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ATM phosphorylates subunit A of PP2A resulting in its nuclear export and spatiotemporal regulation of the DNA damage response

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ATM PHOSPHORYLATES SUBUNIT A OF PP2A RESULTING IN ITS NUCLEAR EXPORT AND SPATIOTEMPORAL REGULATION OF THE DNA DAMAGE RESPONSE

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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List of abbreviations

7AAD	7-Aminoactinomycin D
53BP1	p53 Binding Protein 1
Alt-EJ	Alternative End Joining
A-T	Ataxia-telangiectasia
ATM	Ataxia-telangiectasia mutated
ATR	A-T and RAD3-related
CRM1	Chromosome Region Maintenance 1
CDK	Cyclin Dependent Kinases
DSB	DNA Damage Response
Gy	Gray
h	hour
HEAT	Huntington/Elongation/A-subunit/Tor
HRR	Homologous Recombination Repair
IR	Ionizing Radiation
kDa	Kilodalton
LCMT1	Leucine Carboxyl MethyltransfeRASe-1
LMB	Leptomycin B
MEF	Mouse Embryonic Fibroblast
MRN	Mre11/Rad50/Nbs1 complex

NHEJ	Non-Homologous End Joining
NES	Nuclear Export Signal
OA	Okadaic acid
PARP	Poly (ADP-Ribose) PolymeRASe
PBS	Phosphate Buffer Saline
PCR	PolymeRASe Chain Reaction
PIKK	Phosphatidyl-Inositol-3'-Kinase-related Kinase
PME-1	PP2A-specific MethylesteRASe
PP1	Protein Phosphatase 1
PTPA	PP2A specific phosphatase activator
PVDF	Polyvinylidene difluoride
qPCR	quantitaive PCR
RT	Room Temperature
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Sgo-1	Shugoshin 1
SSA	Single Strand Annealing
SSB	Single Strand Breaks
XRCC	X-Ray Cross-Complementing

ABSTRACT

ATM PHOSPHORYLATES SUBUNIT A OF PP2A RESULTING IN ITS NUCLEAR
EXPORT AND SPATIOTEMPORAL REGULATION OF THE DNA DAMAGE
RESPONSE

By Amrita Sule, M.Sc.

A dissertation submitted in partial fulfillment of the requirements for the degree of
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Ataxia telangiectasia mutated (ATM) is a serine-threonine protein kinase and
major regulator of the DNA damage response (DDR). One critical ATM target is protein
phosphatase 2A (PP2A) known to regulate diverse cellular processes such as mitosis and
cell growth as well as dephosphorylation of many proteins during the recovery from the
DDR while returning the cell to normalcy. Interestingly, ATM and PP2A are known to
form an auto-regulatory yin-yang kinase-phosphatase relationship. Herein, we show that
the phosphorylation of the PP2A-Aα structural subunit at S401 by ATM results in nuclear
export, which regulates the DDR at multiple levels and affects genomic stability and cell
growth. We generated PP2A-Aα conditional knockout mouse embryonic fibroblasts
expressing PP2A-Aα-WT, S401A (cannot be phosphorylated), or S401D

phosphomimetic) transgenes by floxing out the endogenous PP2A-Aα alleles with Cre. The S401D mutant cells displayed increased ERK and AKT signaling, resulting in an enhanced growth rate. Phosphorylation of PP2A-Aα at S401 caused the dissociation of ATM with the holoenzyme, an effect that could be recapitulated with S401D. Additionally, the S401A and S401D mutants exhibited significantly more chromosomal aberrations and underwent increased mitotic catastrophe after radiation. Both the S401A and the S401D cells showed impaired DSB repair (Non-homologous end joining and Homologous recombination repair) and exhibited delayed DNA damage recovery, which was reflected in reduced radiation survival. Time-lapse video and cellular localization experiments showed that the PP2A-Aα subunit was exported to the cytoplasm after radiation possibly by CRM1, a nuclear export protein, in line with the very rapid pleiotropic effects seen. In conclusion, our study demonstrates using a genetically defined system that ATM phosphorylation of a single, critical amino acid S401 is essential for regulating DDR. To study how the interplay between ATM and PP2A affects DDR in the brain, we are in the process of generating a brain specific PP2A-Aα conditional knockout mouse. Loss of many DDR related proteins like ATM and PP2A can lead to severe neuropathological effects. This model will be helpful in dissecting the PP2A-Aα/ATM regulatory circuit in the brain in response to DDR.

