predict whether ATM has impact on the aetiology of lung cancer specifically in never smokers.

4.2.1 Therapeutic implications of ATM deficiency in NSCLC

Despite the fact that radiation treatment is used in >60% of NSCLC cases, local tumor control still poses a remarkable challenge (Kong et al., 2014; Arriagada et al., 1991). Assuming the radio-sensitivity demonstrated in H23 was due to ATM inactivation just as previously described in A-T cells, it may be possible to individualise patients for radiotherapy based on ATM status. Although, the efficacy of cell killing is directly correlated with radiation dose delivered, toxicity to central organs limits high dose radiation delivery especially in locally advanced NSCLC even with advancements in technology (Kong et al., 2014; Grills et al., 2003). With ATM deficient NSCLC tumors, it may be possible to strategically deliver therapeutically effective low-dose radiotherapy. Considering that A-T heterozygotes (individuals who carry a single mutation at *ATM* gene locus) are estimated to be common among the general population (Swift, 2001), it is important to note that, radio-sensitivity associated with loss of ATM will favorably translate to clinical applications only when ATM deficiency is limited to malignant clones because of the emerging reports relating functional ATM polymorphisms with the development of severe toxicities to radiation therapy for cancers including NSCLC in individuals who are relatively healthy prior to developing cancers (Zhang et al., 2010; Yang et al., 2011; Xiong et al., 2012 & Raabe et al., 2012).

The observation of AZD2281 (PARPi) sensitivity in NSCLC cell line with ATM disruption (H23) as shown in figure 3.5, may be indicative of synthetic lethal interaction between ATM and PARP in NSCLC cells. To date, few studies have examined the applicability of PARPi in NSCLC (Albert et al., 2007; Senra et al., 2011; Paul et al., 2011; reviewed in O'Sullivan et al., 2014). With just one exception describing BRCA1 deficient NSCLC sensitivity to PARPi as a single agent, (Paul et al., 2011), other studies (pre-clinical and clinical trials) have examined PARPi mostly in combination treatment with radiation and or chemotherapeutic drugs ((Albert et al., 2007; Senra et al., 2011; O'Sullivan et al., 2014). As promising clinically as PARPi may appear, the fact remains that, potential predictive biomarkers of response are still required to inform the use of PARPi in specific targets that will mostly reap its benefit. Thus, it calls for continued research in the utility of PARPi in ATM deficient NSCLC tumors; given the accumulating evidence demonstrating PARPi sensitivity in ATM deficient malignancies (Weston et al., 2010; Williamson et al., 2010; Williamson et al., 2012; Patel et al., 2012). Of course, the possibility of treating ATM deficient NSCLC with PARPi as a monotherapy may minimise side-effects traditionally associated with conventional cytotoxic agents. Importantly, p53 gene is the most commonly mutated gene in lung adenocarcinoma (Ding et al., 2008) and our lab previously reported enhanced response to PARP inhibition in ATM-deficient p53 mutant MCL and gastric cancer cell lines (Williamson et al., 2012; Kubota et.al., 2014). Because p53 disruption was demonstrated in both H23 and H1395, will necessitate considerations to examine the p53 status alongside ATM, in the utility of PARPi as a single agent in our hands. We can perform this experiment with shRNA knock-down of ATM in both H460 and H552 which are ATM-

proficient cells with wild type and mutant p53 respectively and then evaluate their responses to PARP inhibition.

Typically in previous work, PARPi potentiates cisplatin toxicity in NSCLC cells (Michels et al. 2013a; Olaussen et al., 2013; Miknyoczki et al., 2003). Although the precise role of ATM in HR repair is not as of yet clearly defined ((Rass et al., 2013; Shiloh, 2003), because cisplatin can induce some types of DNA damage known to activate HR pathway (Borst et al., 2008) and that PARP inhibition is toxic in the context of HR repair defects (Bryant et al., 2005; Farmer et al., 2005), the hypothesis here could be that, potentiation of cisplatin-induced toxicity by PARP inhibition as seen in H23 (Figure 3.8) was due to ATM disruption impacting on HR repair efficiency. This hypothesis will require confirmation in future studies bearing in mind the limitations of our study.

4.3 Conclusion

In the present work, my findings suggest that loss of ATM seems to impact on the sensitivity to DNA- damaging agent such as IR as well as the targeted agent AZD2281 (PARPi) but warrants validation of the observation in further investigations. It should be noted that, in contrast to the possibility of a favorable response with radiation therapy, ATM inactivation on its own seems to be a potent survival mechanism in malignant clones against chemotherapeutic drugs, since the association between ATM down-regulation and chemo-resistance have been described in malignancies including NSCLC (Lundholm et al., 2013; Sousa et al., 2013; Cheung-Ong et al., 2013; Wang et al., 2012). However, from the point of synthetic lethality approach, ATM loss might be an inherent tumor defect which will predict sensitivity of tumors (including

NSCLC) to a novel combination of PARPi and chemotherapeutics (such as cisplatin) in addition to radiation treatment in the near future.

4.4 Future directions

Having suggested some research ideas above, in this subsequent section, I will add some flesh to the skeleton built during the course of my discussion above, in addition to other considerations for future studies:

As mentioned earlier, ATM loss has been identified in some of our NSCLC cells. In an attempt to understand the mechanism of its loss, our lab found a number of DNA sequence changes (base substitutions) in those NSCLC cells sequenced for *ATM* gene (Petersen et al., unpublished data). Whether these base substitutions are true missense mutations or just benign changes (single nucleotide polymorphisms) remain to be defined. As missense mutations can impact on ATM functions e.g. via dominant negative effects (Lavin et al., 2004), in situ mutagenesis approaches can effectively answers a question like distinguishing deleterious missense mutations from operationally neutral missense variants, as previously documented (Mitui et al., 2009; Barone et al., 2009; Lavin et al., 2004). That is stable transfections into a single A-T cell line e.g. L3 cells to isolate the effects of each allele on the cellular phenotype including intracellular ATM protein levels and cellular radio-sensitivity. Of course, mutational analysis is one experimental approach, it leaves open rooms for methods that can detect epigenetic or microRNA events as these are other potential mechanisms to silence the ATM gene. Whether environmental factors contribute to the loss of ATM in NSCLC would be another issue to resolve, given that smoking and the recently emerging radon exposure are major risk factors in the aetiology of the disease (Darby et al., 2006; Krewski et al., 2006; Dano et al.,

2000). Therefore, characterization of cytogenetic and molecular changes in relation to these environmental carcinogens will be necessary.

Furthermore, our lab found resistance to several other DNA damaging agents in addition to IR in H1395 cells (Appendix Figure E7). It will be reasonable to question whether death signaling pathway is intact in H1395. Although, the preliminary examination of apoptotic death signal pathway in the cell line post irradiation was inconclusive (Appendix figure E4), bearing in mind the short time period we employed in our method, re-examination of apoptosis might worth trying. Alternatively, flow cytometry assessment of mitotic catastrophe states, another death process which is importantly more relevant in IR- induced mechanism of cell death in solid tumors, may be appropriate in H1395 cells (Eriksson & Stigbrand 2010).

In the course of my discussion above, ATM shRNA knock-down was suggested as a feasible approach to determine the precise role of ATM in NSCLC cells. The knock-down cells can be tested for sensitivity to PARPi combinations with other cytotoxic agents in addition to IR and cisplatin. For instance, commonly used chemotherapeutic drugs in NSCLC like the vinka alkaloids (e.g. Topotecan), Taxanes (e.g. paclitaxel) and nucleoside analogues (gemcitabine) can be investigated.

It is noteworthy to mention that H23 cells belong to the adenocarcinoma subtype of NSCLC (Appendix Table A). Largely, many gene mutations or rearrangement of special interest (e.g. EGFR, ALK, KRAS), identified to date in NSCLC are associated with the adenocarcinoma histological subtype (Raparia et al., 2013). Perhaps, evaluating PARP inhibition in conjunction with other targeted therapies like the EGFR tyrosine kinase inhibitors will be interesting, given

the preclinical success that has been associated with this treatment strategy in head and neck and breast cancer (Nowsheen et al., 2011; Nowsheen et al., 2012).

In addition, assessment of genetic heterogeneity in association with ATM status of NSCLC was suggested above. Whether this will open an avenue for the discovery of new antigens as a result of genomic instability that may make NSCLC amenable to immune therapy is a possibility.

Of course, it goes without saying that an extension of the above mentioned future research in in-vivo studies as well as in the design of clinical trials will be desirable.

References

Agarwal, M. L., Agarwal, A., Taylor, W. R., & Stark, G. R. (1995). p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America, 92*(18), 8493-8497.

Albert, J. M., Cao, C., Kim, K. W., Willey, C. D., Geng, L., Xiao, D., Lu, B. (2007). Inhibition of poly(ADP-ribose) polymerase enhances cell death and improves tumor growth delay in irradiated lung cancer models. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research, 13*(10), 3033-3042. doi:10.1158/1078-0432.CCR-06-2872

Arriagada, R., Le Chevalier, T., Quoix, E., Ruffie, P., de Cremoux, H., Douillard, J. Y., Laplanche, A. (1991). ASTRO (american society for therapeutic radiology and oncology) plenary: Effect of chemotherapy on locally advanced non-small cell lung carcinoma: A randomized study of 353 patients. GETCB (groupe d'etude et traitement des cancers bronchiques), FNCLCC (federation nationale des centres de lutte contre le cancer) and the CEBI trialists. International Journal of Radiation Oncology, Biology, Physics, 20(6), 1183-1190.

Audeh, M. W., Carmichael, J., Penson, R. T., Friedlander, M., Powell, B., Bell-McGuinn, K. M., Tutt, A. (2010). Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and recurrent ovarian cancer: A proof-of-concept trial. *Lancet, 376*(9737), 245-251. doi: 10.1016/S0140-6736(10)60893-8; 10.1016/S0140-6736(10)60893-8.

Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C. W., Chessa, L., Ziv, Y. (1998). Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science (New York, N.Y.), 281*(5383), 1674-1677.

Bakkenist, C. J., & Kastan, M. B. (2003). DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature, 421*(6922), 499-506. doi:10.1038/nature01368.

Barone, G., Groom, A., Reiman, A., Srinivasan, V., Byrd, P. J., & Taylor, A. M. (2009). Modeling ATM mutant proteins from missense changes confirms retained kinase activity. *Human Mutation, 30*(8), 1222-1230. doi:10.1002/humu.21034; 10.1002/humu.21034.

Bartkova, J., Horejsi, Z., Koed, K., Kramer, A., Tort, F., Zieger, K., Bartek, J. (2005). DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature, 434*(7035), 864-870. doi: 10.1038/nature03482.

Bassing, C. H., & Alt, F. W. (2004). The cellular response to general and programmed DNA double strand breaks. *DNA Repair, 3*(8-9), 781-796. doi: 10.1016/j.dnarep.2004.06.001

Basu, A., & Krishnamurthy, S. (2010). Cellular responses to cisplatin-induced DNA damage. *Journal of Nucleic Acids, 2010*, 10.4061/2010/201367. doi: 10.4061/2010/201367; 10.4061/2010/201367.

Batey, M. A., Zhao, Y., Kyle, S., Richardson, C., Slade, A., Martin, N. M., Curtin, N. J. (2013). Preclinical evaluation of a novel ATM inhibitor, KU59403, in vitro and in vivo in p53 functional and dysfunctional models of human cancer. *Molecular Cancer Therapeutics*, doi: 10.1158/1535-7163.MCT-12-0707.

Bekker-Jensen, S., Rendtlew Danielsen, J., Fugger, K., Gromova, I., Nerstedt, A., Lukas, C., Mailand, N. (2010). HERC2 coordinates ubiquitin-dependent assembly of DNA repair factors on damaged chromosomes. *Nature Cell Biology*, *12*(1), 80-6; sup pp 1-12. doi: 10.1038/ncb2008; 10.1038/ncb2008.

Bergman, P., Brodin, D., Lewensohn, R., & de Petris, L. (2012). Validation of the 7th TNM classification for non-small cell lung cancer: A retrospective analysis on prognostic implications for operated node-negative cases. *Acta Oncologica (Stockholm, Sweden)*, doi: 10.3109/0284186X.2012.742960.

Biankin, A. V., Waddell, N., Kassahn, K. S., Gingras, M. C., Muthuswamy, L. B., Johns, A. L., et al. (2012). Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. Nature, 491(7424), 399-405.

Bijnsdorp, I. V., Giovannetti, E., & Peters, G. J. (2011). Analysis of drug interactions. *Methods in Molecular Biology (Clifton, N.J.), 731*, 421-434. doi: 10.1007/978-1-61779-080-5_34; 10.1007/978-1-61779-080-5_34.

Bishop, D. K., Ear, U., Bhattacharyya, A., Calderone, C., Beckett, M., Weichselbaum, R. R., & Shinohara, A. (1998). Xrcc3 is required for assembly of Rad51 complexes in vivo. *The Journal of Biological Chemistry, 273*(34), 21482-21488.

Boehler, C., Gauthier, L. R., Mortusewicz, O., Biard, D. S., Saliou, J. M., Bresson, A., Dantzer, F. (2011). Poly(ADP-ribose) polymerase 3 (PARP3), a newcomer in cellular response to DNA damage and mitotic progression. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(7), 2783-2788. doi:10.1073/pnas.1016574108;10.1073/pnas.1016574108.

