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Interaction between ATM Kinase and p53 in determining glioma radiosensitivity

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INTERACTION BETWEEN ATM KINASE AND P53 IN DETERMINING GLIOMA

RADIOSENSITIVITY

A Thesis submitted in partial fulfillment of the requirement for the degree of Master of Science at Virginia Commonwealth University

By

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LIST OF ABBREVIATIONS

53BP1	p53 Binding Protein 1
APC/C	Anaphase-promoting complex
ANOVA	Analysis of variance
ATM	Ataxia-Telangiectasia Mutated
ATMi	ATM inhibitor
ATR	ATM and Rad3-related Protein
ATRIP	ATR-interacting Protein
AURKA	Aurora Kinase A
BRCA1	Breast cancer 1, early onset
BSA	Bovine serum albumin
Cdc	Cell division cycle
CDK	Cyclin-dependent Kinase
Chk	Checkpoint kinase
CO_2	Carbon dioxide
Cs	Cesium
C-terminal/terminus	Carboxy-terminal/terminus
DAPI	4',6-diamidino-2-phenylindole
DDA	DNA-damaging agent
DDR	DNA damage response
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

DSB	Double strand break
E2F	E2 Transcription Factor
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
FANCD2	Fanconi anemia, complementation group D2
GADD45	Growth arrest and DNA damage inducible
GAPDH	Glyceraldehyde phosphate dehydrogenase
GBM	Glioblastoma multiforme
Gy	Gray
H2AX	Histone family H2A, member X
H2B	Histone H2B
HDAC	Histone deacetylase
hr	Hour(s)
Ig	Immunoglobulin
IR	Ionizing radiation
Kap1	KRAB-associated protein 1
luc	Luciferase
MAPK	Mitogen-activated protein kinase
MDM2	Mouse Double Minute 2 Homolog
MEF	Mouse embryonic fibroblast
MEK3/6	Mitogen-activated protein kinase kinase 3/6
min	Minute(s)
MK2	MAP kinase-activated protein kinase 2

μL	Microliter
mL	Milliliter
μM	Micromolar
mM	Millimolar
Mp53	Mutant p53
M-phase	Mitosis
Mre11	Meiotic Recombination 11 Homolog
MRN	Mre11-Rad50-Nbs1
mRNA	Messenger ribonucleic acid
Myt1	Myelin Transcription Factor 1
Nbs1	Nijmegen Breakage Syndrome 1
N-terminal/terminus	Amino-terminal/terminus
p-	Phospho-
p21	Cyclin-dependent kinase inhibitor 1
p38	Mitogen-activated protein kinase 14
p53	Tumor Suppressor TP53
p107	Retinoblastoma-like protein 1
p130	Retinoblastoma-like protein 2
PBS	Phosphate buffered saline
Plk1	Polo-like Kinase 1
PVDF	Polyvinylidene fluoride
Rad50	Family of RADiation sensitive genes 50 homolog
Rb	Retinoblastoma protein

RFP	Red fluorescent protein
RPA	Replication protein A
Rpm	Revolutions per minute
s	Second(s)
sh	Short-hairpin knockdown
SMC1	Structural maintenance of chromosomes 1
SSB	Single strand break
TBS	Tris-buffered saline
TBST	Tris-buffered saline with 0.1% Tween 20
TAOK	Thousand-and-one kinase
UV	Ultraviolet
wt	Wild type

ABSTRACT

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By: Syed Farhan Ahmad, B.A. Chemistry & Psychology, M.S. Commerce

A Thesis submitted in partial fulfillment of the requirement for the degree of Master of
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Virginia Commonwealth University, 2015

Advisor: Dr. C. Kristoffer Valerie, Department of Radiation Oncology

Glioblastoma multiforme (GBM) is the most common primary brain tumor.
Studies have shown that targeting the DNA damage response can sensitize cancer cells to
DNA damaging agents. Ataxia telangiectasia mutated (ATM) is involved in signaling
DNA double strand breaks. Our group has previously shown that ATM inhibitors
(ATMi) sensitize GBM cells and tumors to ionizing radiation. This effect is greater when
the tumor suppressor p53 is mutated.

The goals of this work include validation of a new ATM inhibitor, AZ32, and
elucidation of how ATMi and p53 status interact to promote cell death after radiation.
We propose that ATMi and radiation induce mitotic catastrophe in p53 mutants by
overriding cell cycle arrest. We tested this hypothesis in human colon carcinoma and
glioma cells that differ only in p53 status.

We found that AZ32 effectively inhibits phosphorylation of ATM targets. In addition, AZ32 significantly sensitizes glioma cells to ionizing radiation. While HCT116 colon carcinoma cells fail to arrest the cell cycle after radiation, their response to ATMi differs from that in gliomas. Indeed, wild type HCT116 cells were more sensitive than p53 mutants to ionizing radiation in the presence of ATMi. In contrast, ATMi significantly radiosensitized glioma cells in which p53 is knocked down. Live cell imaging confirmed that radiation and ATMi preferentially induce mitotic catastrophe in p53-deficient cells. We conclude that p53-deficient cells rely on ATM signaling for G2/M cell cycle arrest. We propose a model of G2/M arrest whereby ATM and p53-dependent signaling pathways converge to ultimately inhibit Cdc25 phosphatases.

<center>**I. Introduction**</center>

1.1 Glioblastoma Multiforme

Glioblastoma Multiforme (GBM) is the most common form of primary brain tumor (Dunn et al., 2012). GBM originates from the glial cells in the brain and is among the most debilitating and lethal forms of cancer. First-line treatment consists of surgical resection, radiation, and adjuvant chemotherapy (Stupp et al., 2005). Yet, in addition to being highly resistant to radio- and chemotherapies, GBM tumors are extremely aggressive and readily invade surrounding neural tissue, which limits the utility of surgical intervention (Cuddapah et al., 2014). Not surprisingly, the average GBM patient survives only 12 to 15 months post-diagnosis (Cloughesy et al., 2014; Stupp et al., 2005). Thus, there is a critical need for more efficacious treatments that specifically target tumor cells and yield longer-term improvements in patient survival.

1.2 Targeting the DNA Damage Response in GBM

Radiation and most cytotoxic chemotherapeutics work by inhibiting DNA replication or directly damaging DNA. The major forms of DNA aberrations include base damage, backbone damage, and intra- or inter-strand crosslinks. The process by which cells detect damage and transduce signals to stimulate repair is termed the DNA damage response (DDR). The two major signaling and detection proteins involved in DDR are the *ataxia telangiectasia* mutated (ATM) and ATM/Rad3-related (ATR) kinases (Cimprich and Cortez, 2008; Shiloh and Ziv, 2013). In the absence of damage, ATM exists as an inactive dimer that rapidly dissociates in to active monomers upon

<center>1</center>

autophosphorylation at serine-1981, which stabilizes the protein at the sites of DNA double strand breaks (DSBs) (Bakkenist and Kastan, 2003; So et al., 2009). In general, DSBs—which most often result from ionizing radiation and radiomimetic drugs—are detected by a complex including the proteins Mre11, Rad50, and Nbs1 (MRN complex), which recruits ATM to damage sites and stimulates its autophosphorylation (Lavin, 2007). UV-induced base damage, crosslinks, and single-strand breaks (SSBs), on the other hand, induce phosphorylation and activation of ATR, which associates directly at the sites of DNA lesions (Bomgarden et al., 2004; Cimprich and Cortez, 2008; Liu et al., 2011). Once activated, ATM and ATR go on to phosphorylate a variety of substrates involved in slowing cell growth, halting cell division, and stimulating DNA repair (Discussed in later sections).

It is thought that cancer can manipulate the DDR to enhance radio- and chemo-resistance and promote survival (Bao et al., 2006; Bouwman and Jonkers, 2012). As such, it may be possible to increase cancer's sensitivity to DNA damaging agents (DDAs) by disrupting the DDR. It is widely documented that cells lacking ATM (i.e., A-T cells) are highly sensitive to ionizing radiation (IR) (Littlefield et al., 1981; McKinnon, 1987; Nayler et al., 2012; Taylor et al., 1975). Members of our group hypothesized that pharmacological inhibition of ATM would sensitize glioma cells to IR (Golding et al., 2009). They found that treatment with an ATM inhibitor (ATMi), KU-60019, prior to IR significantly radiosensitized multiple human glioma cell lines. They also showed that KU-60019 effectively reduced *in-vitro* invasion and migration in the same cells. Another study found that extremely low concentrations of KU-60019 were sufficient to sensitize

U1242 and U373 human glioma cells to IR (Golding et al., 2012). Of particular interest

is that both U1242 and U373 harbor mutations in p53, a prominent ATM substrate.

The p53 tumor suppressor, widely considered the "guardian of the genome", is a

393-amino acid protein involved in a variety of cellular functions, including cell cycle

arrest, DNA repair, apoptosis, and senescence, among others (Freed-Pastor and Prives,

2012; Muller and Vousden, 2013, 2014; Wei et al., 2006). Its structure consists of two

acidic N-terminal transactivation domains, a proline-rich region, a central DNA-binding

domain, an oligomerization domain, and a basic C-terminal regulatory domain. Wild-

type p53 exists as a tetrameric transcription factor that binds DNA and modulates

expression of many genes involved in cell growth and division (Wei et al., 2006). p53

also transactivates genes that regulate its own signaling, such as MDM2, an E3-ubiquitin

ligase that marks p53 for degradation and binds its N-terminus to reduce transcription of

target genes (Barak et al., 1993; Kubbutat et al., 1997; Momand et al., 1992).

p53 is the most prevalently mutated gene in human cancers (Freed-Pastor and

Prives, 2012; Kandoth et al., 2013; Muller and Vousden, 2013, 2014). Nonsense and

truncation mutants that fail to express any p53 protein are common; however, the bulk of

mutations occur as single-amino acid missense substitutions. Such modifications occur at

the highest frequency within p53's central DNA binding domain, and the most commonly

mutated residues include R248, R273, R175, G245, R249, and R282 (Freed-Pastor and

Prives, 2012; Harris and Hollstein, 1993). Mutant forms of p53 can exert dominant

negative effects, possibly through their ability to oligomerize with the wild-type protein.

In addition, loss-of-heterozygosity at the p53 locus is common among mutants, suggesting possible selection pressures that favor the development of mutations at both alleles (Burns et al., 1991; Campo et al., 1991; Nigro et al., 1989; Shetzer et al., 2014).

It has been reported that overexpression of mutant p53 can transform cells, and individuals with germline mutations in p53 suffer from Li-Fraumeni syndrome, an autosomal-dominant disorder characterized by increased susceptibility to a variety of malignancies (Donninger et al., 2008; Eliyahu et al., 1984). More than 50% of all human tumors have been found to carry mutations in p53, and p53-null and mutant mice are significantly more likely to develop tumors than their wild-type counterparts (Donehower, 1996; Nigro et al., 1989; Olive et al., 2004; Vogelstein et al., 2000). Evidence suggests that p53 mutants display many characteristics that may contribute to enhanced tumorigenicity. For example, studies have associated expression of mutant p53 with increased tumor cell proliferation, enhanced resistance to ionizing radiation and chemotherapy, heightened levels invasion and metastasis, and reduced patient survival (Fiorini et al., 2015; Hundley et al., 1997; Muller et al., 2011; Murakami et al., 2000; Reles et al., 2001). p53 mutations have also been linked to genetic instability and cell cycle defects (Fukasawa et al., 1997; Hundley et al., 1997; Kuerbitz et al., 1992; Livingstone et al., 1992) . Indeed, it may be possible to exploit the unique characteristics of p53 mutants to develop targeted cancer therapies.

1.3 Synthetic Lethality in p53 Mutant GBMs

Synthetic lethality refers to the phenomenon whereby disruption of two or more genes results in a cell with an unviable phenotype. The concept originates from microbial genetic theory, but it has recently received attention for its applications to treating cancer. Assuming cancer cells harbor one or more known mutations—and that most "normal" cells do not—it should be possible to kill malignant cells and spare functional tissue with therapies targeting mutated genes and proteins. Theoretically, it may be possible to identify treatments that induce synthetic lethality only in p53 mutants.

As noted earlier, pharmacological inhibition of ATM sensitizes glioma cells to ionizing radiation (Golding et al., 2009, 2012). It is interesting to consider whether this effect varies between p53 mutant and wild-type tumors. Indeed, our group has shown both *in vitro* and *in vivo* that the radiosensitizing effects of ATMi are greater in gliomas overexpressing mutant p53 relative to parental, p53 wild-type cells (Biddlestone-Thorpe et al., 2013; Golding et al., 2009). Similarly, dual therapy with radiation and ATMi significantly reduced tumor growth and increased survival of orthotopic p53-mutant-glioma model mice, but not untreated mice or those with p53-wild type gliomas. Thus, it may be possible to sensitize p53 mutant cells to DDAs using drugs that disrupt the normal DDR.

While such results are promising, the mechanism by which ATMi radiosensitizes p53 mutant gliomas is unclear. It is possible that the observed effects are due to variations in cell cycle regulation between p53 wild-type and defective cells. The

following sections describe the cell cycle and its relation to the DNA damage checkpoints and p53 signaling. The chapter concludes with a discussion of how p53 and ATM may interact within the cell cycle to determine glioma radiosensitivity.

1.4 The Cell Cycle

The eukaryotic cell cycle is characterized by four distinct stages: G1, S, G2 and M phases. Cells typically spend most of their life cycles in G1 and it is in this stage that they synthesize biomolecules necessary to their functions within active tissue. In terminally differentiated, non-dividing cells this phase of the cell cycle is often referred to as G0. G1 is also when cells synthesize the biomolecules necessary in S-phase, during which DNA is replicated. S-phase is followed by G2, which is characterized by rapid growth and protein synthesis. Cells contain four copies of each chromosome during G2, so the total DNA content is twice of what it is G1 or G0. The G1/G0, S, and G2 phases are collectively referred to as interphase. The period during which a cell actually divides is referred to as mitosis, or M-phase. M-phase immediately follows G2 and proceeds through a series of four sub-phases. Chromosomes condense and the nuclear envelope begins to break down during prophase. Chromosomes align along the midline of the cell during metaphase and migrate to opposite poles during anaphase. Telophase is when the cell membrane furrows and divides along the midline to form two new, genetically identical daughter cells. At the end of mitosis, each daughter cell contains two identical copies of every chromosome.

The transitions between each stage of the cell cycle are characterized by distinct signaling mechanisms. The primary proteins mediating these transitions include the cyclins and cyclin-dependent kinases (CDKs). CDKs phosphorylate different cell-cycle proteins through hydrolysis of ATP and their function depends upon association with specific cyclins (Arellano and Moreno, 1997; Hochegger et al., 2008). Intracellular CDK levels remain fairly stable throughout the cell cycle, while cyclin levels typically fluctuate, and different CDK/cyclin pairs are active at different stages of the cell cycle. Levels of D-type cyclins and CDK 4 and 6 activity are highest during G1 phase and decrease as the cell approaches the S-phase transition. Cyclin E and CDK2 activity begins to increase during late G1 and peaks at the beginning of S-phase. Cyclin E is degraded in S-phase as cyclin A expression and Cdc2 (human homolog of CDK1) activity begin to increase. In contrast, cyclin A degradation and increased cyclin B expression is concomitant with the beginning of M-phase. Cdc2 activity and cyclin B levels remain high until the end of metaphase, when activation of the anaphase-promoting complex (APC/C) results in cyclin B ubiquitination and degradation (Buschhorn and Peters, 2006). Thus, B-type cyclins are depleted upon completion of mitosis and the cell cycle starts anew.