CHAPTER 1:

General introduction

DNA damage response

Cells encounter thousands of events everyday, which can cause genomic stress and ultimately result in DNA damage. Damage could be caused by endogenous agents, like reactive oxygen species generated in cell, or during replication due to stalled replication forks, as well as damaged telomeres. Exogenous agents like radiation, radiomimetic and genotoxic drugs also contribute to DNA damage in cells. Over the years, our cells have evolved to elicit a response to cope with such damage (Ciccia and Elledge, 2010; Jackson and Bartek, 2009). This response initiated by the cell is termed as DNA damage response (DDR), which is a collective term for several cellular signaling pathways, which are set-off by detection of DNA damage.

DNA damage can be of different types. Replication errors, which result in mismatches, deletions and insertions. Base modifications due to oxidation, deamination or depurination are some of the common modifications that result in damaged DNA. There are instances when exogenous chemical agents cause bulky adducts or cause one DNA strand to covalently bind to the other strand causing intra-strand or inter-strand crosslinks. UV exposure can induce or single strand breaks (SSBs). Free radicals produced as a by- product of endogenous processes or exogenous agents like ionizing radiation (IR) can induce the formation of double-strand breaks (DSBs). These different kinds of damage evoke distinct repair mechanisms. The cell resolves most of the damage

using the DNA repair machinery. However, failure to do so can be deleterious to the cell. Of all the types of DNA damage, DSBs are the most lethal (O'Connor, 2015).

When cells encounter damaged DNA, sensor proteins initiate signaling to assess the type of damage and elicit an appropriate response by modulating downstream proteins. The three well-studied damage sensing proteins in humans belong to the phosphatidyl-inositol-3'-kinase-related kinase (PI3KK) family of proteins. In an event of DNA damage, Ataxia Telangiectasia mutated (ATM), ATM and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK) get activated and localize at the sites of DNA breaks and mediate DNA repair via modulating survival and death signaling (Shiloh and Ziv, 2013). These PI3KKs direct a coordinated DDR (Valerie and Povirk, 2003) by acting at several levels, including cell cycle checkpoints, DNA double-strand break repair and apoptosis (Abraham, 2001; Shiloh, 2003).

ATM, ATR and DNA-PK have similar structure with a variable number of HEAT repeats at the N-terminus, a FAT domain and a FATC domain at the C-terminus. These kinases phosphorylate their substrates on signature Ser /Thr residues followed by a Gln residue (SQ/TQ). Despite some similarities in structure and functionality, these kinases play distinct roles in DDR regulation. DNA-PK primarily promotes repair of DSBs by non homologous end joining (NHEJ). ATM is an important checkpoint regulator and activates repair by homologous recombination repair (HRR). SSBs, which arise due to collapse of a replication fork or UV irradiation induced stress, activate ATR (Ceccaldi et al., 2016)

Cell Cycle Checkpoint activation

In addition to facilitating efficient DNA repair, DDR also tightly regulates cell cycle progression in an event of damage. The cell cycle is controlled by cyclin-dependent kinases (CDK). DDR effector proteins limit CDK activity to prevent premature entry into the respective stages of cell cycle. The checkpoint kinases Chk1 and Chk2 are direct substrates of ATR and ATM respectively (Bartek and Lukas, 2003; Smits et al., 2006).

The G1/S checkpoint is primarily controlled by p53. ATM as well as Chk2 are the main mediators of G1/S checkpoint. Upon DNA damage, p53 is rapidly stabilized as a result of phosphorylation by ATM, ATR, Chk1 and Chk2 kinases. When phosphorylated at S15, p53 upregulates transcription of p21. p21 is a CDK inhibitor, that suppresses cyclins E/A and therefore mediates G1/S arrest (Bartek and Lukas, 2001).