Borst, P., Rottenberg, S., & Jonkers, J. (2008). How do real tumors become resistant to cisplatin? *Cell Cycle (Georgetown, Tex.), 7*(10), 1353-1359.

Bouchard, V. J., Rouleau, M., & Poirier, G. G. (2003). PARP-1, a determinant of cell survival in response to DNA damage. *Experimental Hematology, 31*(6), 446-454.

Bowman, K. J., Newell, D. R., Calvert, A. H., & Curtin, N. J. (2001). Differential effects of the poly (ADP-ribose) polymerase (PARP) inhibitor NU1025 on topoisomerase I and II inhibitor cytotoxicity in L1210 cells in vitro. *British Journal of Cancer, 84*(1), 106-112. doi: 10.1054/bjoc.2000.1555.

Bozhanov, S. S., Angelova, S. G., Krasteva, M. E., Markov, T. L., Christova, S. L., Gavrilov, I. G., et al. (2010). Alterations in p53, BRCA1, ATM, PIK3CA, and HER2 genes and their effect in modifying clinicopathological characteristics and overall survival of bulgarian patients with breast cancer. Journal of Cancer Research and Clinical Oncology, 136(11), 1657-1669.

Bredemeyer, A. L., Sharma, G. G., Huang, C. Y., Helmink, B. A., Walker, L. M., Khor, K. C., Sleckman, B. P. (2006). ATM stabilizes DNA double-strand-break complexes during V(D)J recombination. *Nature, 442*(7101), 466-470. doi: 10.1038/nature04866.

Brown, K. D., Ziv, Y., Sadanandan, S. N., Chessa, L., Collins, F. S., Shiloh, Y., & Tagle, D. A. (1997). The ataxia-telangiectasia gene product, a constitutively expressed nuclear protein that is not up-regulated following genome damage. *Proceedings of the National Academy of Sciences of the United States of America, 94*(5), 1840-1845.

Bryant, H. E., & Helleday, T. (2006). Inhibition of poly (ADP-ribose) polymerase activates ATM which is required for subsequent homologous recombination repair. *Nucleic Acids Research, 34*(6), 1685-1691. doi: 10.1093/nar/gkl108.

Bryant, H. E., Schultz, N., Thomas, H. D., Parker, K. M., Flower, D., Lopez, E., Helleday, T. (2005). Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature, 434*(7035), 913-917. doi: 10.1038/nature03443.

Budhram-Mahadeo, V. S., Bowen, S., Lee, S., Perez-Sanchez, C., Ensor, E., Morris, P. J., & Latchman, D. S. (2006). Brn-3b enhances the pro-apoptotic effects of p53 but not its induction of cell cycle arrest by cooperating in trans-activation of bax expression. *Nucleic Acids Research, 34*(22), 6640-6652. doi: 10.1093/nar/gkl878.

Campalans, A., Kortulewski, T., Amouroux, R., Menoni, H., Vermeulen, W., & Radicella, J. P. (2013). Distinct spatiotemporal patterns and PARP dependence of XRCC1 recruitment to single-strand break and base excision repair. *Nucleic Acids Research, 41*(5), 3115-3129. doi: 10.1093/nar/gkt025; 10.1093/nar/gkt025.

Canman, C. E., & Lim, D. S. (1998). The role of ATM in DNA damage responses and cancer. *Oncogene, 17*(25), 3301-3308. doi: 10.1038/sj.onc.1202577.

Chen, S., Paul, P., & Price, B. D. (2003). ATM's leucine-rich domain and adjacent sequences are essential for ATM to regulate the DNA damage response. *Oncogene, 22*(41), 6332-6339. doi:10.1038/sj.onc.1206760.

Cheung-Ong, K., Giaever, G., & Nislow, C. (2013). DNA-damaging agents in cancer chemotherapy: Serendipity and chemical biology. *Chemistry & Biology, 20*(5), 648-659. doi: 10.1016/j.chembiol.2013.04.007; 10.1016/j.chembiol.2013.04.007.

Collis, S. J., Swartz, M. J., Nelson, W. G., & DeWeese, T. L. (2003). Enhanced radiation and chemotherapy-mediated cell killing of human cancer cells by small inhibitory RNA silencing of DNA repair factors. *Cancer Research, 63*(7), 1550-1554.

Curtin, N. J., Wang, L. Z., Yiakouvaki, A., Kyle, S., Arris, C. A., Canan-Koch, S., Newell, D. R. (2004). Novel poly(ADP-ribose) polymerase-1 inhibitor, AG14361, restores sensitivity to

temozolomide in mismatch repair-deficient cells. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research, 10*(3), 881-889.

Daniel, J. A., Pellegrini, M., Lee, B. S., Guo, Z., Filsuf, D., Belkina, N. V., Nussenzweig, A. (2012). Loss of ATM kinase activity leads to embryonic lethality in mice. *The Journal of Cell Biology, 198*(3), 295-304. doi: 10.1083/jcb.201204035.

Darby, S., Hill, D., Deo, H., Auvinen, A., Barros-Dios, J. M., Baysson, H., Doll, R. (2006). Residential radon and lung cancer--detailed results of a collaborative analysis of individual data on 7148 persons with lung cancer and 14,208 persons without lung cancer from 13 epidemiologic studies in europe. *Scandinavian Journal of Work, Environment & Health, 32 Suppl 1*, 1-83.

Deckbar, D., Stiff, T., Koch, B., Reis, C., Lobrich, M., & Jeggo, P. A. (2010). The limitations of the G1-S checkpoint. *Cancer Research, 70*(11), 4412-4421. doi: 10.1158/0008-5472.CAN-09-3198; 10.1158/0008-5472.CAN-09-3198.

Ding, L., Getz, G., Wheeler, D. A., Mardis, E. R., McLellan, M. D., Cibulskis, K., et al. (2008). Somatic mutations affect key pathways in lung adenocarcinoma. Nature, 455(7216), 1069-1075

Donawho, C. K., Luo, Y., Luo, Y., Penning, T. D., Bauch, J. L., Bouska, J. J., Frost, D. J. (2007). ABT-888, an orally active poly(ADP-ribose) polymerase inhibitor that potentiates DNA-damaging agents in preclinical tumor models. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research, 13*(9), 2728-2737. doi: 10.1158/1078-0432.CCR-06-3039.

Dungey, F. A., Loser, D. A., & Chalmers, A. J. (2008). Replication-dependent radiosensitization of human glioma cells by inhibition of poly(ADP-ribose) polymerase: Mechanisms and

therapeutic potential. *International Journal of Radiation Oncology, Biology, Physics, 72*(4), 1188-1197. doi: 10.1016/j.ijrobp.2008.07.031; 10.1016/j.ijrobp.2008.07.031.

Eriksson, D., & Stigbrand, T. (2010). Radiation-induced cell death mechanisms. *Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine, 31*(4), 363-372. doi:10.1007/s13277-010-0042-8; 10.1007/s13277-010-0042-8.

Espinosa, E., Zamora, P., Feliu, J., & Gonzalez Baron, M. (2003). Classification of anticancer drugs--a new system based on therapeutic targets. *Cancer Treatment Reviews, 29*(6), 515-523.

Falck, J., Coates, J., & Jackson, S. P. (2005). Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage. *Nature, 434*(7033), 605-611. doi: 10.1038/nature03442

Farmer, H., McCabe, N., Lord, C. J., Tutt, A. N., Johnson, D. A., Richardson, T. B., Ashworth, A. (2005). Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature, 434*(7035), 917-921. doi: 10.1038/nature03445.

Fenton, A. L., Shirodkar, P., Macrae, C. J., Meng, L., & Koch, C. A. (2013). The PARP3- and ATM-dependent phosphorylation of APLF facilitates DNA double-strand break repair. *Nucleic Acids Research,* doi: 10.1093/nar/gkt134.

Filippi, A. R., Franco, P., Galliano, M., & Ricardi, U. (2006). Peripheral blood complete remission after splenic irradiation in mantle-cell lymphoma with 11q22-23 deletion and ATM inactivation. *Radiation Oncology (London, England), 1,* 35. doi: 10.1186/1748-717X-1-35.

Fisher, A. E., Hochegger, H., Takeda, S., & Caldecott, K. W. (2007). Poly(ADP-ribose) polymerase 1 accelerates single-strand break repair in concert with poly(ADP-ribose) glycohydrolase. *Molecular and Cellular Biology, 27*(15), 5597-5605. doi:10.1128/MCB.02248-06.

Fong, P. C., Boss, D. S., Yap, T. A., Tutt, A., Wu, P., Mergui-Roelvink, M., de Bono, J. S. (2009). Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. *The New England Journal of Medicine, 361*(2), 123-134. doi: 10.1056/NEJMoa0900212; 10.1056/NEJMoa0900212.

Fong, P. C., Yap, T. A., Boss, D. S., Carden, C. P., Mergui-Roelvink, M., Gourley, C., Kaye, S. B. (2010). Poly(ADP)-ribose polymerase inhibition: Frequent durable responses in BRCA carrier ovarian cancer correlating with platinum-free interval. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology, 28*(15), 2512-2519. doi: 10.1200/JCO.2009.26.9589.

Gately, D. P., Hittle, J. C., Chan, G. K., & Yen, T. J. (1998). Characterization of ATM expression, localization, and associated DNA-dependent protein kinase activity. *Molecular Biology of the Cell, 9*(9), 2361-2374.

Gatti, R. A., Berkel, I., Boder, E., Braedt, G., Charmley, P., Concannon, P., Lange, K. (1988). Localization of an ataxia-telangiectasia gene to chromosome 11q22-23. *Nature, 336*(6199), 577-580. doi: 10.1038/336577a0.

Gerber, D. E., & Minna, J. D. (2010). ALK inhibition for non-small cell lung cancer: From discovery to therapy in record time. *Cancer Cell, 18*(6), 548-551. doi: 10.1016/j.ccr.2010.11.033; 10.1016/j.ccr.2010.11.033.

Girard, P. M., Riballo, E., Begg, A. C., Waugh, A., & Jeggo, P. A. (2002). Nbs1 promotes ATM dependent phosphorylation events including those required for G1/S arrest. *Oncogene, 21*(27), 4191-4199. doi: 10.1038/sj.onc.1205596.

Goodarzi, A. A., & Lees-Miller, S. P. (2004). Biochemical characterization of the ataxia-telangiectasia mutated (ATM) protein from human cells. *DNA Repair, 3*(7), 753-767. doi: 10.1016/j.dnarep.2004.03.041.

Goodarzi, A. A., Jeggo, P., & Lobrich, M. (2010). The influence of heterochromatin on DNA double strand break repair: Getting the strong, silent type to relax. *DNA Repair, 9*(12), 1273-1282. doi: 10.1016/j.dnarep.2010.09.013.

Goodarzi, A. A., Kurka, T., & Jeggo, P. A. (2011). KAP-1 phosphorylation regulates CHD3 nucleosome remodeling during the DNA double-strand break response. *Nature Structural & Molecular Biology, 18*(7), 831-839. doi:10.1038/nsmb.2077; 10.1038/nsmb.2077.

Gottipati, P., Vischioni, B., Schultz, N., Solomons, J., Bryant, H. E., Djureinovic, T., Helleday, T. (2010). Poly(ADP-ribose) polymerase is hyperactivated in homologous recombination-defective cells. *Cancer Research, 70*(13), 5389-5398. doi: 10.1158/0008-5472.CAN-09-4716; 10.1158/0008-5472.CAN-09-4716.

Grills, I. S., Yan, D., Martinez, A. A., Vicini, F. A., Wong, J. W., & Kestin, L. L. (2003). Potential for reduced toxicity and dose escalation in the treatment of inoperable non-small-cell lung cancer: A comparison of intensity-modulated radiation therapy (IMRT), 3D conformal radiation, and elective nodal irradiation. *International Journal of Radiation Oncology, Biology, Physics, 57*(3), 875-890.

Gronbaek, K., Worm, J., Ralfkiaer, E., Ahrenkiel, V., Hokland, P., & Guldberg, P. (2002). ATM mutations are associated with inactivation of the ARF-TP53 tumor suppressor pathway in diffuse large B-cell lymphoma. *Blood, 100*(4), 1430-1437. doi: 10.1182/blood-2002-02-0382.

Groome, P. A., Bolejack, V., Crowley, J. J., Kennedy, C., Krasnik, M., Sobin, L. H., Participating Institutions. (2007). The IASLC lung cancer staging project: Validation of the proposals for revision of the T, N, and M descriptors and consequent stage groupings in the forthcoming (seventh) edition of the TNM classification of malignant tumours. *Journal of Thoracic Oncology : Official Publication of the International Association for the Study of Lung Cancer, 2*(8), 694-705. doi: 10.1097/JTO.0b013e31812d05d5.

Haince, J. F., Kozlov, S., Dawson, V. L., Dawson, T. M., Hendzel, M. J., Lavin, M. F., & Poirier, G. G. (2007). Ataxia telangiectasia mutated (ATM) signaling network is modulated by a novel poly(ADP-ribose)-dependent pathway in the early response to DNA-damaging agents. *The Journal of Biological Chemistry, 282*(22), 16441-16453. doi: 10.1074/jbc.M608406200.