Entry in to S-phase is dependent upon the activity of CDKs 4, 6, and 2. During G1, the retinoblastoma (Rb), p107, and p130 proteins bind E2F-family transcription factors in the HDAC repressor complex, inhibiting entry in to S-phase (Arellano and Moreno, 1997; Cao et al., 1992; Chellappan et al., 1991; Cobrinik et al., 1993; Dick and Rubin, 2013; Hochegger et al., 2008; Hurford et al., 1997). Furthermore,

phosphorylation of the CDKs reduces their ability to bind cyclins and phosphorylate substrates. Increased activity of Cdc25a phosphatase relieves CDK phosphorylation, resulting in hyperphosphorylation of Rb, p107, and p130 by Cyclin D-CDK4/6 and cyclin E-CDK2 in the cell's nucleus (Dick and Rubin, 2013; Giacinti and Giordano, 2006). This releases E2F from its inhibitory complex and allows it to stimulate expression of genes involved in DNA replication.

M-phase entry is stimulated by activation of cyclin B-Cdc2. Inhibitory phosphorylation by kinases Wee1 and Myt1 keeps Cdc2 inactive in non-mitotic cells (Booher et al., 1997; Den Haese et al., 1995; Parker and Piwnica-Worms, 1992). Around the beginning of M-phase, Aurora Kinase A (AURKA) activates Polo-like Kinase 1 (PLK1), which goes on to phosphorylate the phosphatase Cdc25c and promotes its translocation in to the nucleus (Bruinsma et al., 2014; Macůrek et al., 2008; Toyoshima-Morimoto et al., 2002). In addition, Cdc25b is directly phosphorylated and activated by AURKA (Cazales et al., 2005; Dutertre et al., 2004). Phosphorylated Cdc25, in turn, dephosphorylates Cdc2 to promote mitotic entry.

1.5 ATM/ATR Signaling in Cell Cycle Checkpoints

Detection of DNA damage can arrest the cell cycle at the G1/S or G2/M transitions or within S-phase. Figure 1-1 illustrates cell cycle regulation through ATM and ATR signaling. DSBs detected in G1 activate ATM, which goes on to phosphorylate many substrates, including p53 and the checkpoint kinase, Chk2 (Banin et al., 1998; Chaturvedi et al., 1999). SSBs and base damage activate ATR, which in turn

phosphorylates p53 and the checkpoint kinase Chk1 (Smith et al., 2010). Phosphorylated

Chk1 and Chk2 then phosphorylate Cdc25a, which inactivates the phosphatase and marks

it for ubiquitination and degradation (Busino et al., 2004; Mailand et al., 2000; Smith et

al., 2010). Since Cdc25a activity is necessary for activation of CDKs 2, 4, and 6,

phosphorylation by Chk1 and Chk2 prevents the transition from G1 to S phase, resulting

in cell cycle arrest.

DSBs detected in S-phase initiate a similar response as those detected in G1,

whereby phosphorylation of Cdc25a inhibits CDK2 function. Inhibition of CDK2

prevents activation of Cdc45, which binds origins of replication and is necessary for

initiation of DNA synthesis (Falck et al., 2002). ATM can also phosphorylate histone

H2AX, 53BP1, BRCA1, FANCD2, NBS1, and SMC1, which form distinct foci at sites of

DSBs (Burma et al., 2001; Gatei et al., 2001; Harding et al., 2011; Lim et al., 2000;

Taniguchi et al., 2002; Yazdi et al., 2002). Interestingly, ionizing radiation and chemical

stressors slow down DNA replication in normal cells; however, ATM-deficient cells

maintain continuous rates of DNA replication following stress, a phenomenon referred to

as radioresistant DNA synthesis (Painter, 1981).

ATR is involved in signaling replication fork stalling, SSBs, and base damage

during S-phase. ATR associates with ATR interacting protein (ATRIP) and the pair

localize to sites of damage through interactions with replication protein A (RPA), which

associates with single-stranded DNA (Zou and Elledge, 2003). The localized ATR-

ATRIP complex activates Chk1, which phosphorylates Cdc25a and, thus, inhibits DNA

synthesis through disruption of Cdc45 function (Smith et al., 2010). ATR-ATRIP may further reduce replication fork progression by reducing the kinase activity of cdc7-Dbf4, which is involved in loading of Cdc45 on to chromatin (Costanzo et al., 2003).

The G2/M checkpoint similarly proceeds through ATM and ATR and respective checkpoint kinases Chk2 and Chk1. Rather than stimulating Cdc25 degradation, however, activation of the checkpoint kinases in G2 results in phosphorylation of Cdc25B/C, which promotes association with 14-3-3 protein and subsequent nuclear export and sequestration to the cytosol, preventing activation of cyclin B-Cdc2 (Donzelli and Draetta, 2003). The activity of Wee1, which inhibits cyclin B-Cdc2 activity, is also enhanced through phosphorylation by Chk1 (O'Connell et al., 1997). Altogether, it seems that ATM and ATR work in concert to prevent progression from G2 to M-phase.

1.6 Functions of p53 in G1/S and G2/M Checkpoints

DNA damage results in post-transcriptional modification and activation of p53. Evidence indicates that phosphorylation of p53 at serine-15 by ATM, ATR, and other enzymes can arrest the cell cycle to allow for DNA repair (Banin et al., 1998; Lees-Miller et al., 1992; Shieh et al., 2000; Tibbetts et al., 1999). In the event that damage is too extensive, p53 is thought to directly and indirectly stimulate apoptosis (Fridman and Lowe, 2003). Studies show that p53 is involved in maintaining cell cycle arrest at the G1/S transition. Indeed, there is a correlation between expression of wild-type p53 and G1 arrest following gamma irradiation (Kuerbitz et al., 1992; Livingstone et al., 1992). In contrast, G1/S arrest is impaired in cells expressing mutant p53. It appears that p53-

mediated G1/S arrest depends primarily on enhanced expression of the CDK inhibitor p21 (Brugarolas et al., 1995, 1998; Harper et al., 1995). p21 halts entry in to S-phase by associating with and inactivating cyclin A-CDK2 and cyclin E-CDK2 complexes. This prevents hyperphosphorylation of Rb, p107, and p130, thus inhibiting release of E2F from the HDAC repressor complex. p53 has also been shown to contribute to maintenance of G2/M arrest. It has been reported that irradiated HCT116 cells with wild-type p53 can effectively sustain G2/M arrest, while isogenic p53-null cells appear to continue cycling through mitosis (Bunz, 1998). Cells null for p21 were also defective in their ability to sustain G2/M arrest after IR. Furthermore, activated p53 induces expression of 14-3-3σ protein, which binds Cdc25 phosphatases, preventing their relocation to the nucleus and inhibiting activation of the cyclin B1/Cdc2 complex (Hermeking et al., 1997). p53 also upregulates expression of another protein, GADD45, which has been shown to disrupt the interaction between cyclin B1 and Cdc2 (Jin et al., 2002). A specific form of GADD45, GADD45α, promotes cytosolic sequestration of Cdc25B/C and is upregulated after treatment with DDAs (Reinhardt et al., 2010).

1.7 Cell Cycle Defects in p53 Mutants

Given p53's involvement in maintaining cell cycle arrest, one would predict p53 mutants to continue dividing even in the presence of DNA damage. If damage results in cells with chromosome breaks and if such cells divide to produce nonviable daughters with numerical aberrations, then DDAs should be more harmful to p53 mutants than to wild-type cells. Yet, the relationship is not so simple, and there is evidence that some p53 mutant cells may even display a radioresistant phenotype (Lee and Bernstein, 1993).

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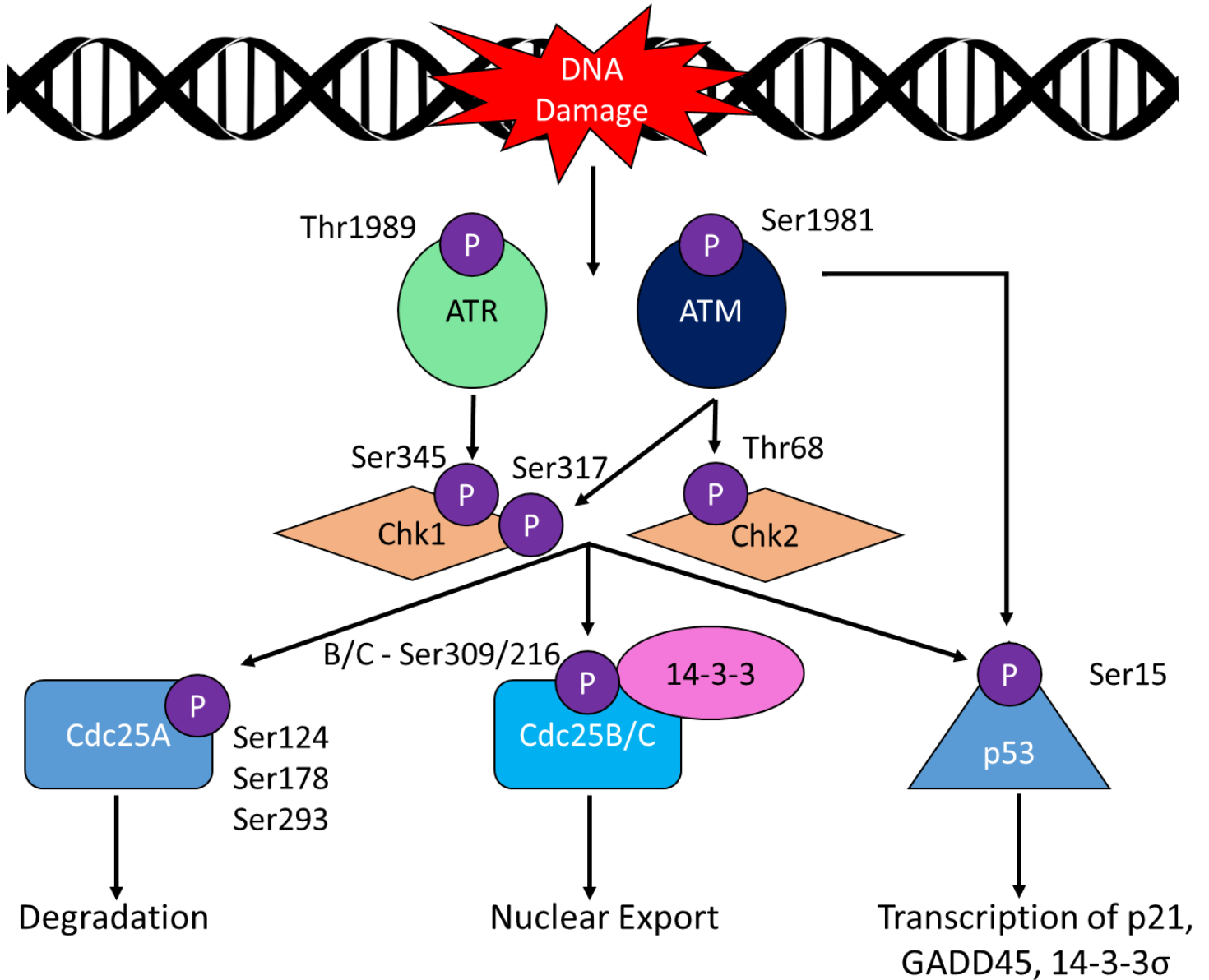
Figure 1-1: Induction of cell cycle arrest through ATM and ATR signaling. DNA damage activates ATR and/or ATM, which phosphorylate and activate p53 and the checkpoint kinases Chk1 and Chk2. Phosphorylation of Cdc25 phosphatases by Chk1/2 leads to their degradation or nuclear export, while phosphorylation of p53 induces transcription of its target genes. These processes work in conjunction to arrest the cell cycle and inhibit cell division.

It is possible that p53 mutants utilize alternative, p53-independent pathways to arrest the cell cycle following DNA damage. Indeed, it has been observed that p53 wild-type glioma cells arrest in G1 following irradiation, while those overexpressing mutant p53 (D281G) tend to arrest in G2/M (Biddlestone-Thorpe et al., 2013). Others have found that inhibition of ATM-dependent signaling through the p38MAPK-MK2 pathway sensitizes p53 mutants to the DDA doxorubicin and induces mitotic catastrophe (Reinhardt et al., 2007). p38 phosphorylates and inactivates Cdc25B and Cdc25C, resulting in their nuclear export through association with 14-3-3 protein (Donzelli and Draetta, 2003). While direct function of ATM in the p38MAPK-MK2 pathway is currently unknown, we expect it is linked through the thousand-and-one kinases (TAOKs) (Beckta et al., 2014). ATM phosphorylates the TAOKs following DNA damage and the TAOKs go on to phosphorylate MEK3 and MEK6, which are the primary enzymes necessary for activating p38 (Raman et al., 2007). Thus, mp53 cells do apparently have alternative mechanisms by which they can inhibit mitosis following DNA damage, and inactivating these mechanisms can promote cell death. Figure 1-2 illustrates an abbreviated model we have proposed to describe G2/M arrest in response to DSBs. According to this model, ATM signaling through Chk2, p53, and p38MAPK-MK2 is responsible for inhibiting mitosis following IR.