The intra-S checkpoint delays progression through S phase by preventing replication of damaged DNA. ATM-mediated phosphorylation of BRCA1, FANCD2, Chk2 and Nbs1 is required for effective intra-S checkpoint. Also, Chk1, activated by ATR, can phosphorylate Cdc25a and promote its proteasome-mediated degradation, subsequently inhibiting CDK activity (Bartek and Lukas, 2003; Mailand et al., 2000).

The G2/M checkpoint ensures that the damage is repaired before proceeding to mitosis and cell division. This checkpoint is activated by ATM/ATR-mediated activation of
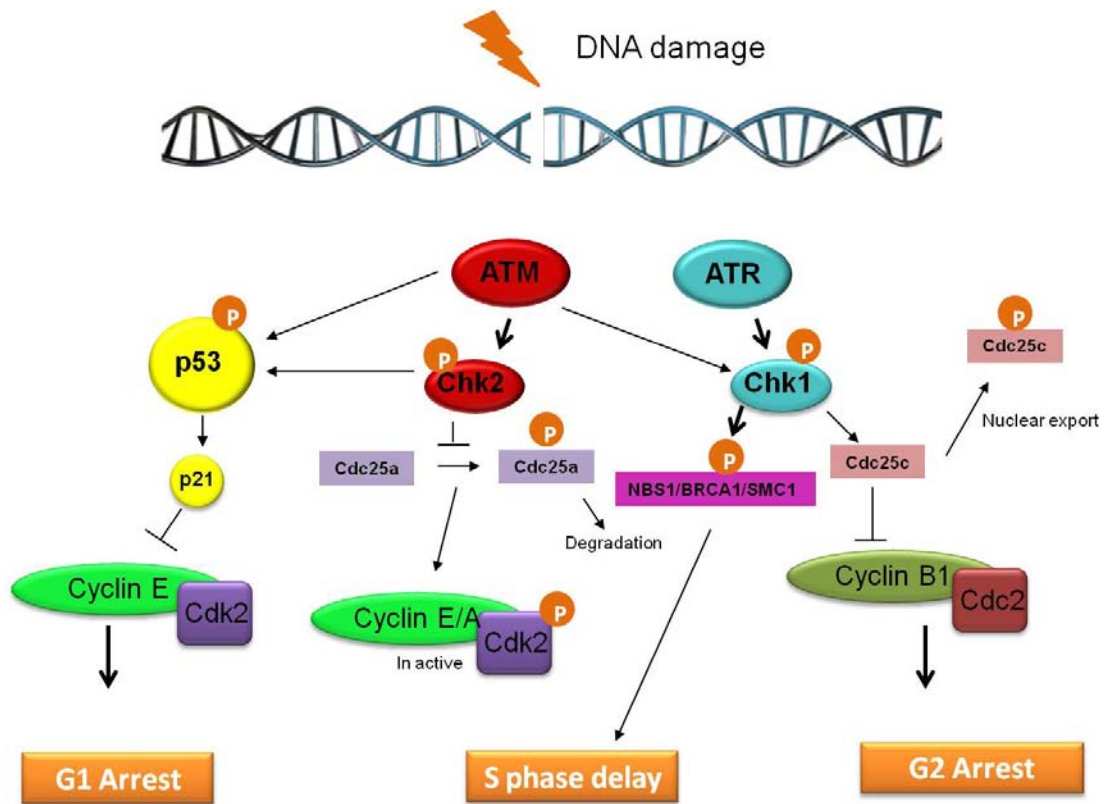
Figure 1-1: Cell cycle checkpoint control post DNA damage

The three main checkpoints initiated post DNA damage occur through G1/S transition, within the S phase and the G2/M transition. Adapted from Abraham (2001).

Chk1. Chk1 phosphorylates Cdc25c and causes its sequestration by 14-3-3 in the cytoplasm. Cdc25c can no longer dephosphorylate cyclin B/Cdc2, thus causing G2/M arrest (Taylor and Stark, 2001).