Haince, J. F., McDonald, D., Rodrigue, A., Dery, U., Masson, J. Y., Hendzel, M. J., & Poirier, G. G. (2008). PARP1-dependent kinetics of recruitment of MRE11 and NBS1 proteins to multiple DNA damage sites. *The Journal of Biological Chemistry, 283*(2), 1197-1208. doi: 10.1074/jbc.M706734200.

Halazonetis, T. D., Gorgoulis, V. G., & Bartek, J. (2008). An oncogene-induced DNA damage model for cancer development. *Science (New York, N.Y.), 319*(5868), 1352-1355. doi: 10.1126/science.1140735; 10.1126/science.1140735.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell, 144*(5), 646-674. doi:10.1016/j.cell.2011.02.013.

Harper, J. W., & Elledge, S. J. (2007). The DNA damage response: Ten years after. *Molecular Cell, 28*(5), 739-745. doi: 10.1016/j.molcel.2007.11.015.

Hay, T., Matthews, J. R., Pietzka, L., Lau, A., Cranston, A., Nygren, A. O., Clarke, A. R. (2009). Poly(ADP-ribose) polymerase-1 inhibitor treatment regresses autochthonous Brca2/p53-mutant mammary tumors in vivo and delays tumor relapse in combination with carboplatin. *Cancer Research, 69*(9), 3850-3855. doi: 10.1158/0008-5472.CAN-08-2388; 10.1158/0008-5472.CAN-08-2388.

Helleday, T. (2011). The underlying mechanism for the PARP and BRCA synthetic lethality: Clearing up the misunderstandings. *Molecular Oncology, 5*(4), 387-393. doi: 10.1016/j.molonc.2011.07.001; 10.1016/j.molonc.2011.07.001.

Hochegger, H., Dejsuphong, D., Fukushima, T., Morrison, C., Sonoda, E., Schreiber, V., Takeda, S. (2006). Parp-1 protects homologous recombination from interference by ku and ligase IV in vertebrate cells. *The EMBO Journal, 25*(6), 1305-1314. doi: 10.1038/sj.emboj.7601015.

Huyen, Y., Zgheib, O., Ditullio, R. A.,Jr, Gorgoulis, V. G., Zacharatos, P., Petty, T. J., Halazonetis, T. D. (2004). Methylated lysine 79 of histone H3 targets 53BP1 to DNA double-strand breaks. *Nature, 432*(7015), 406-411. doi: 10.1038/nature03114.

Ikejima, M., Noguchi, S., Yamashita, R., Ogura, T., Sugimura, T., Gill, D. M., & Miwa, M. (1990). The zinc fingers of human poly(ADP-ribose) polymerase are differentially required for the recognition of DNA breaks and nicks and the consequent enzyme activation. other structures recognize intact DNA. *The Journal of Biological Chemistry, 265*(35), 21907-21913.

Ismail, F., Ikram, M., Purdie, K., Harwood, C., Leigh, I., & Storey, A. (2011). Cutaneous squamous cell carcinoma (SCC) and the DNA damage response: PATM expression patterns in pre-malignant and malignant keratinocyte skin lesions. *PloS One, 6*(7), e21271. doi: 10.1371/journal.pone.0021271; 10.1371/journal.pone.0021271.

Jackson, S. P., & Bartek, J. (2009). The DNA-damage response in human biology and disease. *Nature, 461*(7267), 1071-1078. doi: 10.1038/nature08467.

Kang, B., Guo, R. F., Tan, X. H., Zhao, M., Tang, Z. B., & Lu, Y. Y. (2008). Expression status of ataxia-telangiectasia-mutated gene correlated with prognosis in advanced gastric cancer. *Mutation Research, 638*(1-2), 17-25. doi: 10.1016/j.mrfmmm.2007.08.013.

Khanna, K. K., & Jackson, S. P. (2001). DNA double-strand breaks: Signaling, repair and the cancer connection. *Nature Genetics, 27*(3), 247-254. doi: 10.1038/85798.

Khanna, K. K., Keating, K. E., Kozlov, S., Scott, S., Gatei, M., Hobson, K., Lavin, M. F. (1998). ATM associates with and phosphorylates p53: Mapping the region of interaction. *Nature Genetics, 20*(4), 398-400. doi:10.1038/3882.

Kickhoefer, V. A., Siva, A. C., Kedersha, N. L., Inman, E. M., Ruland, C., Streuli, M., & Rome, L. H. (1999). The 193-kD vault protein, VPARP, is a novel poly(ADP-ribose) polymerase. *The Journal of Cell Biology, 146*(5), 917-928.

Kim, S. T., Lim, D. S., Canman, C. E., & Kastan, M. B. (1999). Substrate specificities and identification of putative substrates of ATM kinase family members. *The Journal of Biological Chemistry, 274*(53), 37538-37543.

Kitamoto, Y., Sakurai, H., Mitsuhashi, N., Akimoto, T., & Nakano, T. (2003). Caffeine diminishes cytotoxic effects of paclitaxel on a human lung adenocarcinoma cell line. *Cancer Letters, 191*(1), 101-107.

Kleine, H., Poreba, E., Lesniewicz, K., Hassa, P. O., Hottiger, M. O., Litchfield, D. W., Luscher, B. (2008). Substrate-assisted catalysis by PARP10 limits its activity to mono-ADP-ribosylation. *Molecular Cell, 32*(1), 57-69. doi: 10.1016/j.molcel.2008.08.009; 10.1016/j.molcel.2008.08.009.

Kong, F. M., Zhao, J., Wang, J., & Faivre-Finn, C. (2014). Radiation dose effect in locally advanced non-small cell lung cancer. *Journal of Thoracic Disease, 6*(4), 336-347. doi:10.3978/j.issn.2072-1439.2014.01.23.

Kozlov, S. V., Graham, M. E., Jakob, B., Tobias, F., Kijas, A. W., Tanuji, M., Lavin, M. F. (2011). Autophosphorylation and ATM activation: Additional sites add to the complexity. *The Journal of Biological Chemistry, 286*(11), 9107-9119. doi:10.1074/jbc.M110.204065.

Krewski, D., Lubin, J. H., Zielinski, J. M., Alavanja, M., Catalan, V. S., Field, R. W., Wilcox, H. B. (2006). A combined analysis of north american case-control studies of residential radon and lung cancer. *Journal of Toxicology and Environmental Health.Part A, 69*(7), 533-597. doi:10.1080/15287390500260945.

Kubota, E., Williamson, C. T., Ye, R., Elegbede, A., Petersen, L., Lees-Miller, S. P., & Bebb, D. G. (2014). Low ATM protein expression and depletion of p53 correlates with olaparib sensitivity in gastric cancer cell lines. *Cell Cycle (Georgetown, Tex.), 13*(13).

Kuroda, S., Urata, Y., & Fujiwara, T. (2012). Ataxia-telangiectasia mutated and the Mre11-Rad50-NBS1 complex: Promising targets for radiosensitization. *Acta Medica Okayama, 66*(2), 83-92.

Kurz, E. U., & Lees-Miller, S. P. (2004). DNA damage-induced activation of ATM and ATM-dependent signaling pathways. *DNA Repair, 3*(8-9), 889-900. doi: 10.1016/j.dnarep.2004.03.029.

Langelier, M. F., Riccio, A. A., & Pascal, J. M. (2014). PARP-2 and PARP-3 are selectively activated by 5' phosphorylated DNA breaks through an allosteric regulatory mechanism shared with PARP-1. *Nucleic Acids Research, 42*(12), 7762-7775. doi:10.1093/nar/gku474; 10.1093/nar/gku474.

Lavin, M. F., Scott, S., Gueven, N., Kozlov, S., Peng, C., & Chen, P. (2004). Functional consequences of sequence alterations in the ATM gene. *DNA Repair, 3*(8-9), 1197-1205. doi: 10.1016/j.dnarep.2004.03.011.

Ledermann, J., Harter, P., Gourley, C., Friedlander, M., Vergote, I., Rustin, G., Matulonis, U. (2012). Olaparib maintenance therapy in platinum-sensitive relapsed ovarian cancer. *The New England Journal of Medicine, 366*(15), 1382-1392. doi: 10.1056/NEJMoa1105535; 10.1056/NEJMoa1105535.

Lee, J. H., Goodarzi, A. A., Jeggo, P. A., & Paull, T. T. (2010). 53BP1 promotes ATM activity through direct interactions with the MRN complex. *The EMBO Journal, 29*(3), 574-585. doi: 10.1038/emboj.2009.372; 10.1038/emboj.2009.372.

Lee, J. H., & Paull, T. T. (2005). ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. *Science (New York, N.Y.), 308*(5721), 551-554. doi: 10.1126/science.1108297.

Lee, Y. R., Yu, D. S., Liang, Y. C., Huang, K. F., Chou, S. J., Chen, T. C., Huang, H. S. (2013). New approaches of PARP-1 inhibitors in human lung cancer cells and cancer stem-like cells by some selected anthraquinone-derived small molecules. *PloS One, 8*(2), e56284. doi: 10.1371/journal.pone.0056284; 10.1371/journal.pone.0056284.

Li, M., Lu, L. Y., Yang, C. Y., Wang, S., & Yu, X. (2013). The FHA and BRCT domains recognize ADP-ribosylation during DNA damage response. *Genes & Development, 27*(16), 1752-1768. doi:10.1101/gad.226357.113; 10.1101/gad.226357.113.

Lips, J., & Kaina, B. (2001). DNA double-strand breaks trigger apoptosis in p53-deficient fibroblasts. *Carcinogenesis, 22*(4), 579-585.

Lo, Y. L., Hsiao, C. F., Jou, Y. S., Chang, G. C., Tsai, Y. H., Su, W. C., et al. (2010). ATM polymorphisms and risk of lung cancer among never smokers. Lung Cancer (Amsterdam, Netherlands), 69(2), 148-154.

Lobrich, M., Shibata, A., Beucher, A., Fisher, A., Ensminger, M., Goodarzi, A. A., Jeggo, P. A. (2010). gammaH2AX foci analysis for monitoring DNA double-strand break repair: Strengths, limitations and optimization. *Cell Cycle (Georgetown, Tex.), 9*(4), 662-669.

Loser, D. A., Shibata, A., Shibata, A. K., Woodbine, L. J., Jeggo, P. A., & Chalmers, A. J. (2010). Sensitization to radiation and alkylating agents by inhibitors of poly(ADP-ribose) polymerase is enhanced in cells deficient in DNA double-strand break repair. *Molecular Cancer Therapeutics, 9*(6), 1775-1787. doi:10.1158/1535-7163.MCT-09-1027.

Loseva, O., Jemth, A. S., Bryant, H. E., Schuler, H., Lehtio, L., Karlberg, T., & Helleday, T. (2010). PARP-3 is a mono-ADP-ribosylase that activates PARP-1 in the absence of DNA. *The Journal of Biological Chemistry, 285*(11), 8054-8060. doi: 10.1074/jbc.M109.077834; 10.1074/jbc.M109.077834.

Lu, F., & Zhang, H. T. (2011). DNA methylation and nonsmall cell lung cancer. *Anatomical Record (Hoboken, N.J.: 2007), 294*(11), 1787-1795. doi: 10.1002/ar.21471; 10.1002/ar.21471

Lukas, C., Melander, F., Stucki, M., Falck, J., Bekker-Jensen, S., Goldberg, M., Lukas, J. (2004). Mdc1 couples DNA double-strand break recognition by Nbs1 with its H2AX-dependent chromatin retention. *The EMBO Journal, 23*(13), 2674-2683. doi: 10.1038/sj.emboj.7600269.

Lundholm, L., Haag, P., Zong, D., Juntti, T., Mork, B., Lewensohn, R., & Viktorsson, K. (2013). Resistance to DNA-damaging treatment in non-small cell lung cancer tumor-initiating cells

involves reduced DNA-PK/ATM activation and diminished cell cycle arrest. *Cell Death & Disease, 4*, e478. doi: 10.1038/cddis.2012.211; 10.1038/cddis.2012.211.

Mahaney, B. L., Meek, K., & Lees-Miller, S. P. (2009). Repair of ionizing radiation-induced DNA double-strand breaks by non-homologous end-joining. *The Biochemical Journal, 417*(3), 639-650. doi: 10.1042/BJ20080413; 10.1042/BJ20080413.

Mangerich, A., & Burkle, A. (2011). How to kill tumor cells with inhibitors of poly(ADP-ribosyl)ation. *International Journal of Cancer.Journal International Du Cancer, 128*(2), 251-265. doi: 10.1002/ijc.25683; 10.1002/ijc.25683.

Massarelli, E., Johnson, F. M., Erickson, H. S., Wistuba, I. I., & Papadimitrakopoulou, V. (2013). Uncommon epidermal growth factor receptor mutations in non-small cell lung cancer and their mechanisms of EGFR tyrosine kinase inhibitors sensitivity and resistance. *Lung Cancer (Amsterdam, Netherlands),* doi: 10.1016/j.lungcan.2013.01.018; 10.1016/j.lungcan.2013.01.018.

Masutani, M., Nozaki, T., Nakamoto, K., Nakagama, H., Suzuki, H., Kusuoka, O., Sugimura, T. (2000). The response of parp knockout mice against DNA damaging agents. *Mutation Research, 462*(2-3), 159-166.