We suspect that ATMi overcomes p53-independent modes of cell cycle arrest, allowing cells to divide in the presence of DNA damage. Members of our group have shown that dual treatment of HCT116 human colon carcinoma cells with ATM inhibitor, AZ32, and radiation increases the frequency of condensed and/or malformed nuclei when
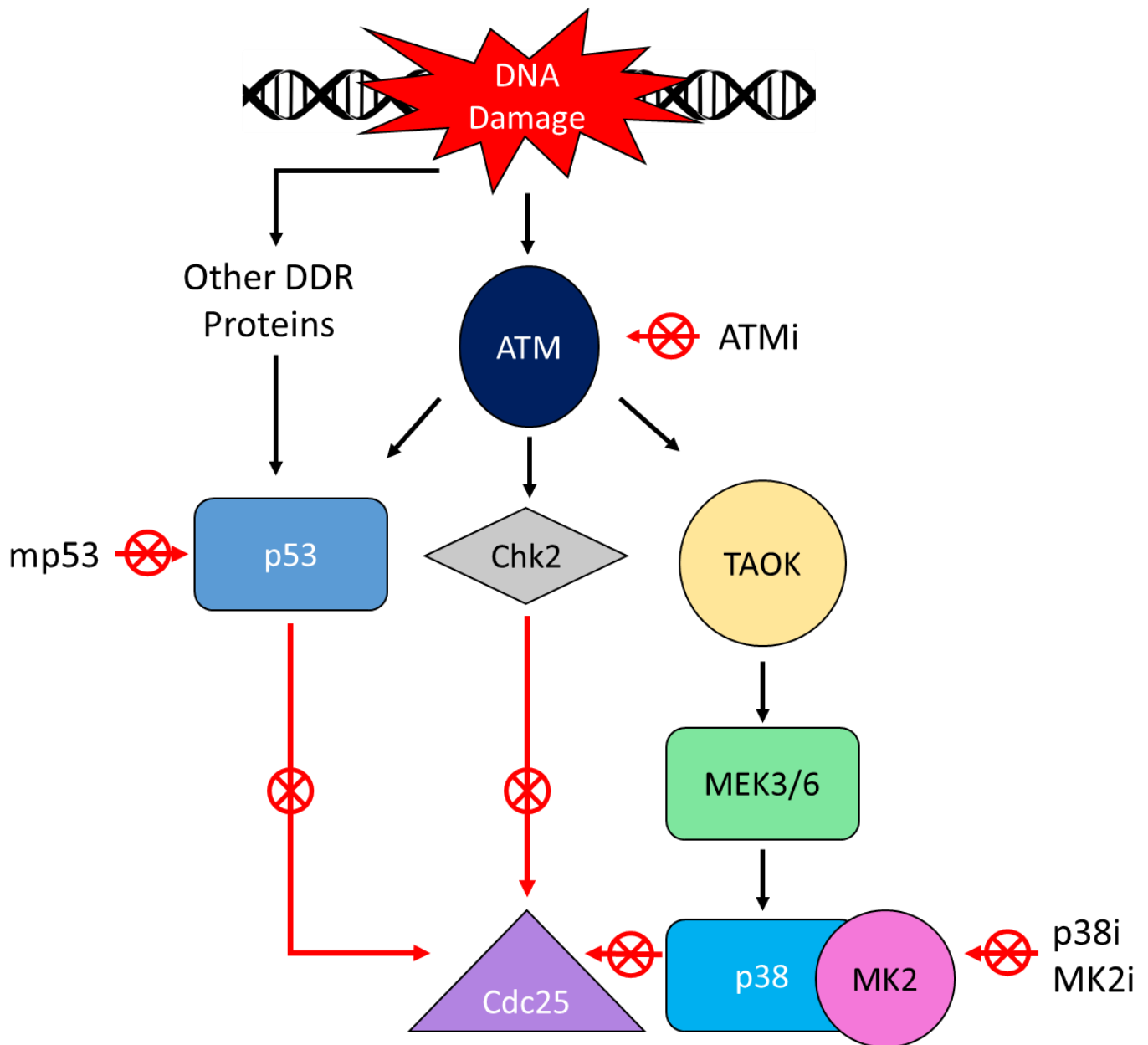
Figure 1-2: A mechanism describing ATM-mediated G2/M arrest. Following DNA damage, ATM activates p53, Chk2, and the TAOKs/p38MAPK-MK2, inducing G2/M arrest through inhibition of Cdc25 phosphatases. p53 activation may be ATM-dependent or independent. p53 mutants depend entirely upon ATM to arrest mitosis. Inactivation of both ATM-dependent and independent pathways results in aberrant mitosis. Arrows: Black = Activation; Red = Inhibition. Adapted from (Beckta et al., 2014).

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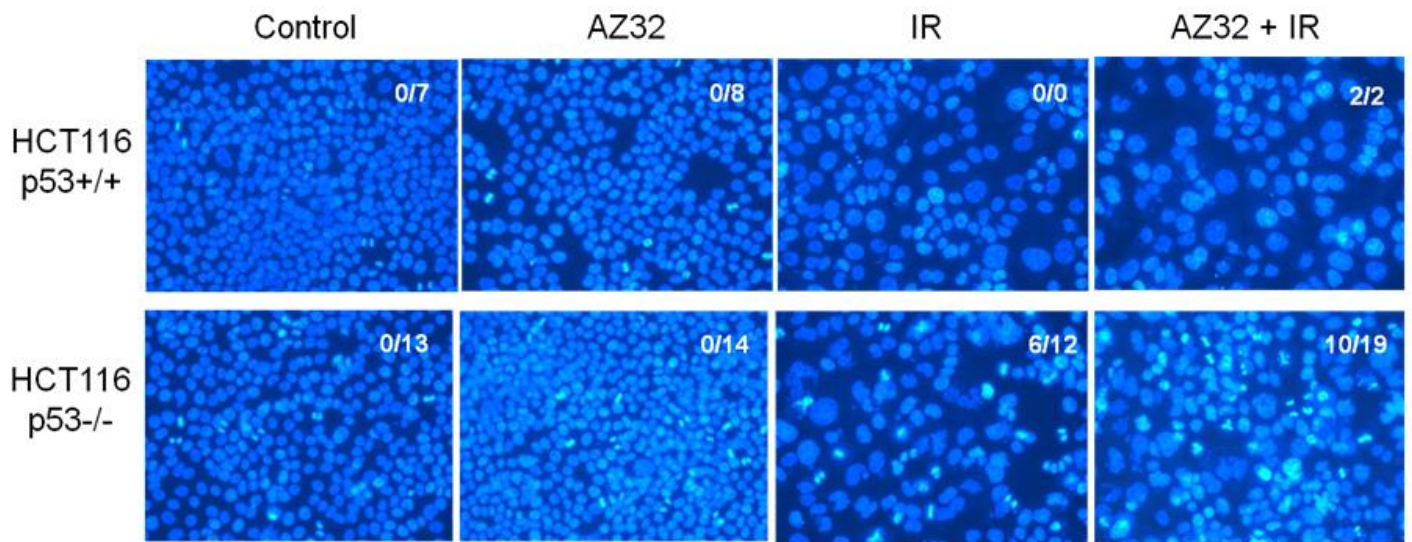
Figure 1-3: ATMi enhances aberrant mitosis in p53-null HCT116 cells. HCT116 p53+/+ and -/- cells were treated with 3 μM AZ32 with or without 4 Gy radiation and 3 days later examined for normal and aberrant mitotic figures by DAPI stain. Cells with condensed chromatin and nuclear morphology indicative of mitosis were counted. Control -/- cells show a 2-fold elevated mitotic frequency relative to +/+ cells, whereas irradiated +/+ cells have no mitotic events after IR. AZ32 with IR increased the frequency of aberrant mitotic figures and polyploidy only in -/- cells. Numerical inserts indicate the proportion of aberrant to total mitoses. (K. Valerie, unpublished)
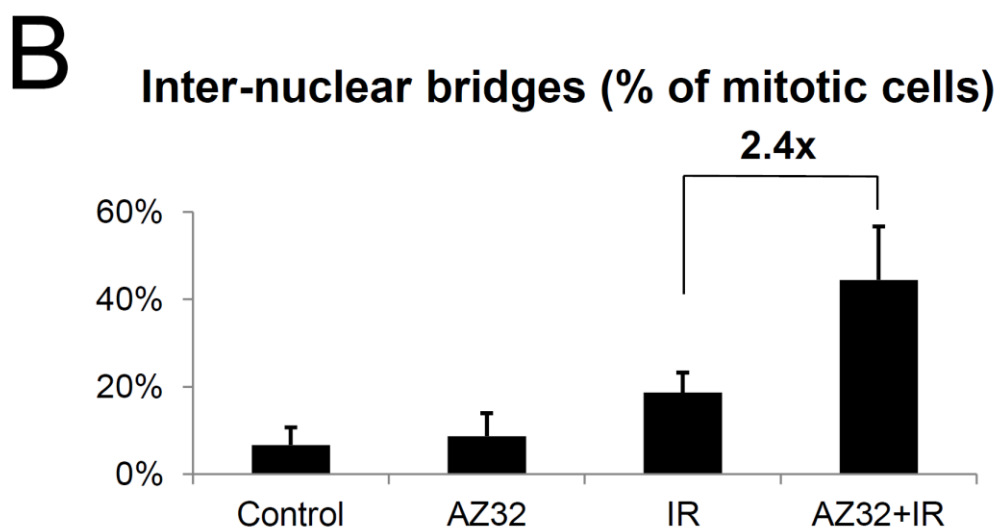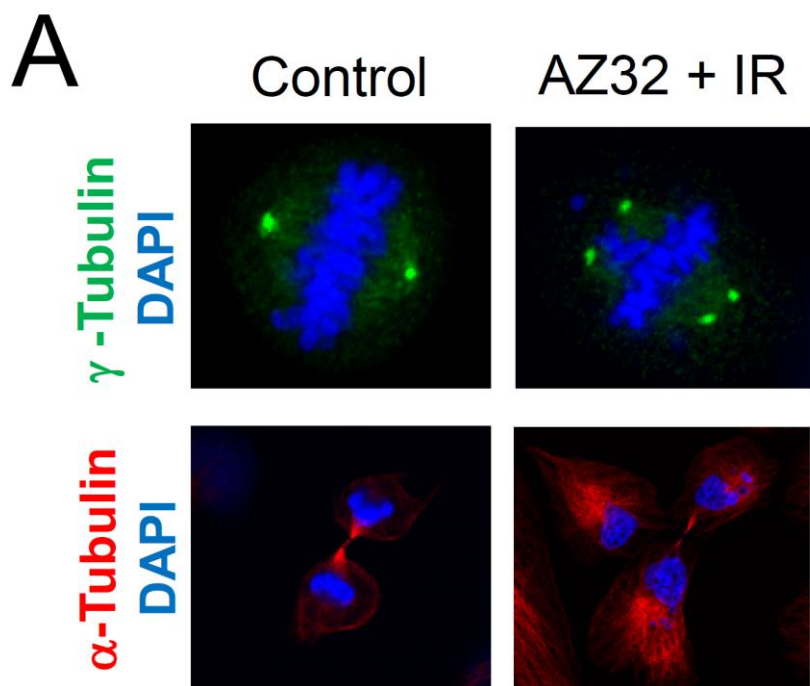
Figure 1-4: Increased mitotic aberrations in glioma cells exposed to ATMi and IR. Irradiated U1242 cells treated with AZ32 show increased centrosomes and anaphase bridges relative to untreated cells (A) and >2-fold over IR alone (B). (J. Beckta, unpublished)

p53 is absent, but not when it is normal (Figure 1-3). This is similar to observations in U1242 human glioma cells—which endogenously carry a p53 (R175H) DNA binding domain mutation—where dual therapy with ATMi and radiation significantly increases the frequency of mitotic defects (Figure 1-4). Yet, while such findings imply that ATMi allows p53 mutant gliomas to overcome G2/M arrest and divide before DNA strand breaks can be repaired, this has not been confirmed.

1.8 Goals of the Current Study

The first objective of the current study was to validate the efficacy of a new, third-generation ATMi, AZ32. The previous generation of ATMi, KU-60019, is a large molecule that is unable to cross the blood brain barrier (BBB), which limits its bioavailability (Vecchio et al., 2015). Thus, its application to glioma therapy would require invasive intracranial administration. AZ32 was identified through a small-molecule screen for its low molecular weight and ability to cross the BBB. We assessed the effects of AZ32 on signaling downstream of ATM by monitoring phosphorylation of various ATM substrates. We also examined the effects of AZ32 on glioma cell survival following radiation. Next, we studied how p53 status affects cell cycling after radiation in otherwise isogenic HCT116 human colon carcinoma cells. We also attempted to generalize the radiosensitizing effects of ATMi on survival in HCT116 cells. Finally, we examined how ATMi affects radiosurvival and mitotic arrest in isogenic glioma cells that differ only in p53 status.

II. Methods

2.1 Antibodies

Primary antibodies include anti-p53 (Calbiochem or Santa Cruz DO-1), anti-p-p53 (Cell Signaling), anti-p-Kap1 (Biosource), anti-Kap1 (Abnova), anti-PLK1 (Novex), anti-p-PLK1 (BD Pharmingen), anti-p-Chk2 (Cell Signaling), anti-Chk2 (Cell Signaling), anti-α tubulin, (Cell Signaling), and anti-GAPDH (EMD Millipore). Secondary antibodies include DyeLight 800 anti-IgG (Rockland Immunochemicals), Alexafluor 680 anti-IgG (Invitrogen), Alexafluor 488 anti-IgG (Invitrogen), Alexafluor 568 anti-IgG (Invitrogen), and Alexafluor 647 anti-IgG (Invitrogen).

2.2 Reagents

AZ32 was provided by Astra Zeneca and dissolved in DMSO to concentrations specified within text. KU-60019 was provided by KuDOS Pharmaceuticals and dissolved in DMSO at a concentration of 3 μM.

2.3 Cell Culture

Table 2-1 lists the cell types and derivatives used in experiments. Cells were grown in complete Dulbecco's Modified Eagles Medium (Gibco) supplemented with 5% FBS and penicillin-streptomycin antibiotic solution. Medium was changed twice a week and cells were passaged once a week in trypsin-EDTA. Cells were grown in incubation at 37ºC and 5% CO_2. Experiments were performed 24-72 hours after passaging.

U87MG	**U87/sh-p53 (Addgene Plasmid #19119 - shp53 pLKO.1 puro)** U87MG cells expressing short-hairpin RNA to knock down p53	**p53 Status**
	U87/luc/RFP U87MG cells expressing luciferase and RFP (Biddlestone-Thorpe et al., 2013)	**Wild-type**
	U87/luc/RFP/sh-p53	**Knockdown**
	U87/H2B-mCherry U87MG cells expressing H2B-mCherry to label chromatin	**Wild-type**
	U87/sh-p53/H2B-mCherry	**Knockdown**
	U87/H2B-mCherry/Centrin 2-EGFP U87MG cells expressing H2B-mCherry to label chromatin and Centrin 2-EGFP to label centrosomes	**Wild-type**
	U87/sh-p53/H2B-mCherry/Centrin 2-EGFP	**Knockdown**
U1242	**U1242/luc/RFP** (Biddlestone-Thorpe et al., 2013)	**Mutant (R175H)**
GL261	(Karlin et al., 2014)	**Mutant (R153P)**
HCT116	**HCT116 p53** +/+ HCT116 cells homozygous for wild-type p53 (Sur et al., 2009)	**Wild-type**
	HCT116 p53 -/- HCT116 cells with p53 knocked out on both alleles (Sur et al., 2009)	**Null**
	HCT116 p53 R248W/- HCT116 with p53 knocked out on one allele and R248W mutant p53 knocked in on the other allele (Sur et al., 2009)	**Mutant (R248W)**
	HCT116/H2B-mCherry	**Wild-type**

Table 2-1: List of cell types and derivatives used in experiments

2.4 Western Blotting

Cells were lysed and harvested in 1x Laemmli buffer (Bio-Rad). Proteins were

resolved on 10% polyacrylamide or precast Criterion^{TM} TGX Any-Kd gradient gels and

transferred on to nitrocellulose or PVDF membranes. Membranes were blocked in casein

blocking buffer (Sigma Aldrich) for at least 2 hours, labeled with primary antibody

(dilution 1:1000 in casein buffer or 5% BSA in TBST) overnight at 4ºC, and secondarily

labeled with IR fluorophore conjugated anti-IgG antibody (DyeLight 800 diluted in

casein buffer at 1:5000 or Invitrogen Alexafluor 680 diluted in casein buffer at 1:10000)

for 2-3 hours at room temperature. Bands were visualized on a Li-cor Odyssey IR imager

and densities analyzed using ImageJ.

2.5 Irradiation

Irradiation was performed using an MDS Nordion Gammacell 40 irradiator with

Cs-137 source at a dose rate of 1.05 Gy/min.