The Spindle assembly checkpoint, also known as M-phase checkpoint, ensures that each of the duplicated chromosomes is attached to mitotic spindles before their separation. At the end of mitosis, APC/C-CDC-20 activity is increased in the cell to degrade all the mitotic determinants. The spindle assembly checkpoint is mediated by early mitotic inhibitor 1 and 2, which inhibit APC/C-CDC-20 activity. Also, Shugoshin 1 (Sgo1) mediated PP2A-B56 recruitment, dephosphorylates Plk1 and prevents dissociation of cohesion and thereby prevents premature segregation of sister chromatids. When cells enter into mitosis despite unrepaired or damaged DNA, apoptotic cell death is triggered, known as mitotic catastrophe.(Castedo et al., 2004).

All these checkpoints are safeguard mechanisms for the cell to progress from one phase to other, ensuring that the damage is resolved before cell cycle progression (**Fig. 1-1**). Timely activation of these check points is essential to maintain genomic stability in an event of DNA damage.

Double-strand break repair

DSBs are detrimental to the cell if left unrepaired (Dasika et al., 1999; Illiakis, 1991). The two primary high fidelity processes used by cells to repair DSBs are the classical non-homologous end joining (c-NHEJ) pathway and the homologous recombination repair (HRR) pathway. Cells also use other DSB repair pathways like alternative end joining (Alt-EJ) and single strand annealing (SSA); however, these pathways are intrinsically mutagenic. These error prone pathways are known to give rise to genomic rearrangements leading to oncogenic transformation (Ceccaldi et al., 2016).

Nonhomologous end joining pathway

DSB repair by c-NHEJ pathway occurs throughout the cell cycle but it is predominant in the G1/G0 phase of cell cycle. NHEJ, though error prone, is extremely efficient in maintaining genomic integrity because of its fast kinetics (Ceccaldi et al., 2016). This repair involves ligation-mediated repair of DSBs and is independent of sequence homology. c-NHEJ is coordinated by the action of DNA-PK, a holoenzyme comprised of Ku heterodimer, Ku70 (70 kDA), Ku80 (86 kDA) and a large catalytic subunit (DNA-PKcs). The DSBs are recognized by heterodimer proteins Ku70/Ku80, that bind to the DSB ends and stabilize them. Ku70/Ku80 proteins act as sensors and recruit DNA-PKcs at the DSBs. At the break, DNA-PK functions as a kinase to synapse the ends and target other NHEJ factors at the DSBs. A multimeric complex consisting of DNA Ligase IV,
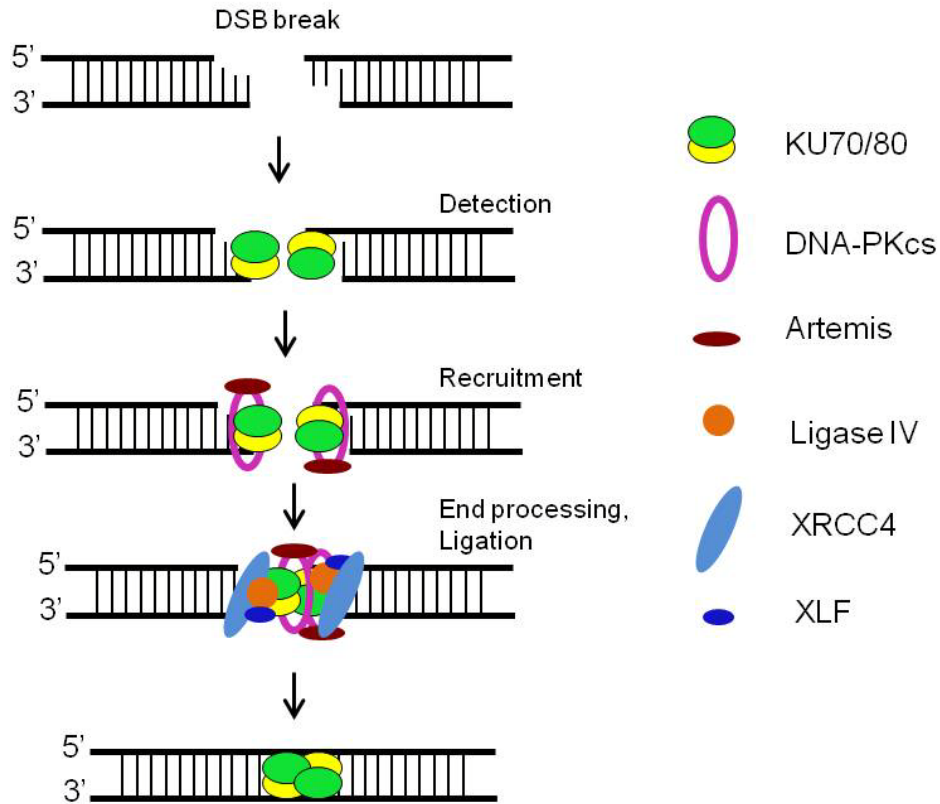
Figure 1-2: Nonhomologous end joining pathway