Mazumder Indra, D., Mitra, S., Roy, A., Mondal, R. K., Basu, P. S., Roychoudhury, S., Panda, C. K. (2011). Alterations of ATM and CADM1 in chromosomal 11q22.3-23.2 region are associated with the development of invasive cervical carcinoma. *Human Genetics, 130*(6), 735-748. doi: 10.1007/s00439-011-1015-8; 10.1007/s00439-011-1015-8.

McCabe, N., Turner, N. C., Lord, C. J., Kluzek, K., Bialkowska, A., Swift, S., Ashworth, A. (2006). Deficiency in the repair of DNA damage by homologous recombination and sensitivity

to poly(ADP-ribose) polymerase inhibition. *Cancer Research, 66*(16), 8109-8115. doi: 10.1158/0008-5472.CAN-06-0140.

McKinnon, P. J. (2012). ATM and the molecular pathogenesis of ataxia telangiectasia. *Annual Review of Pathology, 7*, 303-321. doi: 10.1146/annurev-pathol-011811-132509.

Melander, F., Bekker-Jensen, S., Falck, J., Bartek, J., Mailand, N., & Lukas, J. (2008). Phosphorylation of SDT repeats in the MDC1 N terminus triggers retention of NBS1 at the DNA damage-modified chromatin. *The Journal of Cell Biology, 181*(2), 213-226. doi: 10.1083/jcb.200708210; 10.1083/jcb.200708210.

Menissier de Murcia, J., Ricoul, M., Tartier, L., Niedergang, C., Huber, A., Dantzer, F., de Murcia, G. (2003). Functional interaction between PARP-1 and PARP-2 in chromosome stability and embryonic development in mouse. *The EMBO Journal, 22*(9), 2255-2263. doi:10.1093/emboj/cdg206.

Menisser-de Murcia, J., Mark, M., Wendling, O., Wynshaw-Boris, A., & de Murcia, G. (2001). Early embryonic lethality in PARP-1 atm double-mutant mice suggests a functional synergy in cell proliferation during development. *Molecular and Cellular Biology, 21*(5), 1828-1832. doi: 10.1128/MCB.21.5.1828-1832.2001.

Meyn, M. S. (1995). Ataxia-telangiectasia and cellular responses to DNA damage. *Cancer Research, 55*(24), 5991-6001.

Michels, J., Vitale, I., Senovilla, L., Enot, D. P., Garcia, P., Lissa, D., Kroemer, G. (2013). Synergistic interaction between cisplatin and PARP inhibitors in non-small cell lung cancer. *Cell Cycle (Georgetown, Tex.), 12*(6).

Michels, J., Vitale, I., Galluzzi, L., Adam, J., Olaussen, K. A., Kepp, O., Kroemer, G. (2013). Cisplatin resistance associated with PARP hyperactivation. *Cancer Research, 73*(7), 2271-2280. doi:10.1158/0008-5472.CAN-12-3000; 10.1158/0008-5472.CAN-12-3000.

Miknyoczki, S. J., Jones-Bolin, S., Pritchard, S., Hunter, K., Zhao, H., Wan, W., Ruggeri, B. (2003). Chemopotentiation of temozolomide, irinotecan, and cisplatin activity by CEP-6800, a poly(ADP-ribose) polymerase inhibitor. *Molecular Cancer Therapeutics, 2*(4), 371-382.

Mitui, M., Nahas, S. A., Du, L. T., Yang, Z., Lai, C. H., Nakamura, K., Gatti, R. A. (2009). Functional and computational assessment of missense variants in the ataxia-telangiectasia mutated (ATM) gene: Mutations with increased cancer risk. *Human Mutation, 30*(1), 12-21. doi:10.1002/humu.20805; 10.1002/humu.20805.

Miwa, M., & Masutani, M. (2007). PolyADP-ribosylation and cancer. *Cancer Science, 98*(10), 1528-1535. doi: 10.1111/j.1349-7006.2007.00567.x.

Montoni, A., Robu, M., Pouliot, E., & Shah, G. M. (2013). Resistance to PARP-inhibitors in cancer therapy. *Frontiers in Pharmacology, 4*, 18. doi: 10.3389/fphar.2013.00018; 10.3389/fphar.2013.00018.

Murai, J., Huang, S. Y., Das, B. B., Renaud, A., Zhang, Y., Doroshow, J. H., Pommier, Y. (2012). Trapping of PARP1 and PARP2 by clinical PARP inhibitors. *Cancer Research, 72*(21), 5588-5599. doi: 10.1158/0008-5472.CAN-12-2753; 10.1158/0008-5472.CAN-12-2753.

Nowsheen, S., Bonner, J. A., Lobuglio, A. F., Trummell, H., Whitley, A. C., Dobelbower, M. C., & Yang, E. S. (2011). Cetuximab augments cytotoxicity with poly (adp-ribose) polymerase inhibition in head and neck cancer. *PloS One, 6*(8), e24148. doi:10.1371/journal.pone.0024148; 10.1371/journal.pone.0024148.

Nowsheen, S., Cooper, T., Stanley, J. A., & Yang, E. S. (2012). Synthetic lethal interactions between EGFR and PARP inhibition in human triple negative breast cancer cells. *PloS One, 7*(10), e46614. doi:10.1371/journal.pone.0046614; 10.1371/journal.pone.0046614.

O'Connor P.M, Jackman J., Bae I., Myers T.G, Fan S., Mutor M et al., (1997). Characterization of the p53 Tumor Suppressor pathway in cell lines of the National Cancer Institute Anticancer Drug Screen and Correlations with the Growth-Inhibitory potency of 123 Anticancer agents. Cancer Res. 57: 4285-4300.

O'Sullivan, C. C., Moon, D. H., Kohn, E. C., & Lee, J. M. (2014). Beyond breast and ovarian cancers: PARP inhibitors for BRCA mutation-associated and BRCA-like solid tumors. *Frontiers in Oncology, 4*, 42. doi:10.3389/fonc.2014.00042.

Patel, A. G., De Lorenzo, S. B., Flatten, K. S., Poirier, G. G., & Kaufmann, S. H. (2012). Failure of iniparib to inhibit poly(ADP-ribose) polymerase in vitro. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research, 18*(6), 1655-1662. doi: 10.1158/1078-0432.CCR-11-2890; 10.1158/1078-0432.CCR-11-2890.

Paul, I., Savage, K. I., Blayney, J. K., Lamers, E., Gately, K., Kerr, K., Fennell, D. A. (2011). PARP inhibition induces BAX/BAK-independent synthetic lethality of BRCA1-deficient non-small cell lung cancer. *The Journal of Pathology, 224*(4), 564-574. doi:10.1002/path.2925; 10.1002/path.2925.

Perry, J., & Kleckner, N. (2003). The ATRs, ATMs, and TORs are giant HEAT repeat proteins. *Cell, 112*(2), 151-155.

Pikor, L., Thu, K., Vucic, E., & Lam, W. (2013). The detection and implication of genome instability in cancer. *Cancer Metastasis Reviews, 32*(3-4), 341-352. doi:10.1007/s10555-013-9429-5; 10.1007/s10555-013-9429-5.

Raabe, A., Derda, K., Reuther, S., Szymczak, S., Borgmann, K., Hoeller, U., Dikomey, E. (2012). Association of single nucleotide polymorphisms in the genes ATM, GSTP1, SOD2, TGFB1, XPD and XRCC1 with risk of severe erythema after breast conserving radiotherapy. *Radiation Oncology (London, England), 7*, 65-717X-7-65. doi: 10.1186/1748-717X-7-65; 10.1186/1748-717X-7-65.

Rainey, M. D., Charlton, M. E., Stanton, R. V., & Kastan, M. B. (2008). Transient inhibition of ATM kinase is sufficient to enhance cellular sensitivity to ionizing radiation. *Cancer Research, 68*(18), 7466-7474. doi: 10.1158/0008-5472.CAN-08-0763; 10.1158/0008-5472.CAN-08-0763.

Raparia, K., Villa, C., DeCamp, M. M., Patel, J. D., & Mehta, M. P. (2013). Molecular profiling in non-small cell lung cancer: A step toward personalized medicine. *Archives of Pathology & Laboratory Medicine, 137*(4), 481-491. doi:10.5858/arpa.2012-0287-RA; 10.5858/arpa.2012-0287-RA.

Rass, E., Chandramouly, G., Zha, S., Alt, F. W., & Xie, A. (2013). Ataxia telangiectasia mutated (ATM) is dispensable for endonuclease I-SceI-induced homologous recombination in mouse embryonic stem cells. *The Journal of Biological Chemistry, 288*(10), 7086-7095. doi: 10.1074/jbc.M112.445825; 10.1074/jbc.M112.445825.

Reiman, A., Srinivasan, V., Barone, G., Last, J. I., Wootton, L. L., Davies, E. G., Taylor, A. M. (2011). Lymphoid tumours and breast cancer in ataxia telangiectasia; substantial protective effect

of residual ATM kinase activity against childhood tumours. British Journal of Cancer, 105(4), 586-591. doi: 10.1038/bjc.2011.266; 10.1038/bjc.2011.266.

Reina-San-Martin, B., Chen, H. T., Nussenzweig, A., & Nussenzweig, M. C. (2004). ATM is required for efficient recombination between immunoglobulin switch regions. *The Journal of Experimental Medicine, 200*(9), 1103-1110. doi: 10.1084/jem.20041162.

Riabinska, A., Daheim, M., Herter-Sprie, G. S., Winkler, J., Fritz, C., Hallek, M., Reinhardt, H. C. (2013). Therapeutic targeting of a robust non-oncogene addiction to PRKDC in ATM-defective tumors. *Science Translational Medicine, 5*(189), 189ra78. doi:10.1126/scitranslmed.3005814; 10.1126/scitranslmed.3005814.

Riballo, E., Kuhne, M., Rief, N., Doherty, A., Smith, G. C., Recio, M. J., Lobrich, M. (2004). A pathway of double-strand break rejoining dependent upon ATM, artemis, and proteins locating to gamma-H2AX foci. *Molecular Cell, 16*(5), 715-724. doi: 10.1016/j.molcel.2004.10.029.

Richardson, C., Horikoshi, N., & Pandita, T. K. (2004). The role of the DNA double-strand break response network in meiosis. *DNA Repair, 3*(8-9), 1149-1164. doi: 10.1016/j.dnarep.2004.05.007.

Richardson, C., & Jasin, M. (2000). Frequent chromosomal translocations induced by DNA double-strand breaks. *Nature, 405*(6787), 697-700. doi:10.1038/35015097.

Riley, T., Sontag, E., Chen, P., & Levine, A. (2008). Transcriptional control of human p53-regulated genes. *Nature Reviews.Molecular Cell Biology, 9*(5), 402-412. doi: 10.1038/nrm2395; 10.1038/nrm2395.

Rogakou, E. P., Pilch, D. R., Orr, A. H., Ivanova, V. S., & Bonner, W. M. (1998). DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *The Journal of Biological Chemistry, 273*(10), 5858-5868.

Rooney, S., Chaudhuri, J., & Alt, F. W. (2004). The role of the non-homologous end-joining pathway in lymphocyte development. *Immunological Reviews, 200*, 115-131. doi: 10.1111/j.0105-2896.2004.00165.x.

Roschke, A. V., Tonon, G., Gehlhaus, K. S., McTyre, N., Bussey, K. J., Lababidi, S., Kirsch, I. R. (2003). Karyotypic complexity of the NCI-60 drug-screening panel. *Cancer Research, 63*(24), 8634-8647.

Rossi, D., & Gaidano, G. (2012). ATM and chronic lymphocytic leukemia: Mutations, and not only deletions, matter. *Haematologica, 97*(1), 5-8. doi: 10.3324/haematol.2011.057109; 10.3324/haematol.2011.057109.

Rottenberg, S., Jaspers, J. E., Kersbergen, A., van der Burg, E., Nygren, A. O., Zander, S. A., Jonkers, J. (2008). High sensitivity of BRCA1-deficient mammary tumors to the PARP inhibitor AZD2281 alone and in combination with platinum drugs. *Proceedings of the National Academy of Sciences of the United States of America, 105*(44), 17079-17084. doi: 10.1073/pnas.0806092105; 10.1073/pnas.0806092105.

Rulten, S. L., Fisher, A. E., Robert, I., Zuma, M. C., Rouleau, M., Ju, L., Caldecott, K. W. (2011). PARP-3 and APLF function together to accelerate nonhomologous end-joining. *Molecular Cell, 41*(1), 33-45. doi: 10.1016/j.molcel.2010.12.006; 10.1016/j.molcel.2010.12.006.

Safar, A. M., Spencer, H.,3rd, Su, X., Coffey, M., Cooney, C. A., Ratnasinghe, L. D., Fan, C. Y. (2005). Methylation profiling of archived non-small cell lung cancer: A promising prognostic

system. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research, 11*(12), 4400-4405. doi: 10.1158/1078-0432.CCR-04-2378.

Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Shiloh, Y. (1995). A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science (New York, N.Y.), 268*(5218), 1749-1753.

Schiller, J. H., Harrington, D., Belani, C. P., Langer, C., Sandler, A., Krook, J., Eastern Cooperative Oncology Group. (2002). Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. *The New England Journal of Medicine, 346*(2), 92-98. doi: 10.1056/NEJMoa011954.