2.6 Confocal Microscopy

Cells were grown on 4-chamber slides (Lab-Tek) to 90% confluency. Cells were

fixed in 4% paraformaldehyde for 15 min, washed twice in PBS for 5 min, permeabilized

in 0.5% Triton X-100 for 10 min, and blocked for at least 2 hours in casein/2% goat

serum. Primary antibody (dilution 1:500 in casein/2% goat serum) labeling was

performed at 4ºC overnight. Slides were washed in PBS, and secondary antibody

(dilution 1:500 in casein/2% goat serum) labeling was performed at room temperature for

2-3 hours.   Cell nuclei were counterstained with DAPI (1 mg/mL) and mounted in

VECTASHIELD mounting medium (Vector Laboratories). Imaging was performed on a Zeiss LSM 710 laser scanning confocal microscope and images were analyzed using Zeiss Zen software.

2.7 Colony Forming Assay

Cells were grown to confluency in a 6 cm tissue culture dish and passaged in trypsin. Single cells were transferred in to 5 mL of culture media, 0.5 mL of cell solution was added to 0.5 mL of trypan blue, and approximate cell concentration was measured by counting on a hemocytometer. Cells were seeded in 6 cm dishes or 6-well plates at appropriate numbers. Cells were allowed 4-5 hours to attach and were either left untreated or treated with 3 μM AZ32. Cells were irradiated 30 min to 1 hour after addition of AZ32. Medium was changed 16 hours after irradiation. Medium was discarded two to three weeks after irradiation, cells were labelled in 0.5% crystal violet in 25% methanol, and colonies were counted. The average colony counts between replicates were recorded and compared between different treatment conditions. Dose response curves were constructed by plotting surviving fraction vs. radiation dose. Statistical significance of results was calculated using ANOVA in IBM SPSS Statistics 22 or in GraphPad Prism.

2.8 Live Cell Imaging

U87/H2B-mCherry/Centrin 1-EGFP, U87/sh-p53/H2B-mCherry/Centrin 1-EGFP, U87/H2B-mCherry, or U87/sh-p53/H2B-mCherry cells were seeded on 4-chamber, glass-bottom CELLview tissue culture dishes (Grenier Bio-One) and allowed to

grow for 48-72 hours. For drug and radiation treatment conditions, 3 µM AZ32 was

added 30 min to 1 hour before irradiation at 5 Gy. Still images were taken every 7 min

for a total of 16 hours beginning 2 hours after irradiation. Cells were incubated at 37ºC

and 5% CO_2 throughout the imaging period.

2.9 Identification of Aberrant Mitoses

Aberrant mitoses were identified visually by morphological abnormalities in

DNA and/or centrosomes. DNA was visualized by DAPI stain or by expression of a

fluorescent histone H2B-mCherry fusion protein. Centrosomes were fluorescently

labelled with antibodies against α-tubulin or with a Centrin 2-EGFP fusion protein.

Abnormal mitoses were identified as previously described (Amé et al., 2009; Lengauer,

2001; Plug-DeMaggio et al., 2004; Wonsey and Follettie, 2005). For still images, mitotic

cells with obvious chromosome breaks, abnormal chromatin morphology, and/or

chromatin bridges were scored as aberrant. Mitotic cells with more than two centrosomes

were also scored as aberrant. For live-cell imaging, nuclei that failed to complete mitosis

or appeared to fragment during or after mitosis were scored as aberrant.

III. Results

3.1 AZ32 Inhibits Phosphorylation of ATM Substrates

It has been shown that two early generation ATM inhibitors, KU-55933 and KU-60019, inhibit phosphorylation of p53 (S15) in glioma cells following radiation (Golding et al., 2009; Hickson et al., 2004). In order to assess the ability of AZ32 to inhibit ATM, U1242 glioma cells were irradiated at 5 Gy following treatment with AZ32 at concentrations of 0, 0.3, 1, or 3 µM. It was found that AZ32 inhibits phosphorylation of p53 in a dose-dependent manner (Figure 3-1). Furthermore, AZ32 is an equally potent inhibitor of p53 phosphorylation as KU-60019. Following DNA damage, ATM rapidly phosphorylates KRAB-associated-protein-1 (Kap1) at serine-824 (White et al., 2012, 2006). We examined phosphorylation of p53 and Kap1 in GL261 mouse glioma cell extracts. Western blot shows that AZ32 yields stable inhibition of Kap1 phosphorylation even 3 hours after radiation at 3 Gy (Figure 3-2). In contrast, GL261 cells display elevated basal levels of p53 phosphorylation that is not reduced by AZ32, and IR-induced phosphorylation of p53 recovers after 3 hours. This is consistent with the fact that p53 is phosphorylated by kinases other than ATM. Sustained inhibition of ATM signaling by AZ32 was also confirmed in human glioma cells. AZ32-treated and untreated U1242 human glioma cells were irradiated at 5 Gy and harvested 30 min to 6 hours afterward. Phosphorylation of Kap1 (S824) and Chk2 (T68) was assessed by Western blot (Figure 3-3). As expected, phosphorylation of Kap1 and Chk2 persists several hours after radiation in untreated cells. In contrast, AZ32 inhibits phosphorylation by ATM even 6 hours after radiation. Overall levels of Kap1 and Chk2 were unaffected by AZ32 or IR.
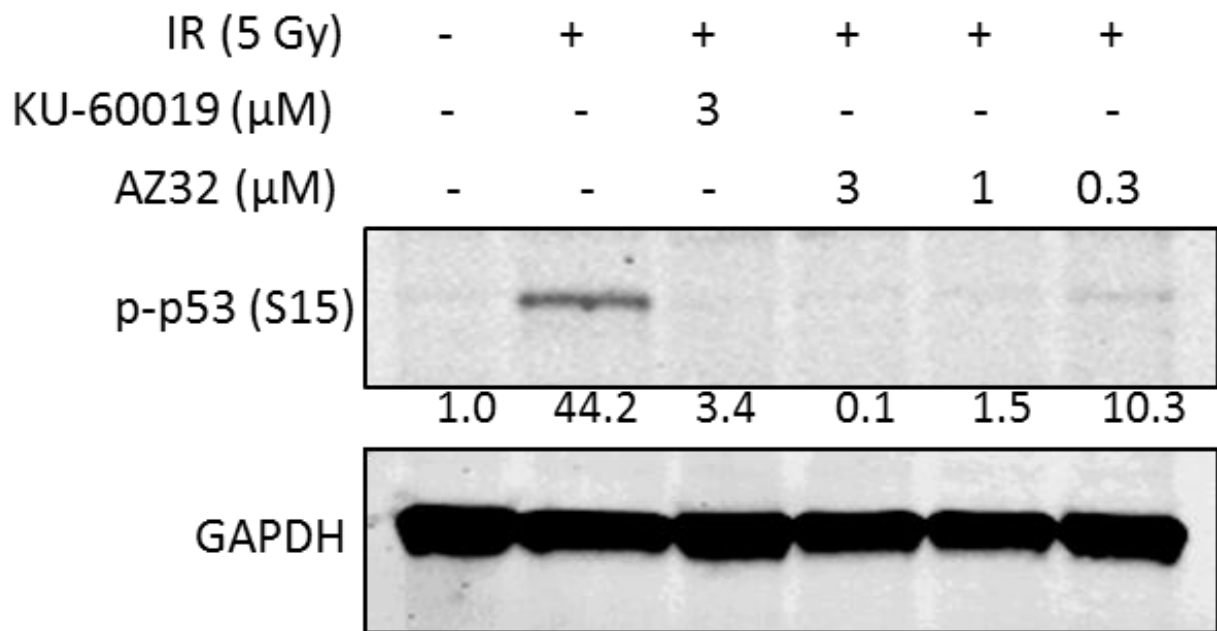
Figure 3-1: AZ32 inhibits phosphorylation of p53 in a dose-dependent manner in U1242 human glioma cells. AZ32 was administered at doses of 0, 0.3, 1, and 3μM and phosphorylation of p53 was monitored 30 minutes after irradiation at 5 Gy. Protein band densities for phospho-p53 were normalized to levels of GAPDH
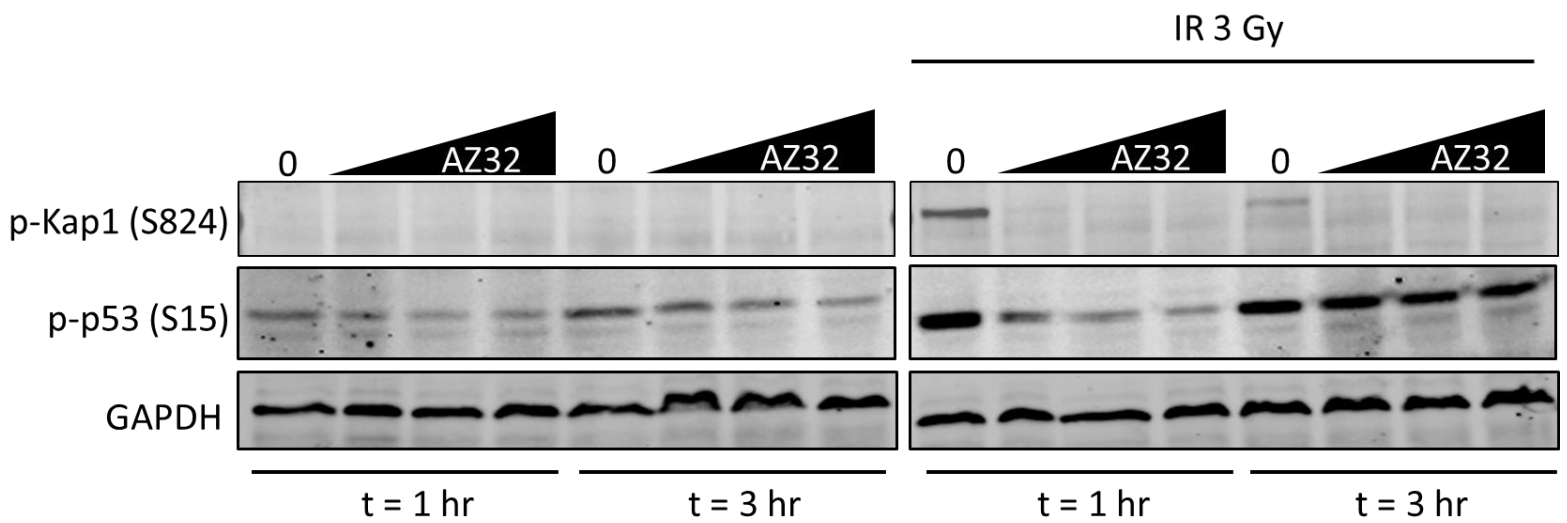
Figure 3-2: AZ32 inhibits phosphorylation of Kap1 and p53 in a dose-dependent manner in GL261 mouse glioma cells. AZ32 was administered at doses of 0, 0.3, 1, and 3 μM 30 min prior to irradiation at 3 Gy. Phosphorylation of ATM targets Kap1 (S824) and p53 (S15) was monitored 1 and 3 hours after IR.
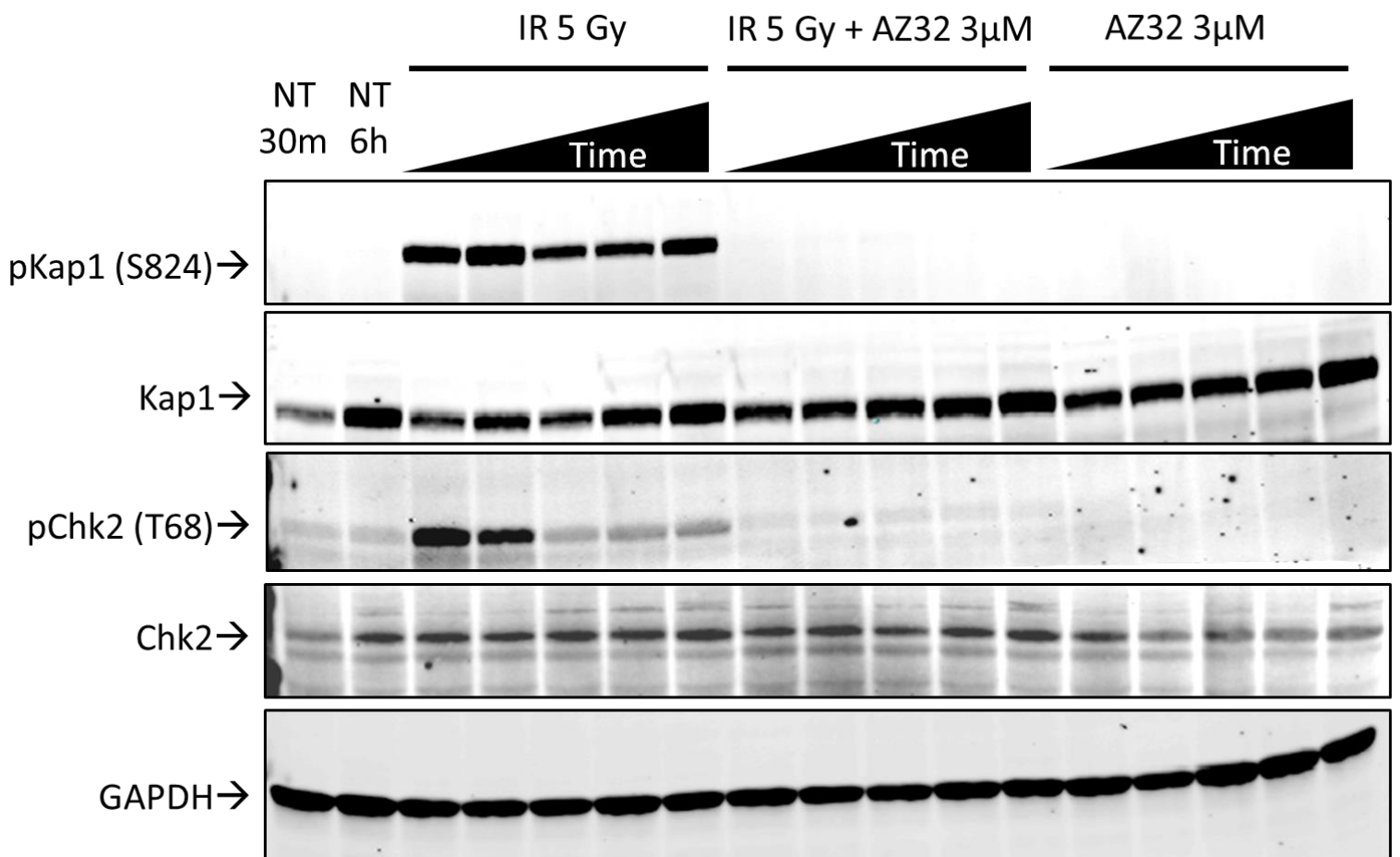
Figure 3-3: AZ32 inhibits ATM signaling several hours after irradiation in U1242 glioma cells. U1242 human glioma cells were treated with AZ32 at 3μM 30 min prior to irradiation at 5 Gy. Cells were harvested 30 min, 1 hr, 2 hrs, 4 hrs, or 6 hrs after irradiation. AZ32 effectively inhibits downstream phosphorylation of Kap1 (S824) and Chk2 (T68) several hours after irradiation. NT, No Treatment.