The DSB is recognized by KU70/KU80 heterodimer and recruits DNA-PKcs. The XLF protein, DNA ligase IV and XRCC5 complex is recruited at the DSBs. Artemis carries out end processing on the unligatable ends before they are joined. Adapted from Valerie and Povirk (2003)

XRCC4 and XLF are recruited and activated in a DNA-PKcs dependent manner (**Fig. 1-2**). Artemis functions as an endonuclease that mediates end-processing of the DNA overhangs in case of unligatable termini (Povirk et al., 2007). DNA ligase IV mediates DNA-strand joining and exists in a complex with XRCC4 (Critchlow et al., 1997; Robins and Lindahl, 1996). Pol μ and λ polymeRASes are associated with c-NHEJ to fill in nucleotide overhangs (Ma et al., 2004). XLF stimulates gap filling (Akopiants et al., 2009). These NHEJ players have mechanistic flexibility, which permits NHEJ to function on a wider range of DNA starting structures.

Homologous recombination repair

HRR is a critical DSB repair pathway. It repairs the DSBs using a sister chromatid or homologous chromosomes and hence is restricted to the S/G2 phase of cells cycle. It is regulated by ATM at DSBs and ATR at SSBs (stalled/collapsed replication forks), which have the potential to be converted to DSBs (Golding et al., 2004; Wang et al., 2004). HRR is initiated by 5′ -3′ resection of the DNA ends resulting in two single DNA strands. The heterotrimeric complex Mre11-Rad50-Nbs1 (MRN) binds to the DNA ends (Williams et al., 2009). The single-stranded DNA coated with Rad51 begins strand invasion and searches for a homologous region within the DNA duplex and forms a D-loop structure (Barzel and Kupiec, 2008). DNA polymeRASe extends the invading strand to form a Holliday Junction. The second DSB enters the D loop and a double Holliday
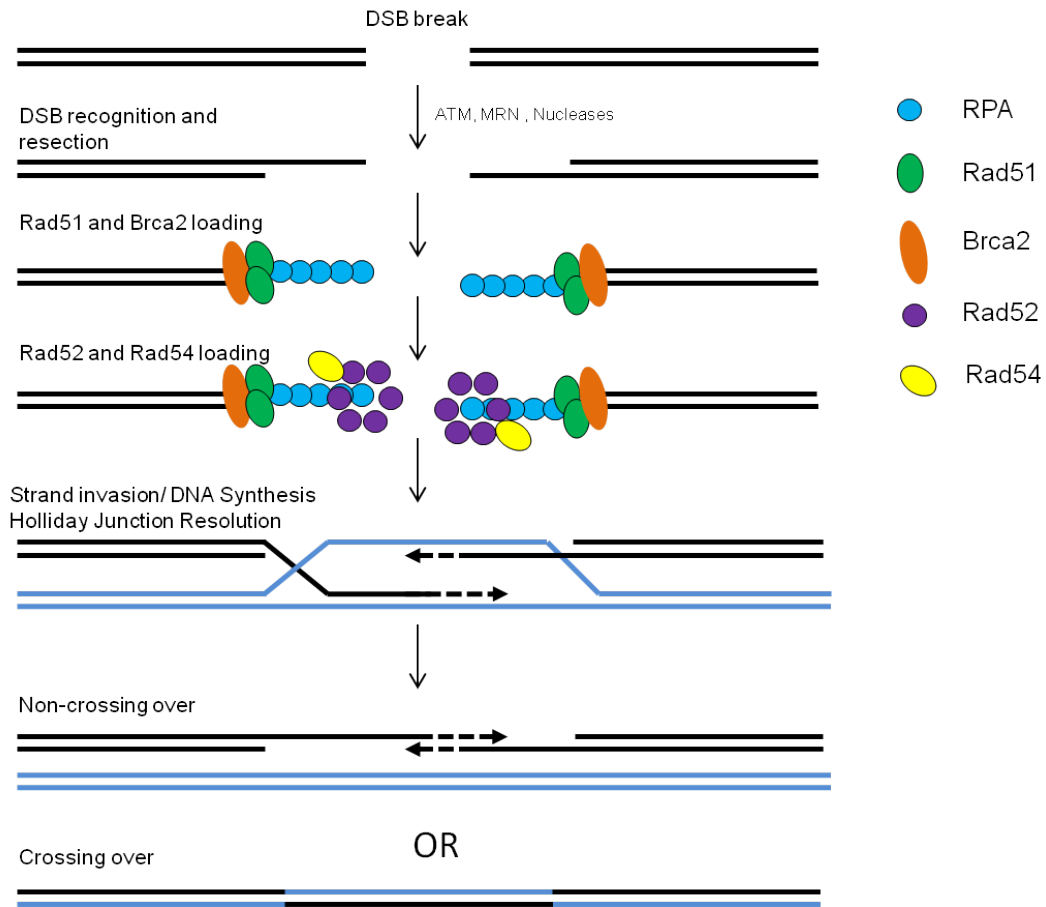
Figure 1-3: Homologous recombination repair pathway