Schreiber, V., Dantzer, F., Ame, J. C., & de Murcia, G. (2006). Poly(ADP-ribose): Novel functions for an old molecule. *Nature Reviews.Molecular Cell Biology, 7*(7), 517-528. doi: 10.1038/nrm1963.

Senra, J. M., Telfer, B. A., Cherry, K. E., McCrudden, C. M., Hirst, D. G., O'Connor, M. J., Stratford, I. J. (2011). Inhibition of PARP-1 by olaparib (AZD2281) increases the radiosensitivity of a lung tumor xenograft. *Molecular Cancer Therapeutics, 10*(10), 1949-1958. doi:10.1158/1535-7163.MCT-11-0278.

Shafman, T., Khanna, K. K., Kedar, P., Spring, K., Kozlov, S., Yen, T., Lavin, M. F. (1997). Interaction between ATM protein and c-abl in response to DNA damage. *Nature, 387*(6632), 520-523. doi:10.1038/387520a0.

Shen L, Yin Z.H, Wan Y, Zhang Y, Li K, Zhou B.S. (2012). Association between ATM polymorphisms and cancer risk: a meta-analysis. Mol Biol Rep 39:5719–5725.

Shibata, A., Barton, O., Noon, A. T., Dahm, K., Deckbar, D., Goodarzi, A. A., Jeggo, P. A. (2010). Role of ATM and the damage response mediator proteins 53BP1 and MDC1 in the maintenance of G(2)/M checkpoint arrest. *Molecular and Cellular Biology, 30*(13), 3371-3383. doi: 10.1128/MCB.01644-09; 10.1128/MCB.01644-09.

Shiloh, Y. (1995). Ataxia-telangiectasia: Closer to unraveling the mystery. *European Journal of Human Genetics : EJHG, 3*(2), 116-138.

Shiloh, Y. (2003). ATM and related protein kinases: Safeguarding genome integrity. *Nature Reviews.Cancer, 3*(3), 155-168. doi: 10.1038/nrc1011.

Shiloh, Y., & Kastan, M. B. (2001). ATM: Genome stability, neuronal development, and cancer cross paths. *Advances in Cancer Research, 83*, 209-254.

Shiloh, Y., & Ziv, Y. (2012). The ATM protein: The importance of being active. *The Journal of Cell Biology, 198*(3), 273-275. doi: 10.1083/jcb.201207063.

Sousa, M. M., Zub, K. A., Aas, P. A., Hanssen-Bauer, A., Demirovic, A., Sarno, A., Slupphaug, G. (2013). An inverse switch in DNA base excision and strand break repair contributes to melphalan resistance in multiple myeloma cells. *PloS One, 8*(2), e55493. doi:10.1371/journal.pone.0055493; 10.1371/journal.pone.0055493.

Staibano, S., Pepe, S., Lo Muzio, L., Somma, P., Mascolo, M., Argenziano, G., De Rosa, G. (2005). Poly(adenosine diphosphate-ribose) polymerase 1 expression in malignant melanomas from photoexposed areas of the head and neck region. *Human Pathology, 36*(7), 724-731. doi: 10.1016/j.humpath.2005.04.017.

Stankovic, T., Kidd, A. M., Sutcliffe, A., McGuire, G. M., Robinson, P., Weber, P., Taylor, A. M. (1998). ATM mutations and phenotypes in ataxia-telangiectasia families in the british isles:

Expression of mutant ATM and the risk of leukemia, lymphoma, and breast cancer. *American Journal of Human Genetics, 62*(2), 334-345. doi:10.1086/301706.

Statistics Canada (2011). Percentage distribution of cancer incidence and mortality of the four most commonly diagnosed cancers versus all other types, Canada, 2007. Retrieved on Jan. 20, 2013 from: http://www.statcan.gc.ca/pub/82-624-x/2011001/article/chart/11596-01-chart1-eng.htm.

Stewart, G. S., Last, J. I., Stankovic, T., Haites, N., Kidd, A. M., Byrd, P. J., & Taylor, A. M. (2001). Residual ataxia telangiectasia mutated protein function in cells from ataxia telangiectasia patients, with 5762ins137 and 7271T-->G mutations, showing a less severe phenotype. *The Journal of Biological Chemistry, 276*(32), 30133-30141. doi:10.1074/jbc.M103160200.

Stiff, T., O'Driscoll, M., Rief, N., Iwabuchi, K., Lobrich, M., & Jeggo, P. A. (2004). ATM and DNA-PK function redundantly to phosphorylate H2AX after exposure to ionizing radiation. *Cancer Research, 64*(7), 2390-2396.

Stucki, M., Clapperton, J. A., Mohammad, D., Yaffe, M. B., Smerdon, S. J., & Jackson, S. P. (2005). MDC1 directly binds phosphorylated histone H2AX to regulate cellular responses to DNA double-strand breaks. *Cell, 123*(7), 1213-1226. doi: 10.1016/j.cell.2005.09.038.

Swift, M. (2001). Public health burden of cancer in ataxia-telangiectasia heterozygotes. *Journal of the National Cancer Institute, 93*(2), 84-85.

Tentori, L., Leonetti, C., Scarsella, M., D'Amati, G., Vergati, M., Portarena, I., Graziani, G. (2003). Systemic administration of GPI 15427, a novel poly(ADP-ribose) polymerase-1 inhibitor, increases the antitumor activity of temozolomide against intracranial melanoma,

glioma, lymphoma. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research, 9*(14), 5370-5379.

Thu, K. L., Vucic, E. A., Chari, R., Zhang, W., Lockwood, W. W., English, J. C., Lam, W. L. (2012). Lung adenocarcinoma of never smokers and smokers harbor differential regions of genetic alteration and exhibit different levels of genomic instability. *PloS One, 7*(3), e33003. doi:10.1371/journal.pone.0033003; 10.1371/journal.pone.0033003.

Tommiska, J., Bartkova, J., Heinonen, M., Hautala, L., Kilpivaara, O., Eerola, H., et al. (2008). The DNA damage signalling kinase ATM is aberrantly reduced or lost in BRCA1/BRCA2-deficient and ER/PR/ERBB2-triple-negative breast cancer. Oncogene, 27(17), 2501-2506.

Tomoda, T., Kurashige, T., Moriki, T., Yamamoto, H., Fujimoto, S., & Taniguchi, T. (1991). Enhanced expression of poly(ADP-ribose) synthetase gene in malignant lymphoma. *American Journal of Hematology*, *37*(4), 223-227.

Tramontano, F., Malanga, M., & Quesada, P. (2007). Differential contribution of poly(ADP-ribose)polymerase-1 and -2 (PARP-1 and -2) to the poly(ADP-ribosyl)ation reaction in rat primary spermatocytes. *Molecular Human Reproduction, 13*(11), 821-828. doi: 10.1093/molehr/gam062.

Tutt, A., Robson, M., Garber, J. E., Domchek, S. M., Audeh, M. W., Weitzel, J. N., Carmichael, J. (2010). Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and advanced breast cancer: A proof-of-concept trial. *Lancet, 376*(9737), 235-244. doi: 10.1016/S0140-6736(10)60892-6; 10.1016/S0140-6736(10)60892-6.

Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M., & Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions, 160*(1), 1-40. doi: 10.1016/j.cbi.2005.12.009.

Wang, T. H., Wang, H. S., & Soong, Y. K. (2000). Paclitaxel-induced cell death: Where the cell cycle and apoptosis come together. *Cancer, 88*(11), 2619-2628.

Wang, M., Wu, W., Wu, W., Rosidi, B., Zhang, L., Wang, H., & Iliakis, G. (2006). PARP-1 and ku compete for repair of DNA double strand breaks by distinct NHEJ pathways. *Nucleic Acids Research, 34*(21), 6170-6182. doi: 10.1093/nar/gkl840.

Wang, C., & Lees-Miller, S. P. (2013). Detection and repair of ionizing radiation-induced DNA double strand breaks: New developments in nonhomologous end joining. *International Journal of Radiation Oncology, Biology, Physics, 86*(3), 440-449. doi:10.1016/j.ijrobp.2013.01.011; 10.1016/j.ijrobp.2013.01.011.

Weston, V. J., Oldreive, C. E., Skowronska, A., Oscier, D. G., Pratt, G., Dyer, M. J., Stankovic, T. (2010). The PARP inhibitor olaparib induces significant killing of ATM-deficient lymphoid tumor cells in vitro and in vivo. *Blood, 116*(22), 4578-4587. doi: 10.1182/blood-2010-01-265769

World Health Organization (WHO), (February, 2012). Cancer. Retrieved from: http://www.who.int/mediacentre/factsheets/fs297/en/.

Williamson, C. T., Muzik, H., Turhan, A. G., Zamo, A., O'Connor, M. J., Bebb, D. G., & Lees-Miller, S. P. (2010). ATM deficiency sensitizes mantle cell lymphoma cells to poly(ADP-ribose) polymerase-1 inhibitors. *Molecular Cancer Therapeutics, 9*(2), 347-357. doi: 10.1158/1535-7163.MCT-09-0872.

Williamson, C. T., Kubota, E., Hamill, J. D., Klimowicz, A., Ye, R., Muzik, H., Lees-Miller, S. P. (2012). Enhanced cytotoxicity of PARP inhibition in mantle cell lymphoma harbouring mutations in both ATM and p53. *EMBO Molecular Medicine, 4*(6), 515-527. doi:10.1002/emmm.201200229; 10.1002/emmm.201200229.

Xie, A., Puget, N., Shim, I., Odate, S., Jarzyna, I., Bassing, C. H., Scully, R. (2004). Control of sister chromatid recombination by histone H2AX. *Molecular Cell, 16*(6), 1017-1025. doi: 10.1016/j.molcel.2004.12.007.

Xiong, H., Liao, Z., Liu, Z., Xu, T., Wang, Q., Liu, H., Wei, Q. (2012). ATM polymorphisms predict severe radiation pneumonitis in patients with non-small cell lung cancer treated with definitive radiation therapy. *International Journal of Radiation Oncology, Biology, Physics,* doi: 10.1016/j.ijrobp.2012.09.024; 10.1016/j.ijrobp.2012.09.024.

Yamamoto, K., Wang, Y., Jiang, W., Liu, X., Dubois, R. L., Lin, C. S., Zha, S. (2012). Kinase-dead ATM protein causes genomic instability and early embryonic lethality in mice. *The Journal of Cell Biology, 198*(3), 305-313. doi: 10.1083/jcb.201204098.

Yang, M., Zhang, L., Bi, N., Ji, W., Tan, W., Zhao, L., Lin, D. (2011). Association of P53 and ATM polymorphisms with risk of radiation-induced pneumonitis in lung cancer patients treated with radiotherapy. *International Journal of Radiation Oncology, Biology, Physics, 79*(5), 1402-1407. doi: 10.1016/j.ijrobp.2009.12.042.

Yazdi, P. T., Wang, Y., Zhao, S., Patel, N., Lee, E. Y., & Qin, J. (2002). SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint. *Genes & Development, 16*(5), 571-582. doi: 10.1101/gad.970702.

Yelamos, J., Schreiber, V., & Dantzer, F. (2008). Toward specific functions of poly(ADP-ribose) polymerase-2. *Trends in Molecular Medicine, 14*(4), 169-178. doi:10.1016/j.molmed.2008.02.003; 10.1016/j.molmed.2008.02.003.

You, Z., Chahwan, C., Bailis, J., Hunter, T., & Russell, P. (2005). ATM activation and its recruitment to damaged DNA require binding to the C terminus of Nbs1. *Molecular and Cellular Biology*, *25*(13), 5363-5379. doi: 10.1128/MCB.25.13.5363-5379.2005.

You, Z., Shi, L. Z., Zhu, Q., Wu, P., Zhang, Y. W., Basilio, A., Hunter, T. (2009). CtIP links DNA double-strand break sensing to resection. *Molecular Cell, 36*(6), 954-969. doi: 10.1016/j.molcel.2009.12.002.

Zha, S., Sekiguchi, J., Brush, J. W., Bassing, C. H., & Alt, F. W. (2008). Complementary functions of ATM and H2AX in development and suppression of genomic instability. *Proceedings of the National Academy of Sciences of the United States of America, 105*(27), 9302-9306. doi: 10.1073/pnas.0803520105; 10.1073/pnas.0803520105.

Zhang, C. C., Yang, J. M., Bash-Babula, J., White, E., Murphy, M., Levine, A. J., & Hait, W. N. (1999). DNA damage increases sensitivity to vinca alkaloids and decreases sensitivity to taxanes through p53-dependent repression of microtubule-associated protein 4. *Cancer Research, 59*(15), 3663-3670.

Zhang, X., Miao, X., Liang, G., Hao, B., Wang, Y., Tan, W., Lin, D. (2005). Polymorphisms in DNA base excision repair genes ADPRT and XRCC1 and risk of lung cancer. *Cancer Research, 65*(3), 722-726.

Zhang, L., Yang, M., Bi, N., Fang, M., Sun, T., Ji, W., Wang, L. (2010). ATM polymorphisms are associated with risk of radiation-induced pneumonitis. *International Journal of Radiation Oncology, Biology, Physics, 77*(5), 1360-1368. doi: 10.1016/j.ijrobp.2009.07.1675.