3.2 AZ32 Enhances Radiosensitivity of Glioma Cells

Early generation ATMis have been shown to enhance radiation sensitivity of glioma cells (Biddlestone-Thorpe et al., 2013; Golding et al., 2009, 2012). The effects of AZ32 on radiosensitivity were assessed by clonogenic colony forming assay. U1242 cells were treated with AZ32 at 3 µM, irradiated, and allowed to grow for 10 days. The log of surviving fraction was plotted against radiation dose and the data were fit to a second order polynomial (Figure 3-4). It was found that AZ32 significantly radiosensitizes U1242 glioma cells at doses as low as 1 Gy. AZ32 reduced survival by a factor of 1.5 at 1 Gy and 3.2 at 2 Gy.

3.3 Mutant p53 Abrogates Cell Cycle Arrest in HCT116 Cells

Next, we sought to examine how ATMi and p53 status determine radiosensitivity. It was necessary that we work with isogenic cell lines that differ only in p53 status. HCT116 human colon carcinoma cells that vary only in p53 status have been well validated (Bunz, 1998; Bunz et al., 1999, 2002; Sur et al., 2009). These cells have been used previously to examine the effects of p53 status on cytotoxicity following treatment with DDAs (Bunz et al., 1999). Studies have found that knockout of p53 prevents these cells from sustaining G2/M arrest after IR (Bunz, 1998). We studied if arrest is also defective in cells expressing mutant p53 (mp53). Mp53 HCT116 cells harbor the R248W DNA binding domain mutation on one allele and a defective p53 promoter on the other. Western blot shows that both wild-type and mp53 are induced after 2 Gy of radiation (Figure 3-5). In contrast, p53 was not induced in p53-null cells. Mitosis was assessed by immunofluorescence. Wild-type and mp53 HCT116 cells were irradiated at 5 Gy and
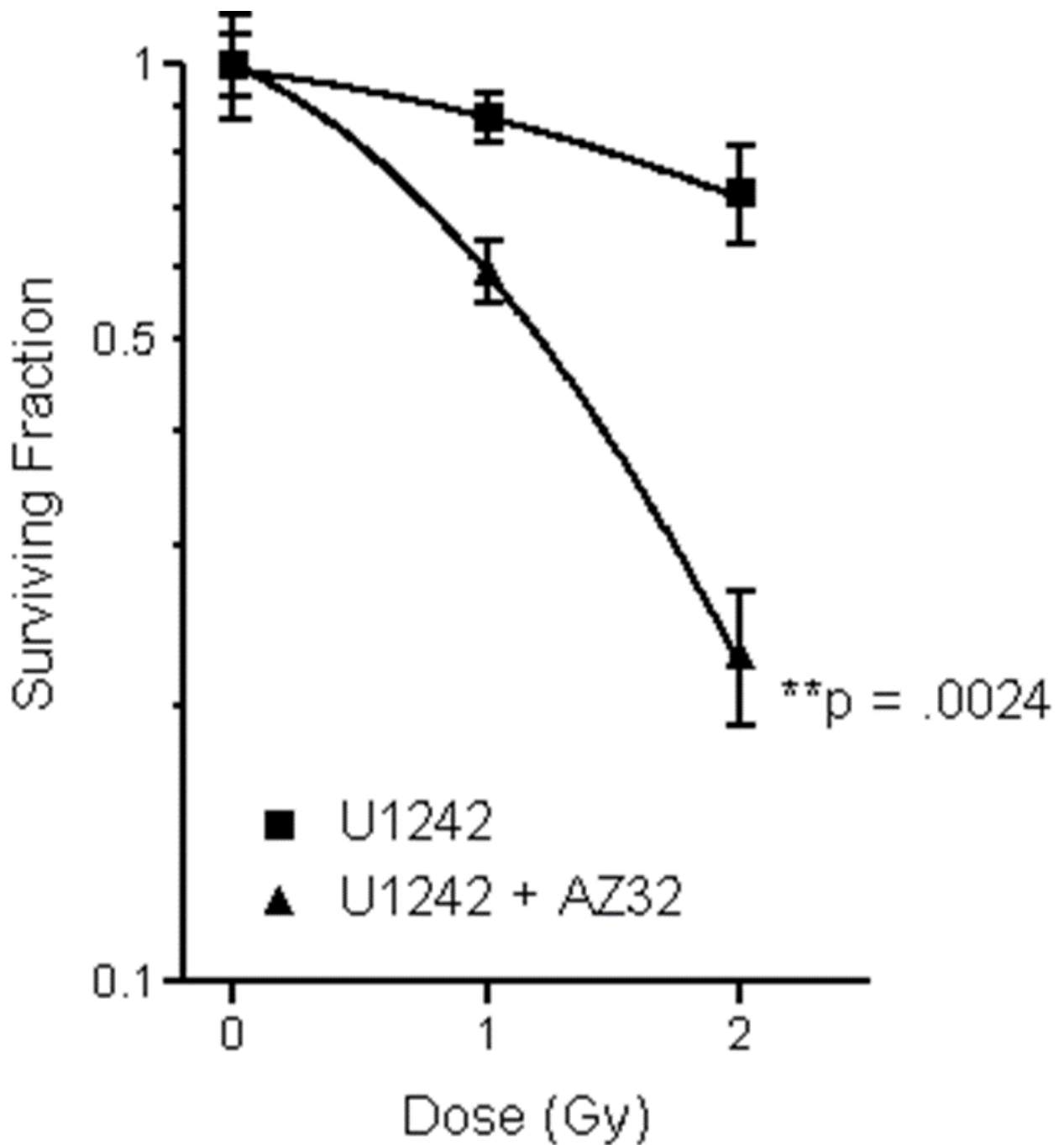
Figure 3-4: ATM inhibition by AZ32 significantly radiosensitizes U1242 glioma cells. Log plot of surviving fraction vs. radiation dose. Colony forming assay confirms that treatment with 3 μM AZ32 significantly reduces survival of U1242 glioma cells following irradiation. The survival reduction factors are 1.5 at 1 Gy and 3.2 at 2 Gy.
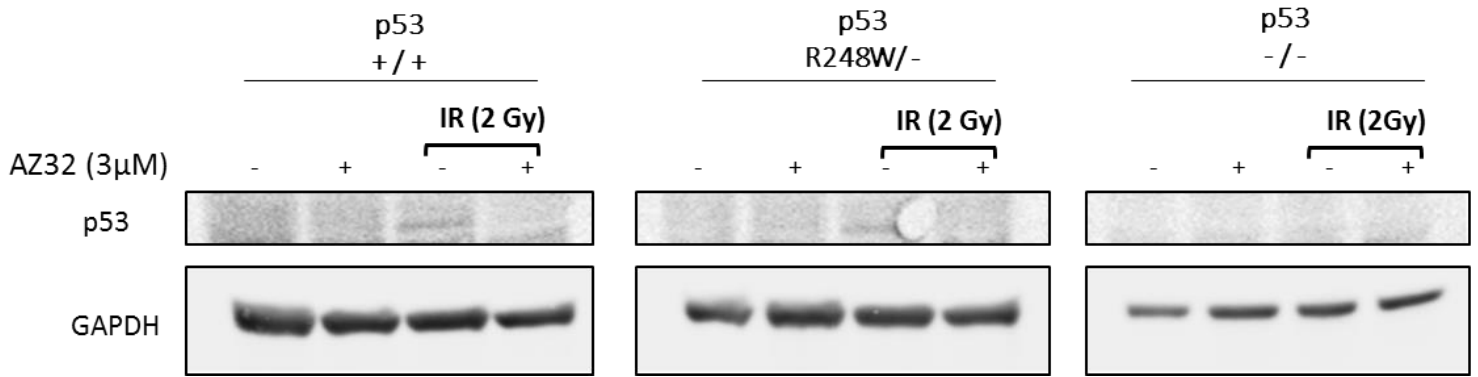
Figure 3-5: Western blot of irradiated HCT116 cells with different p53 status. p53 is induced by irradiation in p53 +/+ and p53 R248W/- HCT116 cells, but not in p53 -/- HCT116 cells. Cells were treated with AZ32 an hour prior to IR at 2 Gy and harvested an hour after IR.
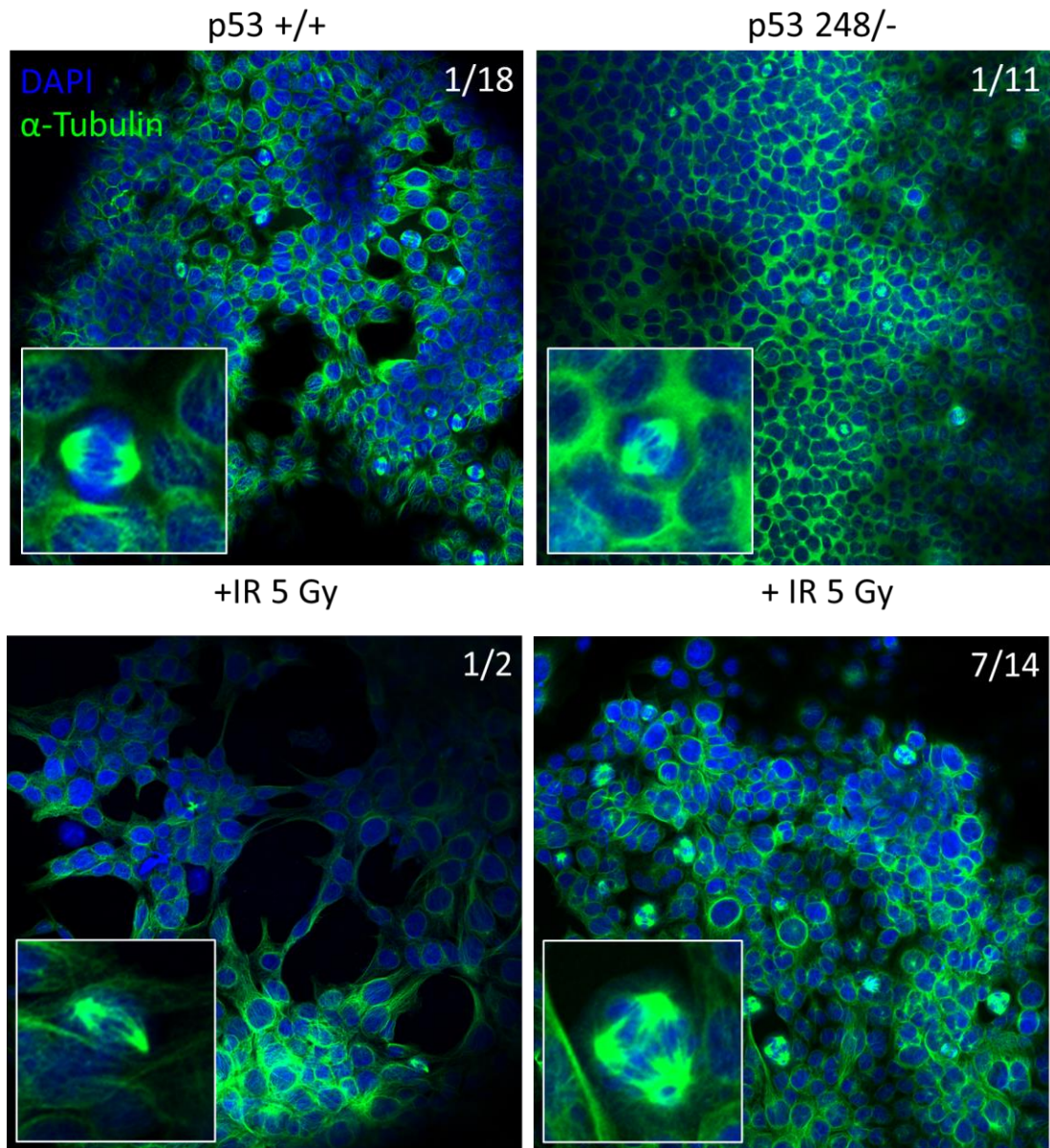
Figure 3-6: p53 mutant HCT116 cells continue cycling after irradiation. p53 +/+ or

mutant p53 R248W/- HCT116 cells were irradiated at 5 Gy and allowed to grow for 48

hours. Cells were fluorescently labelled with DAPI and antibodies against α-tubulin.

While p53 +/+ cells appear to arrest following irradiation, p53 mutant R248W/- cells

continue to undergo mitosis. Numerical inserts indicate the proportion of aberrant to total

mitoses.

fixed after 48 hours. Microtubules were labelled with antibodies against α-tubulin and DNA was stained with DAPI. Mitoses were counted and aberrant events were identified by abnormalities in DNA and centrosome morphology. Mitosis appears normal in unirradiated p53 wild-type and mp53 cells (Figure 3-6). Yet, while p53 wild-type cells arrest after IR, mp53 cells continue dividing with a high frequency of aberrant events.