Zhang, X. P., Liu, F., & Wang, W. (2011). Two-phase dynamics of p53 in the DNA damage response. *Proceedings of the National Academy of Sciences of the United States of America, 108*(22), 8990-8995. doi: 10.1073/pnas.1100600108; 10.1073/pnas.1100600108.

Ziv, Y., Bielopolski, D., Galanty, Y., Lukas, C., Taya, Y., Schultz, D. C., Shiloh, Y. (2006). Chromatin relaxation in response to DNA double-strand breaks is modulated by a novel ATM- and KAP-1 dependent pathway. *Nature Cell Biology*, *8*(8), 870-876. doi: 10.1038/ncb1446.

Appendix A: Pathological characteristics of NSCLC cell lines

Table A: Specific characteristics of the examined NSCLC cell lines
Cell lines information retrieved from ATCC (marked as §); CCLE (marked as ☨); COSMIC Database (marked as **)
*O'Connor et al., 1997; ***Roschke et al., 2003; N/A: not available
Modal chromosome number (M), Number of numerical (N) and structural (S) chromosomal changes

Cell lines	ATM protein expression	*ATM* and TP53 status	**No of identified mutations	*** Karyotype			§Histological subtype	§Sex of origin
				M	N	S		
NCI-H23	Reduced level relative to normal lymphoblastic cells, BT	☨*ATM* mutation : codon 1919, A -> C (glutamine -> proline) ☨TP53 mutation: codon 246, C -> G (isoleucine -> methionine)	494 in 472 genes analyzed	57	12	28	Adenocarcinoma	Male
NCI-H1395	Undetectable level (faint ATM bands in western blot with long film exposure)	**ATM* mutation : codon 2666, A-> G (threonine -> alanine) **TP53 non-mutant	45 in 4670 genes analyzed	N/A	N/A	N/A	Adenocarcinoma	Female
NCI-H226	Detectable level relative to normal lymphoblastic cells, BT	*ATM* status N/A *TP53 mutation: codon 309 (C -> G, phenylalanine -> alanine)	80 in 96 genes analyzed	62	16	17	Squamous cell carcinoma	Male
NCI-H460	Detectable level relative to normal lymphoblastic cells, BT	*ATM* status N/A *Wild type p53	228 in 227 genes analyzed	53	7	8	Large cell carcinoma	Male

Cell lines	ATM protein expression	ATM and TP53 status	**No of identified mutations	***Karyotype			§Histological subtype	§Sex of origin
				M	N	S		
NCI-H522	Detectable level relative to normal lymphoblastic cells, BT	*ATM* status N/A *TP53 mutation: codon 191 deletion, G -> frame shift (proline -> none)	167 in 164 genes analyzed	51	11	9	Adenocarcinoma	Male
HCC4006	Detectable level relative to normal lymphoblastic cells, BT	*ATM & TP53* status N/A	22 in 21 genes analyzed	N/A	N/A	N/A	Adenocarcinoma	Male
NCI-H1793	Detectable level relative to normal lymphoblastic cells, BT	*ATM* status N/A §TP53 mutations codon 209, T -> A (arginine -> none); codon 273, G -> A (arginine -> histidine)	0 in 4 genes analyzed	N/A	N/A	N/A	Adenocarcinoma	Female

Appendix B: Gamma cell 1000 ^{137}Cs source

B

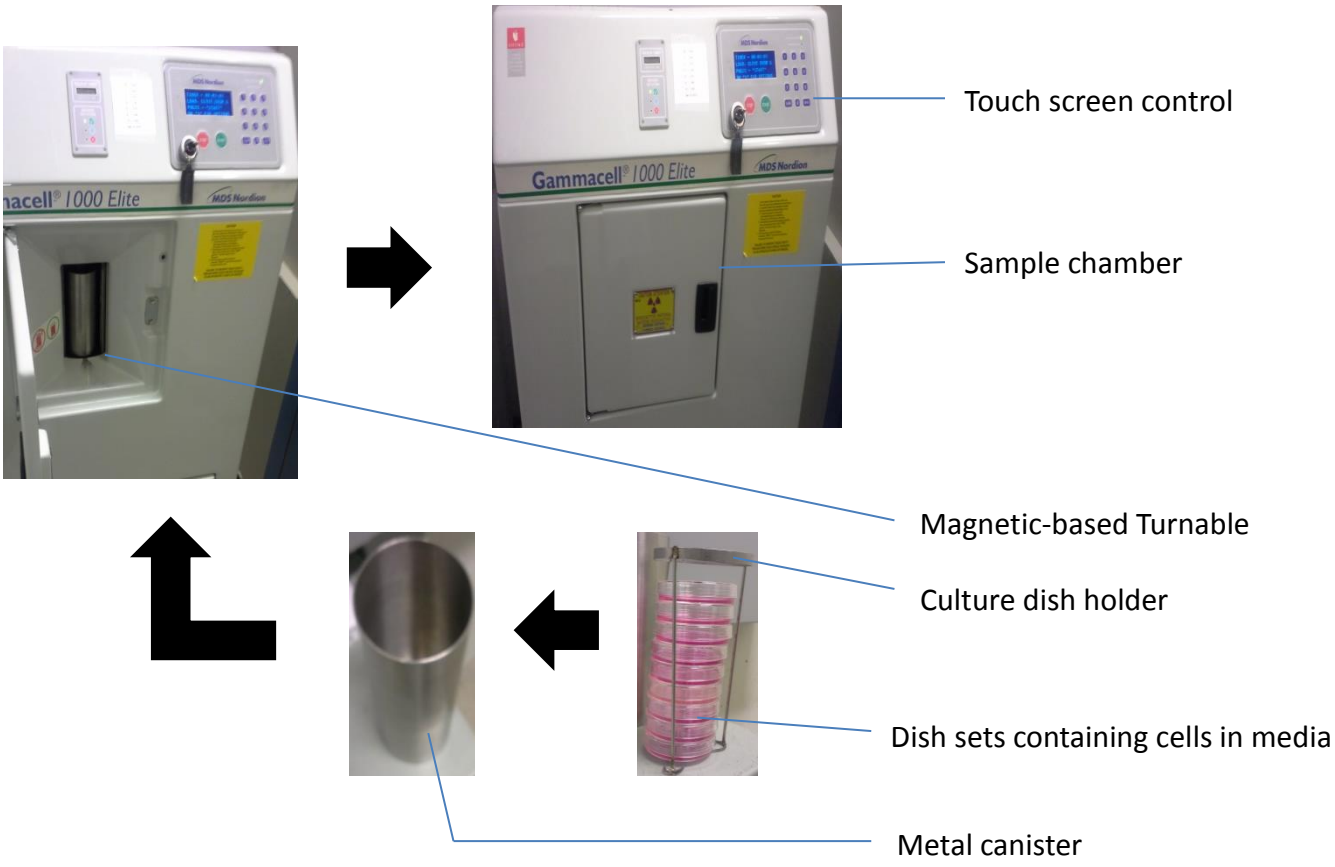


Figure B: Gamma Cell 1000® Elite (irradiator)
Steps involved in irradiating cells using Gamma Cell 1000 irradiator, set of culture dishes are loaded onto a dish holder which is placed in a metal canister. The canister is transferred to the irradiator where it is held in place by a turnable within the sample chamber.

Appendix C: Antibodies information

Table C: Antibody condition for western blot

RT- room temperature, O/N- overnight

a. Epitope is between amino acid residue 1 and 50 of human Tripartite Motif-Containing 28 using the numbering given in entry NP_005753.1 (Gene ID 10155)
b. Epitope is between residues 11-25 of p53 of human origin
c. Epitope is between residue 1175 and the C-terminus of human SMC1 using the numbering given in entry NP_006297.2

◊. The exact epitope not specified by the company

Antigen	Specie	Primary antibody at 4°C O/N	Secondary antibody at RT	Company
◊ATM	Rabbit	1:3000	1:2000, 1hr	Epitomics
◊ p-S1981 ATM	Rabbit	1:5000	1:2000, 1hr	Epitomics
DNA-PKcs (DPK1A)	Rabbit	1:10,000	1:3000, 0.5hr	In house
◊ p-S2056 DNA-PKcs	Rabbit	1:5000	1:2000, 1hr	Epitomics
KAP1[a]	Rabbit	1:2000	1:3000, 0.5hr	Abcam
p-S824 KAP1	Rabbit	2µg/ml	1:3000, 1hr	In house
p53 (DOI)[b]	Mouse	1:2000	1:3000, 1hr	Santa Cruz
◊ p-S15 p53	Rabbit	1:200	1:2000, 1hr	Cell signaling
SMC1[c]	Rabbit	1:2000	1:3000, 1hr	Novus
◊PARP-1	Rabbit	1:3000	1:2000, 1hr	Cell signaling

Appendix D: Gene-specific primers sequences used in RT-PCR

Table D: RT- PCR specific primer sequence

Gene	Forward primers	Reverse primers
GADD45α	GTCGACGTTGAGCAGCTTG	CCGAAAGGATGGATAAGGTG
GUSB	CGTCCCACCTAGAATCTGCT	TTGCTCACAAAGGTCACAGG
p21	AATCTGTCATGCTGGTCTGC	GGAAGACCATGTGGACCTGT
PUMA	GCACCTAATTGGGCTCCATC	GGAGACAAGAGGAGCAGCAG

Appendix E: Supplementary data

E1: DNA damage responsive proteins in NSCLC cell lines

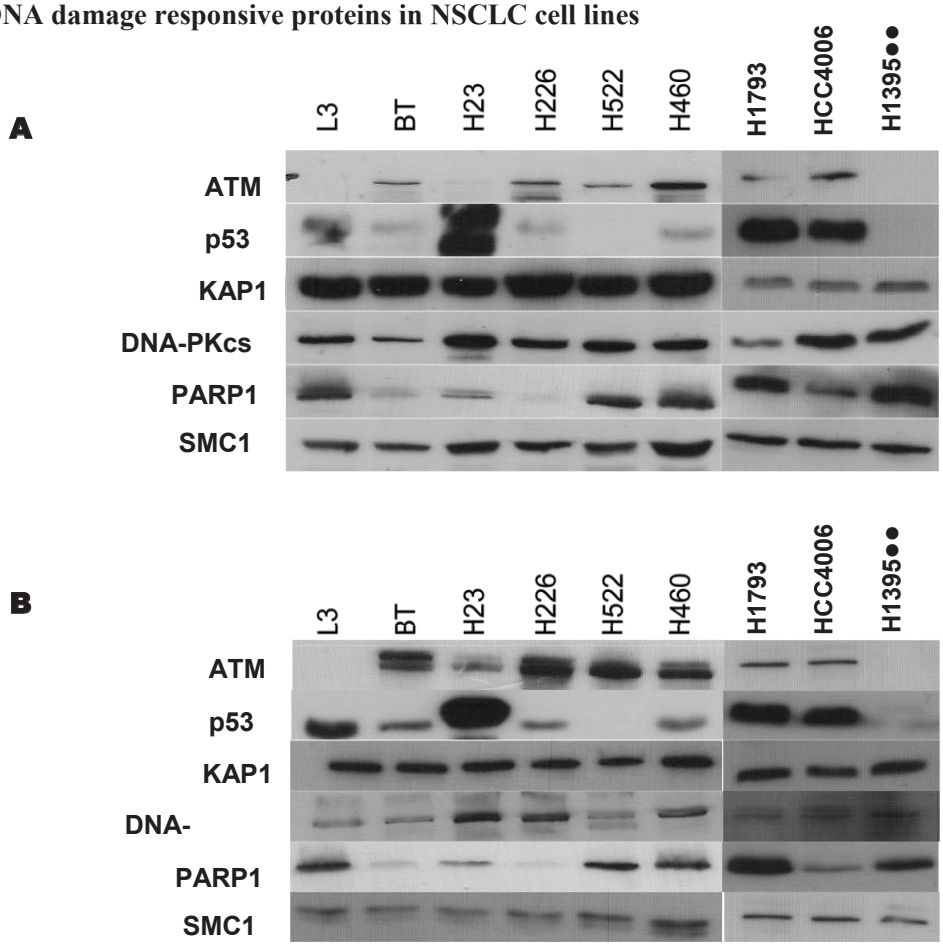
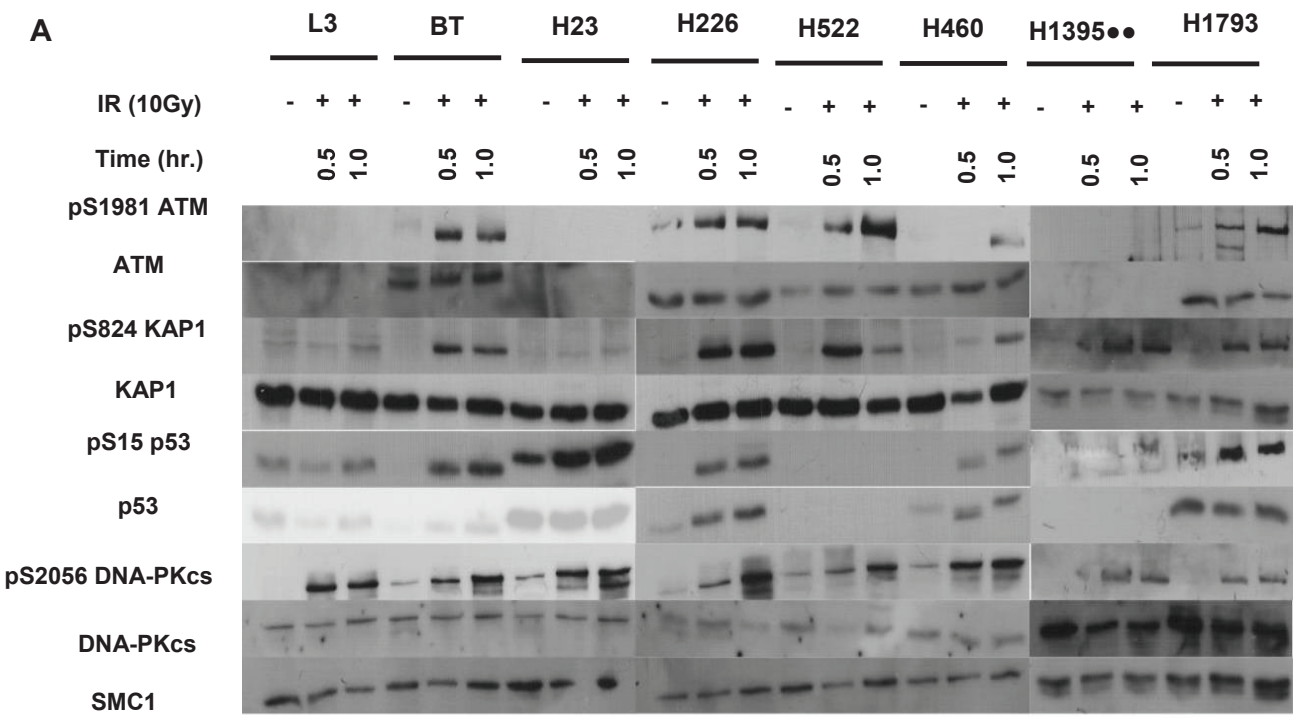
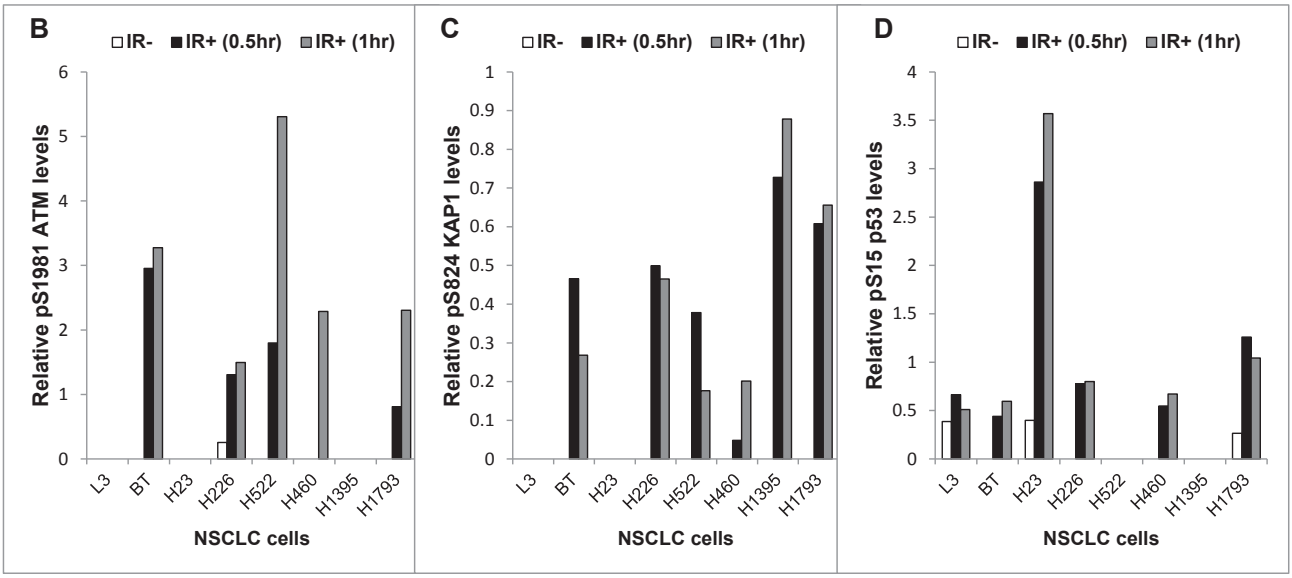


Figure E1: Analysis of DNA damage responsive proteins in NSCLC cell lines
Western blot as in figure 3.1.1, A and B are replicates of figure 3.1.1

E2: Phosphorylation of ATM downstream target in NSCLC cell lines




B
IR-
IR+ (0.5hr)
IR+ (1hr)
Relative pS1981 ATM levels
0
1
2
3
4
5
6
L3
BT
H23
H226
H522
H460
H1395
H1793
NSCLC cells
C
IR-
IR+ (0.5hr)
IR+ (1hr)
Relative pS824 KAP1 levels
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
L3
BT
H23
H226
H522
H460
H1395
H1793
NSCLC cells
D
IR-
IR+ (0.5hr)
IR+ (1hr)
Relative pS15 p53 levels
0
0.5
1
1.5
2
2.5
3
3.5
4
L3
BT
H23
H226
H522
H460
H1395
H1793
NSCLC cells

E

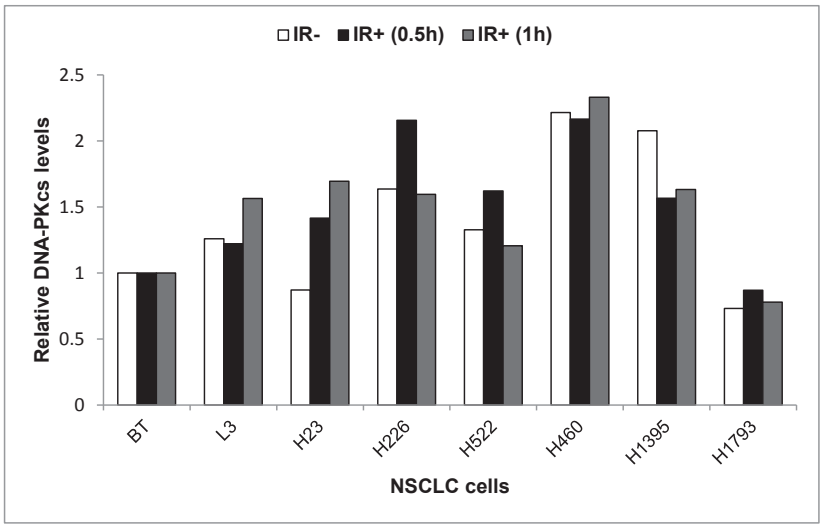
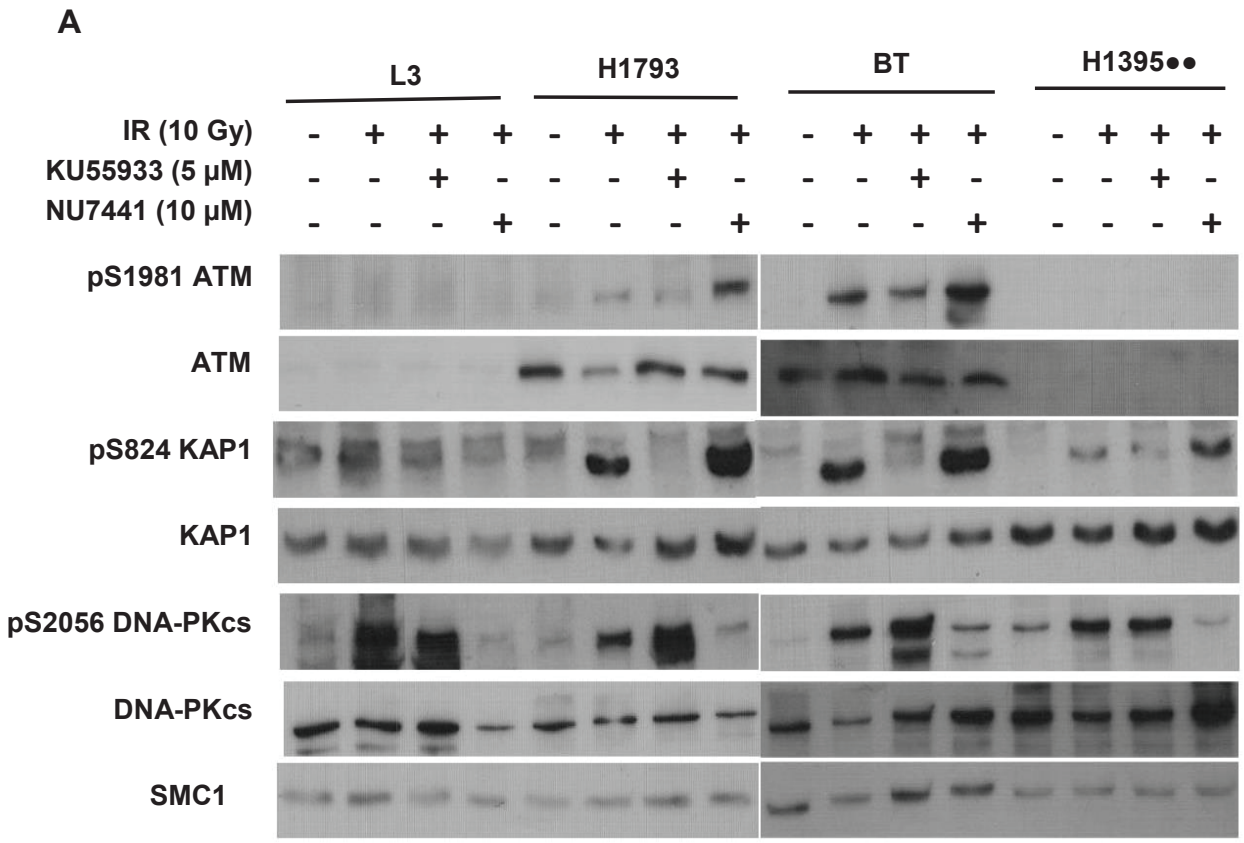
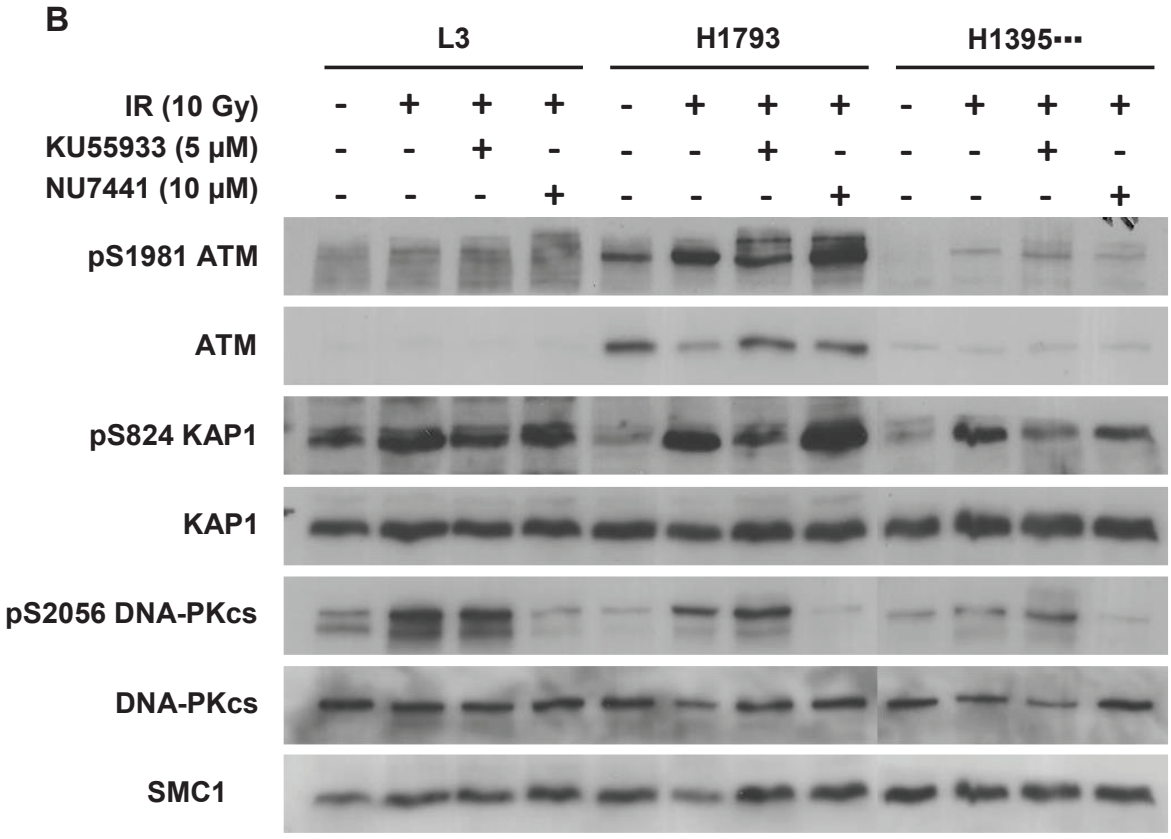


Figure E2: Differential phosphorylation of ATM downstream target in NSCLC cell lines with undetectable ATM
Experimental method as in figure 3.2A. E2A is a replicate of figure 3.2A. The relative pS1981 ATM, pS824 KAP1 and pS15 p53 in E2A were quantified using ImageJ and normalized to ATM, KAP1 and p53 respectively. In addition, DNA-PKcs in E2A was normalized to SMC1 and expressed relative to BT cell as follows: DNA-PKcs in untreated cells (IR-) relative to DNA-PKcs in untreated BT cell i.e. IR- NSCLC cell/ IR- BT cell, DNA-PKcs in the treated cells (IR+ 0.5h or 1h)) relative to DNA-PKcs in treated BT cell i.e. IR+ (0.5h) NSCLC cell/ IR+ (0.5h) BT cell or IR+ (1h) NSCLC cell/ IR+ (1h) BT cell respectively.