3.4 AZ32 Radiosensitizes Wild-type and p53 Mutant HCT116 Cells

The observation that cell cycle arrest is defective in irradiated mp53 HCT116 cells led us to question if IR causes these cells to die by mitotic catastrophe. Previous studies have shown that inhibition of ATM radiosensitizes mp53 gliomas to a greater extent than their wild-type counterparts (Biddlestone-Thorpe et al., 2013). We examined if HCT116 cells respond similarly to ATMi. Results from a colony forming radiosurvival assay of wild-type and mp53 HCT116 cells after treatment with 3 μM AZ32 are plotted in Figure 3-7. It is evident that mp53 HCT116 cells are more resistant to low doses of IR than their wild-type counterparts. Yet, while AZ32 significantly radiosensitizes both cell lines, the effects are greater in wild-type cells. At radiation doses of 1 and 2 Gy, respectively, AZ32 reduces survival by a factor of 2.2 and 7.5 in wild-type cells and 1.7 and 4.6 in mutants. This is in stark contrast to our group's previous findings in glioma cells. It is possible that the difference is due to the fact that glioma cells display much higher levels of endogenous replicative stress than carcinomas (Bartkova et al., 2010). It may be that constant replicative stress selects for glioma cells that are inherently deficient in apoptosis. In contrast, HCT116 cells are known to depend on wild-type p53 for apoptosis following DNA damage (Kaeser et al., 2004).
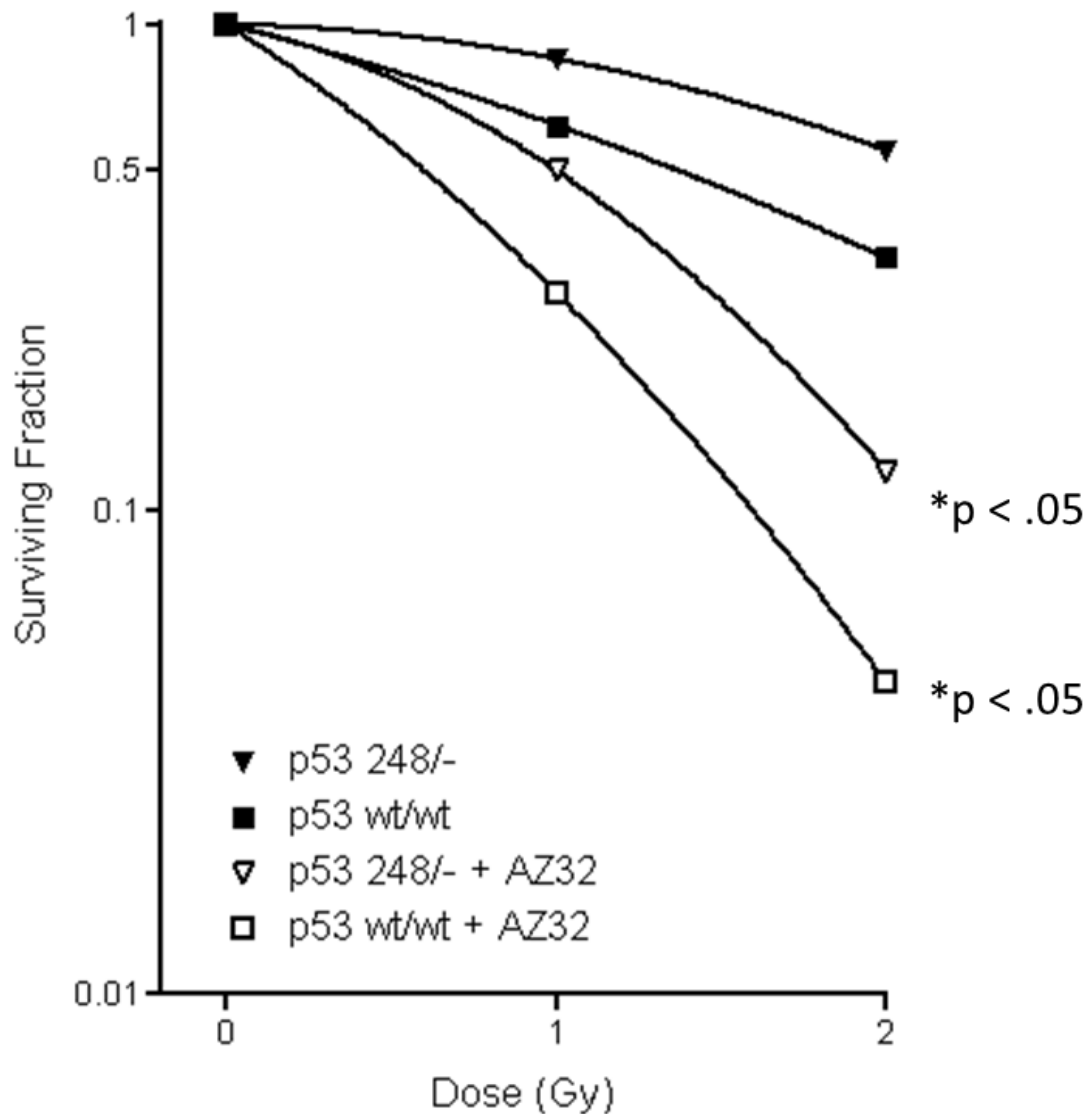
Figure 3-7: ATMi radiosensitizes both wild-type and p53 mutant HCT116/H2B-mCherry cells. Log plot of surviving fraction vs. radiation dose. Colony forming assay shows that treatment with AZ32 radiosensitizes both wild-type and mutant p53 cells. Wild-type HCT116 cells appear to be sensitized to a greater degree than p53 mutants. Survival reduction factors for wild-type cells are 2.2 at 1 Gy and 7.5 at 2 Gy. Survival reduction factors for p53 mutants are 1.7 at 1 Gy and 4.6 at 2 Gy.

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3.5 Short-hairpin RNA Effectively Knocks Down p53 Expression

Since HCT116 cells differ from gliomas in their response to ATMi, we sought to work with isogenic glioma cell lines that differ only in p53 status. While our group has reported that ATMi sensitizes mp53 cells to a greater degree than wild-type p53 gliomas, these studies utilized cell lines in which mp53 was overexpressed from a viral vector (Biddlestone-Thorpe et al., 2013). As such, it is unclear whether the effects of ATMi on radiosurvival are solely a product of p53 status or if they also depend on the levels of mp53 expression. To construct a more appropriate model for study, U87 and U87/luc-RFP cells were infected with a retrovirus expressing a short-hairpin RNA targeting p53 (sh-p53). Western blot confirms that protein levels of p53 are significantly lower in cells expressing sh-p53 (Figure 3-8). Examination of p53 levels in individual U87/sh-p53 clones led to selection of clone D1 for downstream studies (Figure 3-8B). To compare p53 expression and phosphorylation between U87 and D1 U87/sh-p53 clones, cells were irradiated at 5 Gy and extracts were subject to Western blot (Figure 3-9). It was confirmed that post-IR levels of whole and phospho-p53 are significantly reduced in D1 clones relative to their wild-type U87 counterparts.

3.6 p53 Knockdown Enhances Glioma Radiosensitivity with AZ32

We compared the effects of ATMi on radiosurvival between wild-type and p53 knockdown U87 glioma cells. Western blot confirms that phosphorylation of p53 (S15) following irradiation is significantly reduced in U87 cells with wild-type p53 and almost completely abrogated in those expressing short-hairpin RNA against p53 following treatment with 3 μM AZ32 (Figure 3-10). In order to determine if p53 status affects

33
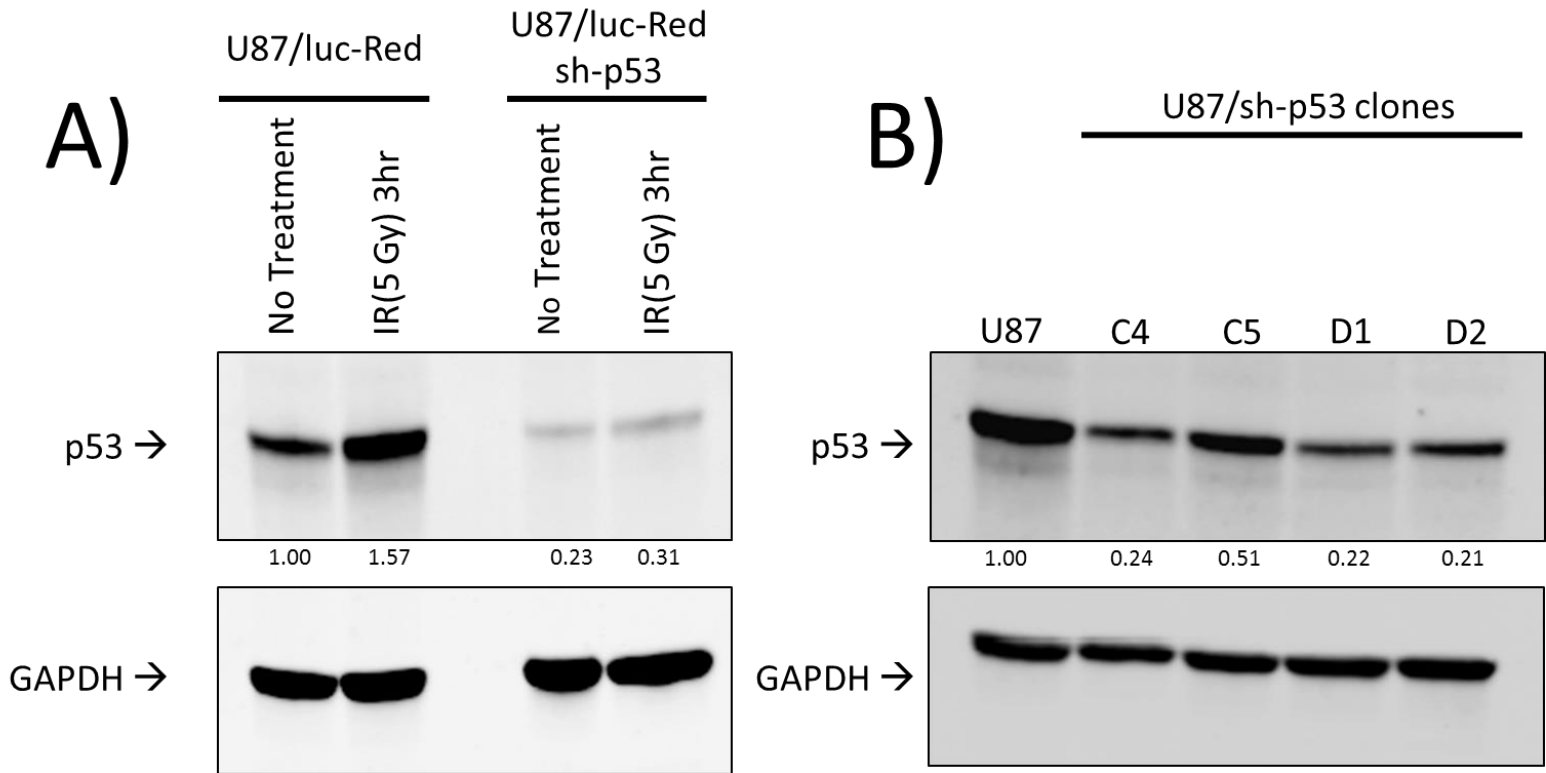
Figure 3-8: Short-hairpin-p53 cells display effective knockdown of p53. Protein levels show that p53 is effectively knocked down in A) U87/luc/RFP/sh-p53 cells, and B) different U87/sh-p53 clones. In (A), cells were irradiated at 5 Gy and harvested 3 hours after IR. Relative densities for p53 are recorded under each lane. Band densities were normalized to levels of GAPDH.
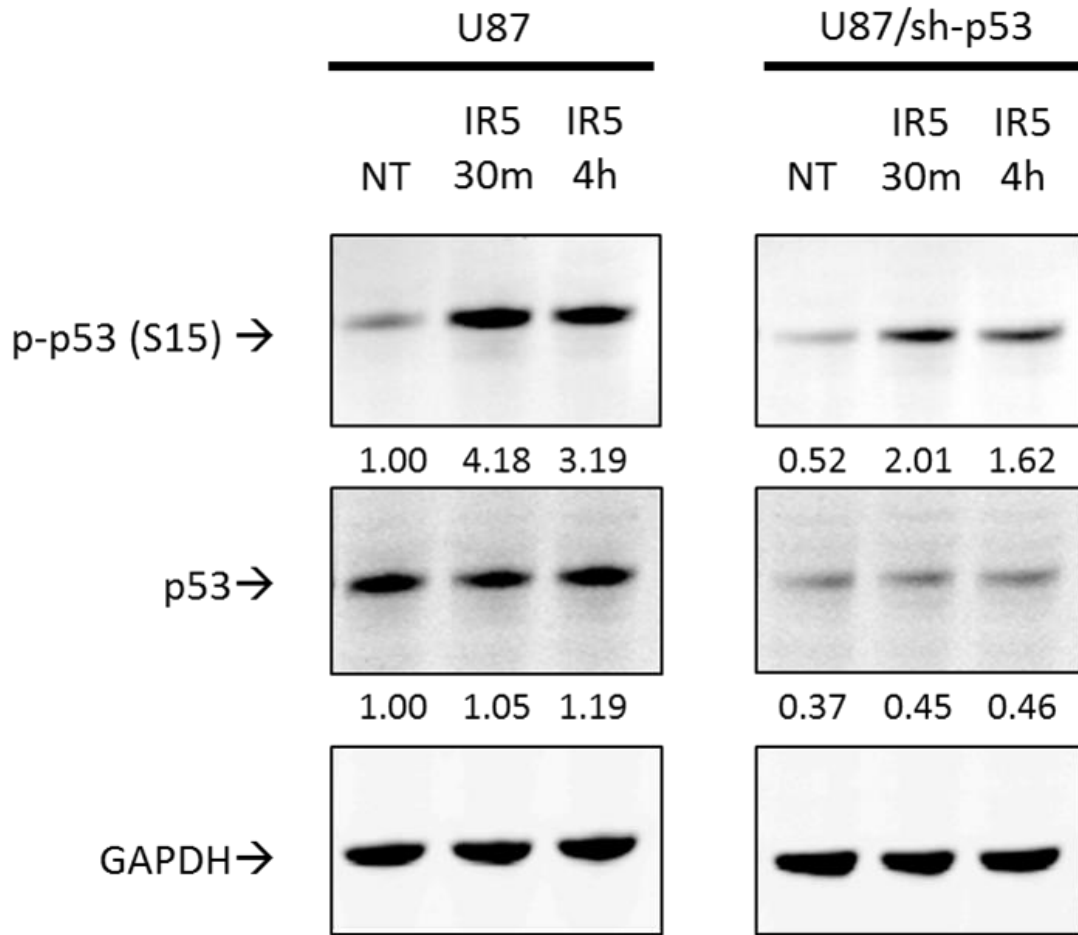
Figure 3-9: Short-hairpin-p53 U87 clone D1 displays effective knockdown of p53.

Protein levels of p53 and phospho-p53 (S15) show that p53 is effectively knocked down in U87/sh-p53 clone D1. Cells were irradiated at 5 Gy and harvested 30 minutes or 4 hours after IR. Relative densities for p53 and phospho-p53 are recorded under each lane. Band densities were normalized to levels of GAPDH.
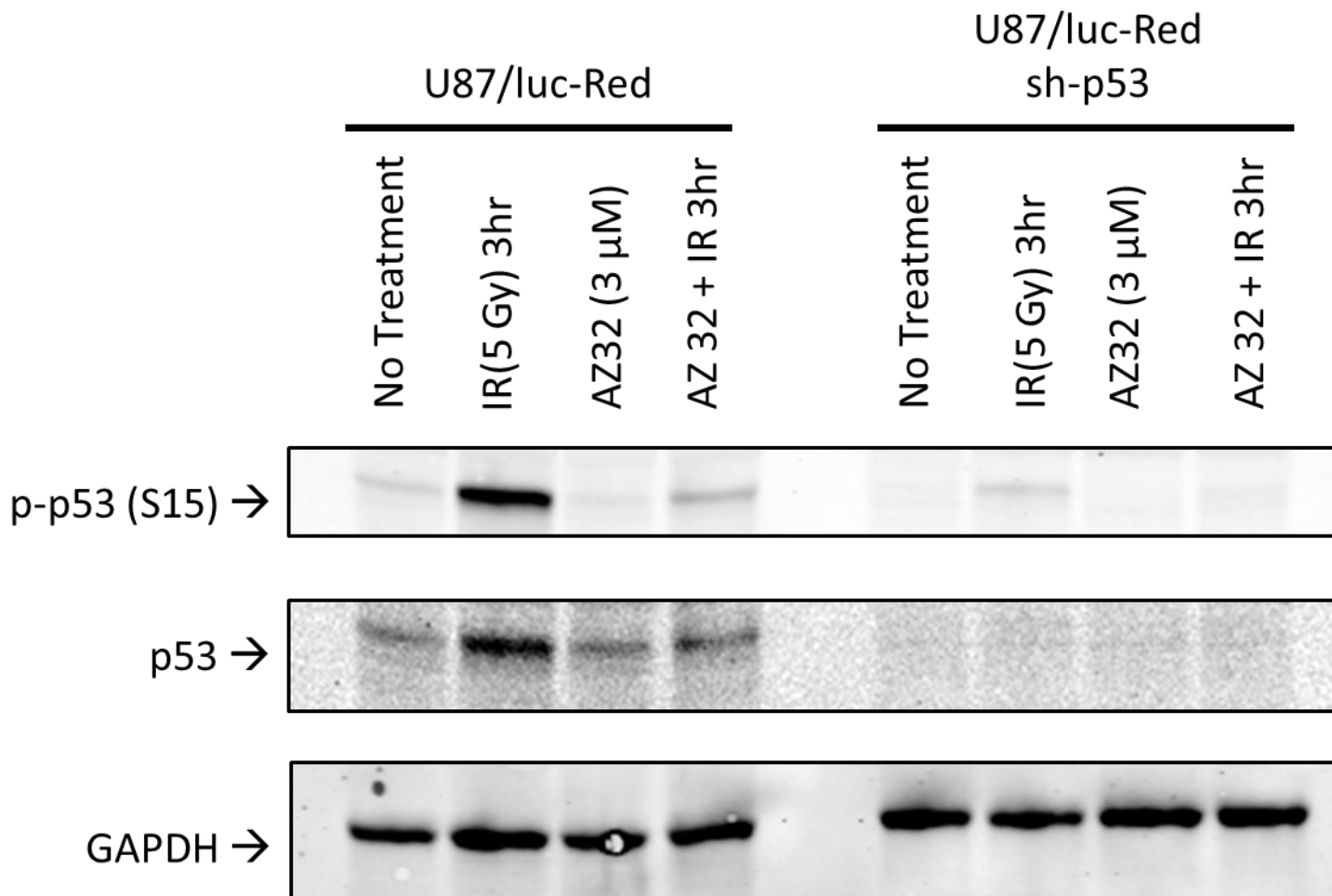
Figure 3-10: AZ32 inhibits S15 phosphorylation of p53 in p53 wild-type and p53-knockdown cells. Protein levels show that p53 is effectively knocked down in U87/luc/RFP/sh-p53 cells and that AZ32 inhibits accumulation of phospho-p53 (S15) following IR. Cells were treated with AZ32 an hour prior to irradiation at 5 Gy and harvested 3 hours after IR.

radiosensitization by ATMi, clonogenic radiosurvival assays were performed on U87 and U87/sh-p53 cells (Figure 3-11). We found that AZ32 radiosensitizes U87/sh-p53 cells to a much greater degree than wild-type U87 cells. In wild-type cells, AZ32 reduced survival by a factor of 1.1 at 1 Gy and 1.3 at 2 Gy. In sh-p53 cells, AZ32 reduced survival by a factor of 2.1 at both 1 and 2 Gy. Thus, the radiosensitizing effects of ATMi depend on p53 status in U87 glioma cells.