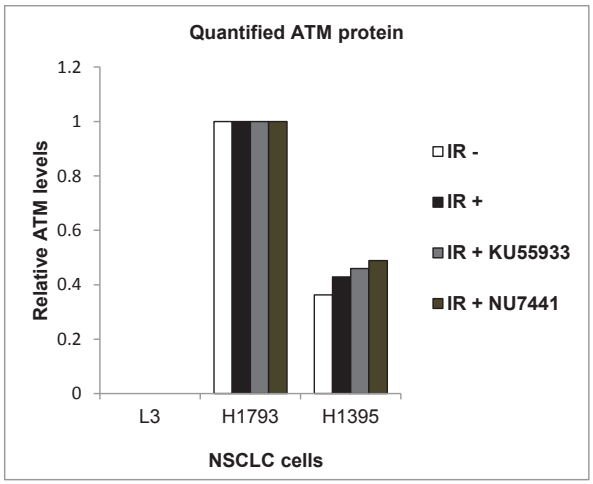
E3: ATM dependent KAP1 phosphorylation



115


B
L3
H1793
H1395
IR (10 Gy) - + + + - + + + - + + +
KU55933 (5 μM) - - + - - - + - - - + -
NU7441 (10 μM) - - - + - - - + - - - +
pS1981 ATM
ATM
pS824 KAP1
KAP1
pS2056 DNA-PKcs
DNA-PKcs
SMC1

C



D

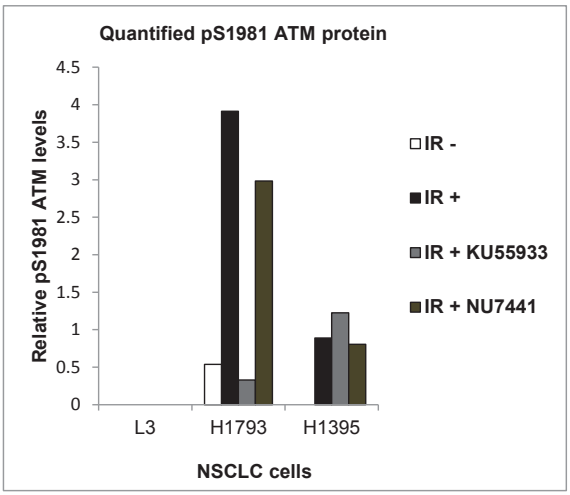
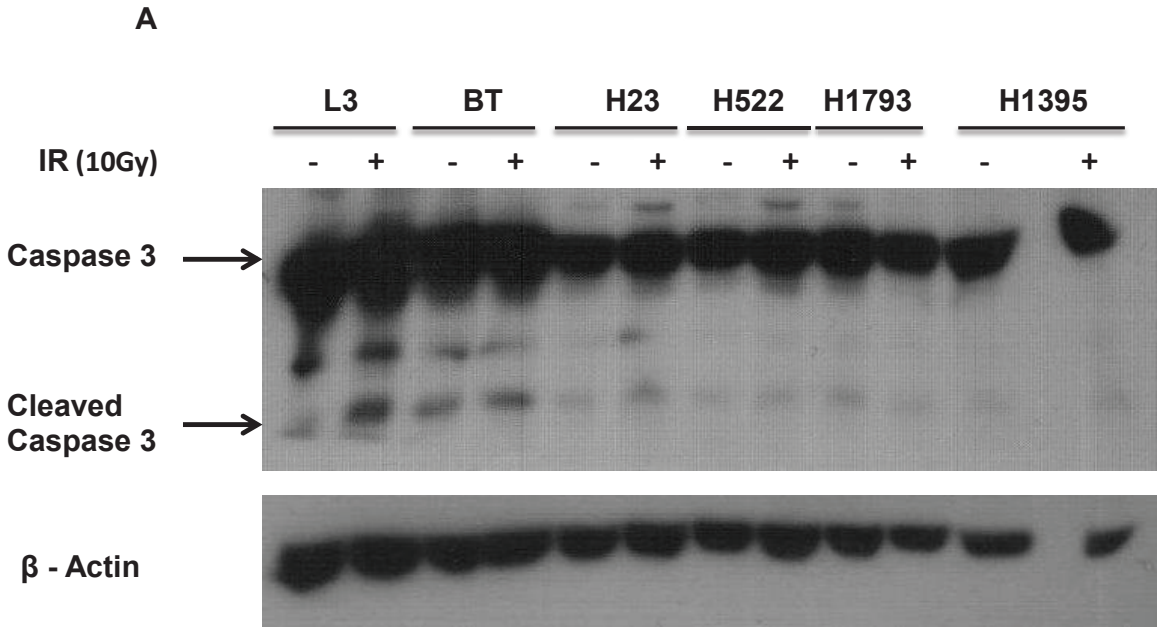


Figure E3: KAP1 phosphorylation at serine 824 is ATM dependent in H1395 cell line
Experimental method as in figure 3.3A, E3A and B are replicates of figure 3.3A. The level of ATM in E3 B was quantified using ImageJ after normalizing to SMC1 and expressed relative to ATM in H1793 cells as follows ATM in untreated cells (IR-) relative to ATM in untreated H1793 i.e. IR- H1395 or L3 cells/ IR- H1793. Similarly, ATM in the treated cells relative to ATM in treated H1793 i.e. IR+ H1395 or L3 cells/ IR+ H1793, IR + KU55933 H1395 or L3 cells/ IR + KU55933 H1793, IR+ NU7441 H1395 or L3 cells/ IR+ NU7441 H1793 respectively (C). pS1981 ATM level normalized to ATM in E3 B is also shown (D). Note that the blot and thus exposure for H1395 in E3 B is distinct from that of L3 and H1793. Additionally, in E3 B, some ATM and pS1981 ATM bands (with long exposure (▪▪▪) as compare to H1395 in E3 A) seem detectable. However, the functionality of ATM (though unaffected by ATM inhibition, E3 B) in H1395 will require further testing.

E4: IR-induced caspase 3 activation



B

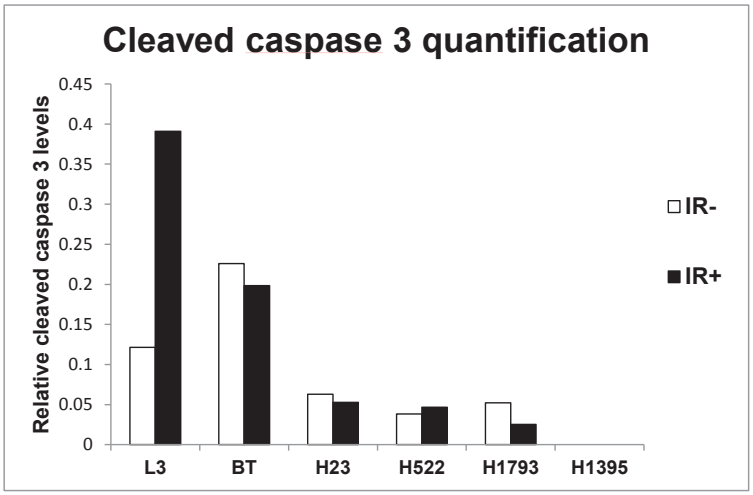
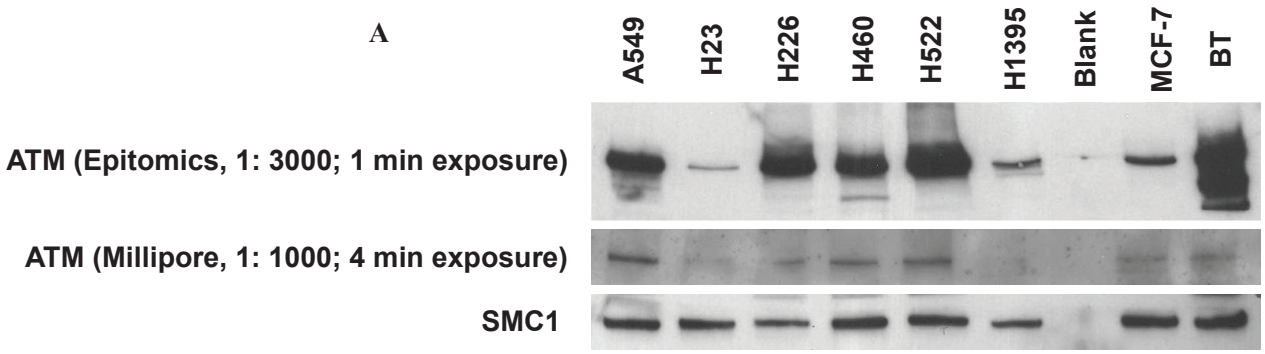


Figure E4: Analysis of IR-induced caspase 3 activation
NSCLC cells were treated with 10 Gy of IR or left untreated. WCE was harvested (as in figure 3.2A) after 2 hours of cell incubation. Western blot was performed as in figure 3.1.1; specific primary antibodies were used to detect target proteins as indicated (E4 A), β-Actin serves as the loading control. Relative levels of the cleaved caspase 3 were quantified using ImageJ after normalizing to caspase 3.

E5: ATM protein detection in NSCLC cells with a second primary antibody

The work on pages 120-121 was carried out by Dr. Petersen L.F, unpublished data.



B

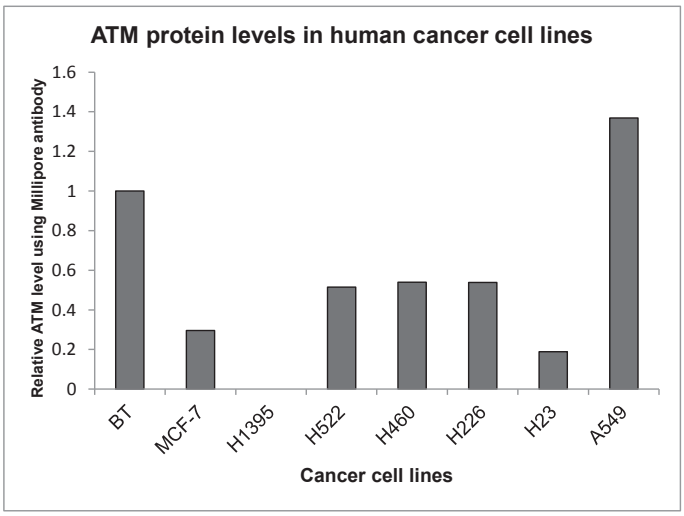


Figure E5: ATM protein detection in NSCLC cells with a different antibody (Millipore)
A.WCE was performed as in figure 3.1.1 and the proteins were resolved on a 4 – 12% gradient gel. ATM was detected using a mouse monoclonal anti-ATM antibody raised against full-length human ATM (Millipore). Note that the epitomics primary antibody was used at 1:3000 dilutions here (and an anti-rabbit secondary 1: 10000, (Sigma) as compared to 1:5000 dilutions and anti-rabbit secondary at 1:2000 (Bio-Rad),(see Table C) used in the rest of this work. B. Quantification of ATM levels detected by the mouse monoclonal primary antibody (Millipore) was performed relative to SMC1 and normalised to BT cells using the ImageJ.

E6: *ATM* gene sequence changes identified in NSCLC cell lines

Table E1: Single nucleotide polymorphisms (SNPs) detected in NSCLC cell lines using next generation sequencing ** denotes previously found SNPs, (The work on this page was performed by Dr. Petersen L.F, unpublished data)

H23 (Reduced *ATM level)*	**H1395** (Reduced *ATM level)*	**H226** (Relatively normal *ATM level)*
G514D H1380Y Q1919P** **N1983S**	**N1983S** T2666A**	**N1983S**

E7: Inhibitory concentration of chemotherapeutic drugs on 50% of NSCLC cells (IC 50).

Table E2: Chemotherapy groups with their IC 50.

Work on this page done by Yee et al., unpublished data.

Chemotherapy drug groups and IC 50 (nM) Not available- NA						
NSCLC Cells	Platinum e.g. Cisplatin	Topoisomerase 1 poison e.g. Topotecan	Taxane e.g. Paclitaxel	Nucleoside analogue e.g. Gemcitabine	Vinka alkaloid e.g. Vinorelbine	Topoisomerase 2 poison e.g. Doxorubicin
H23	1285.07	106.43	11.66	3.79	1.89	46.28
H1395	6375.68	1073.37	578.62	2278.60	1090.66	1964.31
H522	4195.69	591.33	9.43	NA	5.83	NA
H460	1101.62	42.92	9.05	12.25	2.29	137.63