3.7 p53 Knockdown and AZ32 Enhance Mitotic Catastrophe in Irradiated Glioma

The mechanism by which p53 status and ATMi interact to determine glioma radiosensitivity remains unclear. Studies have shown that inhibition of ATM-dependent signaling through the p38MAPK-MK2 pathway induces mitotic catastrophe in p53-defective cells treated with doxorubicin (Reinhardt et al., 2007). Thus, we explored if ATMi results in aberrant cycling in irradiated sh-p53 glioma cells. In order to a follow mitosis, U87 and U87/sh-p53 cells were infected with lentiviruses expressing fluorescently tagged histone H2B and Centrin 2. H2B-mCherry labels chromatin, while Centrin 2-EGFP labels centrosomes. Cells were treated with 3μM AZ32 30 minutes prior to irradiation at 5 Gy. Mitosis was monitored by live-cell imaging on a spinning disc confocal microscope. Total and aberrant mitoses were counted visually and mitotic statistics recorded (Table 3-1). Representative images of normal and aberrant mitoses are provided in Figure 3-12. AZ32 greatly increases the frequency of aberrant mitoses only in cells in which p53 was knocked down. While there were only 1-3 instances of aberrant mitoses among wild-type and untreated U87/sh-p53 cells, 16 aberrant mitoses were identified in U87/sh-p53 cells treated with AZ32.
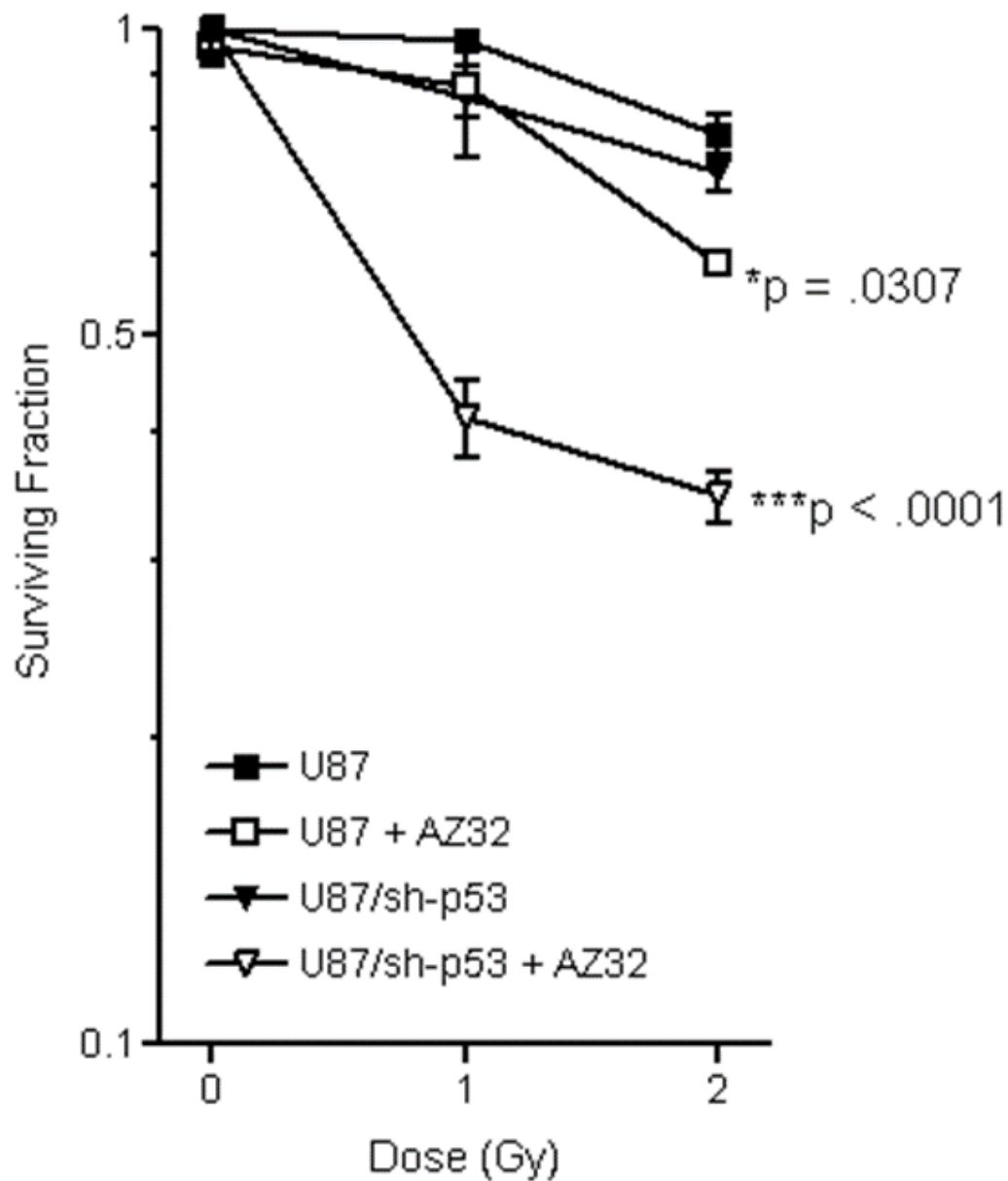
Figure 3-11: ATM inhibition by AZ32 significantly radiosensitizes U87/sh-p53 glioma cells. Log plot of surviving fraction vs. radiation dose. Colony forming assay confirms that treatment with 3 μM AZ32 radiosensitizes U87/sh-p53 glioma cells to a greater degree than wild-type U87 cells. Survival reduction factors for wild-type cells are 1.1 at 1 Gy and 1.3 at 2 Gy. Survival reduction factors for p53 mutants are 2.1 at 1 Gy and 2.1 at 2 Gy.
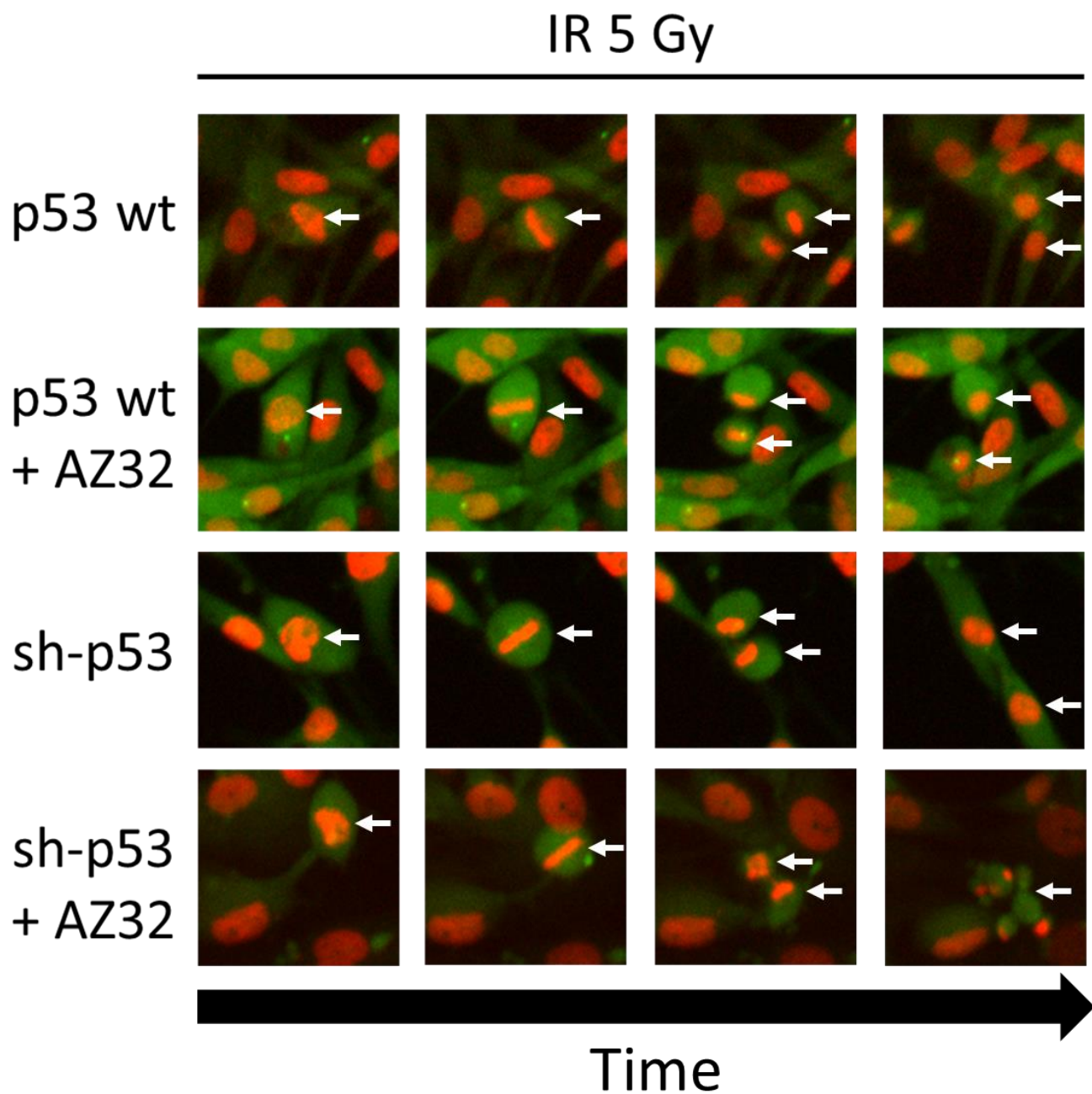
Figure 3-12: ATMi increases the rate of mitotic catastrophe in glioma cells when p53 is knocked down. Time series of mitosis in U87/Centrin-EGFP/H2B-mCherry and U87/sh-p53/Centrin-EGFP/H2B-mCherry cells. Treatment with 3 μM AZ32 does not appear to greatly increase the frequency of aberrant mitoses in p53 wild-type cells, but does in sh-p53 cells.

	Total Mitoses	Aberrant Mitoses	% Aberrant Mitoses	Mitotic Index
p53 wt + IR 5 Gy	37	1	2.7%	0.054
p53 wt + IR 5 Gy + AZ32	28	3	10.7%	0.042
sh-p53 + IR 5 Gy	25	3	12.0%	0.065
sh-p53 + IR 5 Gy + AZ32	35	16	45.7%	0.088

*Total cell counts for p53wt were 681 untreated and 671 with AZ32. Total count for sh-p53 cells were 382 untreated and 396 with AZ32

Table 3-1: Mitotic statistics for irradiated U87/Centrin-EGFP/H2B-mCherry cells

IV. Discussion and Future Directions

4.1 AZ32: From Bench to Clinic

Despite recent advances, survival rates for patients with glioblastoma multiforme remain dismal. Radiotherapy is an essential part of first-line treatment for GBM, but side-effects and inherent neurotoxicity remain significant drawbacks. In addition, gliomas tend to be highly aggressive and resistant to radiation. Indeed, it has been shown that glioma-derived cancer stem cells can preferentially activate DNA damage checkpoints and repair mechanisms to promote radioresistance (Bao et al., 2006). Therefore, targeting proteins involved in the DNA damage response may be a useful strategy for improving the efficacy of radiotherapy.

ATM kinase is the primary protein involved in signaling DNA double-strand breaks. Its substrates include a variety of proteins involved in cell cycle checkpoints and DNA repair (Matsuoka et al., 2007). As discussed in previous sections, pharmacological inhibition of ATM potently sensitizes glioma cells and tumors to ionizing radiation (Biddlestone-Thorpe et al., 2013; Golding et al., 2009, 2012). This is promising, because clinical adoption of ATMi could reduce the effective radiation dose necessary to kill tumor cells. This in turn could yield significant improvements in the length and quality of life for GBM patients. Therefore, the identification and validation of stronger, more specific ATM inhibitors is critical.

A next-generation ATMi, AZ32, was identified in a high-throughput molecular screen as a low-weight compound with enhanced blood brain barrier (BBB) penetration (Karlin et al., 2014). This work has validated the efficacy of AZ32 in human glioma cells by monitoring radiation-induced phosphorylation of Kap1, Chk2, and p53. These proteins are essential components of the DDR and, as such, are rapidly phosphorylated by ATM in response to ionizing radiation (Banin et al., 1998; Ward et al., 2001; White et al., 2012, 2006). We found that ATM inhibition by AZ32 is dose dependent and appears to be prominent even several hours after IR. In addition, AZ32 was found to be an equally potent inhibitor of ATM signaling as its predecessor, KU-60019. This is significant, because while KU-60019 has been established as a strong and specific ATM inhibitor, it is unable to cross the BBB (Vecchio et al., 2015). Thus, AZ32 offers enhanced bioavailability without sacrificing strength. We also confirmed that AZ32 significantly reduces radiosurvival of human glioma cells. Current clinical guidelines for GBM radiotherapy call for administration of daily 2 Gy fractions for a total of 40-60 Gy (Ghose et al., 2010). Our results are promising, because AZ32 significantly enhances glioma radiosensitivity at doses as low as 1 Gy. Such evidence suggests that clinical adoption of AZ32 could potentially lower the radiation dose necessary to combat GBM tumors.

Before transitioning to the clinic, further research is necessary to elucidate upon the pharmacological and biological properties of AZ32. For example, researchers have yet to assess AZ32's stability. Furthermore, it has been reported that serum greatly reduces the potency and bioavailability of the previous-generation ATMi, KU-60019 (Golding et al., 2012). Thus, it is critical to confirm AZ32's serum stability and efficacy.

It is also essential that future studies elaborate on AZ32's *in vivo* pharmacokinetics. This includes measures of bioavailability, circulatory and tissue distribution, BBB penetration, and excretion. It would be particularly valuable to assess if AZ32 can be administered non-invasively without any deleterious off-target side effects. It is also necessary to elaborate on tolerance and relative toxicity. Most importantly, research must validate the *in vivo* efficacy of AZ32 in radiosensitizing GBM tumors. This includes measuring effects on actual tumor size, growth, and persistence, as well as overall survival in animals. Indeed, our group has already shown that AZ32 administered with radiotherapy significantly increases survival in GL261 mouse glioma models and is in the process of further validating its effects *in vivo* (Karlin et al., 2014). Future work should also focus on examining if ATMi treatment can sensitize other cancers to DDAs. In addition, it is crucial to elaborate on how inhibition of ATM affects radiosensitivity of normal tissue, and whether such interactions have implications for clinical adoption of ATMi in cancer therapy. Finally, studies should elaborate on the molecular and physiological mechanisms that underlie radiosensitization by ATMi.

4.2 Linking Mitotic Catastrophe to Radiosurvival

While ATMi effectively sensitizes glioma cells to radiation, how it does so remains a mystery. It is also unclear why defects in p53 enhance these radiosensitizing effects. Studies have shown that ATM-dependent signaling through the p38MAPK-MK2 pathway is essential for G2/M arrest in p53-null mouse embryonic fibroblasts (MEFs), and that disruption of this pathway results in cell death by mitotic catastrophe (Reinhardt et al., 2007). This suggests that ATMi overrides G2/M arrest in cells with defective p53,

thus reducing radiosurvival by promoting mitotic catastrophe. We began testing this hypothesis in isogenic HCT116 cells expressing the R248W DNA-binding-domain mutant form of p53. Others have shown that disruption of p53 sensitizes these cells to IR and other DDAs (Bunz et al., 1999). Another report by the same group indicates that p53-null HCT116 cells fail to arrest the cell cycle after IR (Bunz, 1998). We confirmed that mp53 HCT116 cells are also unable to inhibit mitosis after IR. Contrary to expectations though, aberrant mitotic entry did not translate to reduced radiosurvival. In fact, we found that mp53 cells were significantly more radioresistant than wild-type HCT116 cells. Furthermore, ATMi was more effective at radiosensitizing p53 wild-type cells than their mp53 counterparts.

These observations are in stark contrast to our group's previous findings in glioma cells (Biddlestone-Thorpe et al., 2013). We suspect that this difference may be due to the fact that basal levels of replicative stress are significantly higher in gliomas than in cancers derived from epithelial tissues (Bartkova et al., 2010). Indeed, neither wild-type nor p53-deficient HCT116 cells appear to display any significant degree of inherent genetic instability (Bunz et al., 2002). It may be that wild-type p53 induces apoptosis in response to replicative stress in HCT116 cells, but that such mechanisms are inactive in glioma. Indeed, previous reports indicate that gliomas are often highly resistant to death by apoptosis (Eisele and Weller, 2013; Steinbach and Weller, 2004). Thus, it is possible that ATM inhibition in irradiated HCT116 cells reduces repair of DSBs, stimulating apoptosis through p53-dependent signaling. This would enhanced radioresistance in mp53 background. Yet, the Vogelstein group has shown that deletion of p53 in HCT116

cells sensitizes them to apoptosis after treatment with IR and other DDAs (Bunz et al., 1999). The authors suggest that this is due to reduced p21 expression, but cells expressing R248W-mp53 also appear to be defective in p21 induction and, therefore, should exhibit a phenotype similar to p53-null cells (Song et al., 2007; Sur et al., 2009; Wohak et al., 2014). An alternate theory is that the reduced responsiveness of mp53 HCT116 cells to ATMi is due to the fact that ATM signaling is already partially defective in these cells. Indeed, HCT116 cells harbor an intrinsic mutation in Mre11, which results in defective signaling through ATM and Chk2 (Takemura et al., 2006). It has also been shown that MEFs expressing R248W-mp53 are unable to efficiently recruit ATM to sites of DSBs due to defects in the Mre11-Nbs1-Rad50 complex (Song et al., 2007). Finally, it may be that the observed effects are due to inherent differences in signaling between different p53 mutants. Our group's previous studies utilized glioma cells overexpressing D281G-mp53. It has been documented that different p53 mutants vary in their ability to transactivate target genes and interact with protein binding partners (Freed-Pastor and Prives, 2012; Kang et al., 2013; Muller and Vousden, 2013). Therefore, it may be that structural and functional differences between R248W and D281G-mp53 alter sensitivity to radiation and ATMi.

Given the possibility that DDR signaling and dominant modes of cell death differ between colon carcinomas and gliomas, we sought to work with isogenic glioma cells that differ only in p53 status. Previous results from our group indicate that mp53 gliomas are more sensitive to IR than their wild-type counterparts in the presence of ATMi, but these studies were performed in cells in which mp53 was overexpressed. Thus, it

remains unclear if ATMi's radiosensitizing effects are due to p53 status, or if

overexpression is a confounding variable. Therefore, we selected glioma cell clones

expressing short-hairpin RNA against p53. The parental U87 glioma cell line is the same

one used in the previous study showing ATMi preferentially sensitizes p53 mutants to IR.

We found that radiosurvival does not vary significantly between sh-p53 and wild-type

U87 glioma cells. It was also confirmed that radiosensitization by ATMi is enhanced in

cells in which p53 is knocked down. Finally, time-lapse imaging following IR revealed

that ATMi does preferentially increase the rate of mitotic catastrophe in sh-p53 cells. As

previously mentioned, our group has shown that G1/S arrest is defective in mp53

gliomas, while G2/M arrest appears intact (Biddlestone-Thorpe et al., 2013). The current

findings support the theory that ATM is necessary for p53-independent G2/M arrest.

The molecular mechanisms underlying our observations remain unclear, but it has

been suggested by the Yaffe group that ATM-mediated activation of the p38MAPK-MK2

pathway is necessary for G2/M arrest in p53-deficient cells (Reinhardt et al., 2007).

Furthermore, inactivation of MK2 results in mitotic catastrophe in p53-null MEFs

following treatment with DDAs cisplatin and doxorubicin. The same group showed that

doxorubicin results in translocation of the p38/MK2 complex from the nucleus to the

cytoplasm within an hour of treatment (Reinhardt et al., 2010). Specifically,

phosphorylation of MK2 (T334) appears to be necessary for exposing a nuclear export

signal (Ben-Levy et al., 1998; Meng et al., 2002; White et al., 2007). Once in the

cytoplasm, MK2 phosphorylates a variety of proteins involved in binding and degrading

GADD45α mRNA, increasing its expression. GADD45α, in turn, binds to Cdc25B and

Cdc25C, sequestering them to the cytosol and preventing aberrant mitotic entry. ATM's direct involvement with this pathway remains unclear, but it has been suggested that signaling through the thousand-and-one kinases (TAOKs) may underlie the interaction (Beckta et al., 2014). The TAOKs directly activate MAPK kinases MEK3 and MEK6, which phosphorylate and active p38MAPK. Indeed, TAOKs themselves are directly phosphorylated by ATM after IR (Raman et al., 2007). Thus, ATM-mediated activation of p38 by the TAOKs may contribute to efficient G2/M arrest in p53-deficient cells.

Intriguingly, it has been reported that nuclear signaling through p38 and MK2 is required for proper mitosis through an interaction with Plk1 (Tang et al., 2008). The authors found that Plk1 colocalizes with p38 and MK2 at spindle poles during prophase and metaphase and that MK2 phosphorylates Plk1 at serine-326 both *in vitro* and *in vivo*. In addition, p38 or MK2 depletion or expression of a Plk1 mutant (S326A) was sufficient to induce mitotic arrest. Mitotic arrest by p38 or MK2 inactivation was reversed only in cells expressing the Plk1 phospho-mimetic mutant (S326E). As mentioned previously, Plk1 promotes mitotic entry by facilitating nuclear translocation of Cdc25c and Cdc25b phosphatases (Lobjois et al., 2009; Toyoshima-Morimoto et al., 2002). Plk1 also appears to be necessary for recovery from G2/M arrest and its expression is elevated in cells lacking wild-type p53 (Macůrek et al., 2008; Sur et al., 2009). Furthermore, p53 represses Plk1 expression by directly binding its promoter, and a physical interaction between Plk and p53's DNA binding domain reduces transcription of p53-target genes (Ando et al., 2004; McKenzie et al., 2010).

In conclusion, our findings and the literature suggest that cell cycle arrest in response to DNA damage depends on a complex interplay between ATM, the TAOKs, p38MAPK, MEK3/6, MK2, p53, and Plk1. Signaling through these pathways converges on the Cdc25 phosphatases, resulting in their cytosolic sequestration and inhibition of mitosis. Figure 4-1 outlines a possible model of the various signaling components that contribute to G2/M arrest. Theoretically, disruption of signaling through ATM, TAOKs, MEK3/6, p38, or MK2 should abrogate G2/M arrest in p53-deficient cells. Importantly, it is necessary to assess if targeting TAOKs and downstream MEK3/6 can sensitize cancer cells to IR and other DDAs. In addition, future studies should elaborate upon the role of Plk1 in G2/M arrest and checkpoint reversal. Our group has already begun work in glioma cell lines overexpressing Aurora kinase A (AURKA), the kinase necessary for activating Plk1 and stimulating mitosis and checkpoint recovery (Bruinsma et al., 2014; Macůrek et al., 2008). We expect that cells overexpressing AURKA will display increased signaling through Plk1, causing them to behave more like p53 mutants in their sensitivity to DDAs and cell cycling. Finally, it is necessary to elaborate upon the variation between glioma and colon carcinoma cells in their response to ATMi. It would be interesting to explore how these cells differ in p53-dependent apoptosis. Specifically, it would be useful to analyze how expression of p53 targets involved in apoptosis varies between p53-null and mutant U87 and HCT116 cells. It would also be of value to assess the functionality and fidelity of the MRN-complex in recruiting ATM to sites of DSBs in glioma cells, since such mechanisms appear to be defective in colon carcinoma. Indeed, elucidating upon how various signaling mechanisms interact to determine cell death in different cancers would be useful for identifying pathways to target for new therapies.
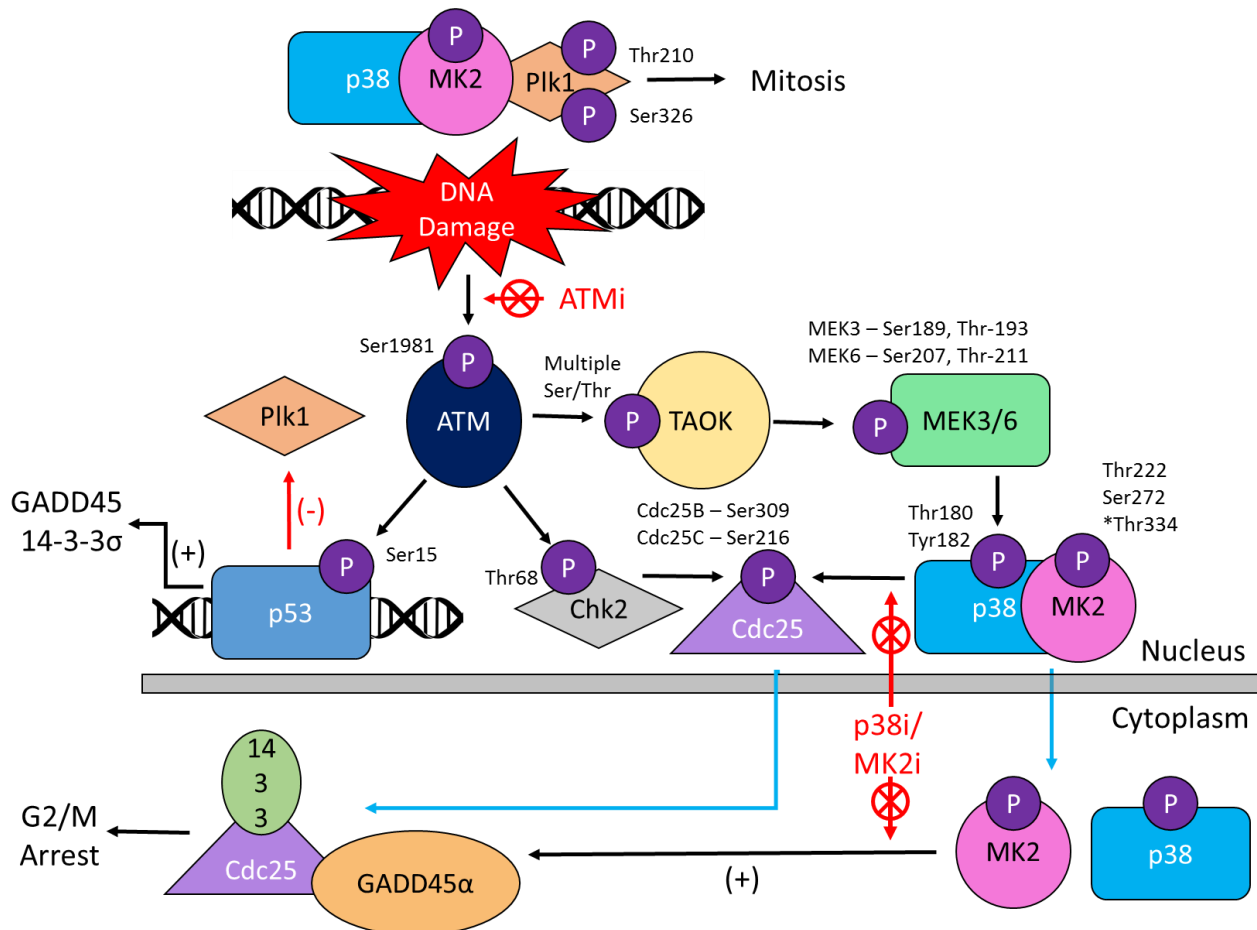
Figure 4-1: An expanded model of G2/M Arrest following DNA damage. Both p53 and ATM contribute to G2/M arrest in normal cells. ATM signaling ultimately results in phosphorylation of Cdc25, which stimulates binding by 14-3-3 and subsequent nuclear export. Activation of p38 by MEK3/6 results in phosphorylation of MK2 on threonine 334, exposing a nuclear export signal. Cytoplasmic MK2 promotes expression of GADD45α, which binds and inhibits Cdc25. p53 inhibits transcription of Plk1 and stimulates transcription of proteins that sequester Cdc25 to the cytosol, sustaining mitotic arrest. In the absence of p53, Plk1 levels remain high and nuclear export of p38 and MK2 is necessary to sustain G2/M arrest. ATM or p38/MK2 inhibition in p53 mutants promotes mitotic catastrophe. Arrows: Black = Activation/Phosphorylation; Red = Inhibition; Blue = Translocation

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