Post detection of DNA damage, HRR proceeds to resect the DSB ends exposing a 3'
single-stranded DNA. RPA coats the single strand of the DNA, which is then replaced by
Rad51. Strand invasion takes place after locating the homologous sequence and results in
formation of a D-loop structure. The invading 3' strand is extended by polymeRASe.
Resolution of the Holliday junction can cause crossing over and swapping of arms
between the interacting chromatids.

Junction is formed (Barzel and Kupiec, 2008). The resolution of two-cross strand or Holliday Junctions should result in intact double-stranded products (**Fig. 1-3**).

Choice of DSB repair pathways

The choice of DSB repair pathway depends on many factors, one of the determining factors being extent of end resection. A balance between BRACA1 and 53BP1 decides which pathway is ultimately chosen. BRCA1 binds to CtIP and MRN complex at DSBs and promotes repair by HRR (Hu et al., 2014). Conversely, 53BP1 blocks resection by preventing access of CtIP at the breaks, thereby directing repair via c-NHEJ. This function of 53BP1 is dependent on its phosphorylation by ATM (Bunting et al., 2010).

There are other pathways that also mediate DSB repair. Once resection has occurred, it leaves the choice of repair by HRR or by the other homology-based pathways SSA and Alt-EJ. Although Alt-EJ has always been thought to be a backup pathway for c-NHEJ, recent studies have shown that it competes with HRR for repair of DSBs. (Ceccaldi et al., 2016). In Alt-EJ pathway, PARP1 is thought to function in recruitment of repair factors like Polθ and mediate the microhomology dependent repair. Loss of PARP1 in HRR-compromised cells caused synthetic lethality suggesting that PARP-mediated Alt-EJ compensates for HRR deficiency (Bryant et al., 2005; Farmer et al., 2005).

Phosphatases and Kinases

The DDR is a synchronized effort of many phosphatases and kinases. The PI3KKs (ATM, ATR and DNA-PK) can phosphorylate hundreds of proteins in response to exogenous DNA damage. DDR is a coordinated phosphorylation-signaling cascade, which involves tightly regulated post-translational modifications of proteins, resulting in their inhibition, activation or change in subcellular localization. Studies are being carried out to examine the dynamics of phosphorylation events during and after DDR (Bennetzen et al., 2010). Therefore, to bring the cell to its native state, reversible phosphorylation is of utmost importance (Hunter, 1995; Pawson and Scott, 1997). Phosphatases are the evident candidates that can aid in regulating balance of the phosphorylation events post DDR. Thus, phosphorylation and dephosphorylation can act as an ON or OFF switch for the activity of the target protein. However, phosphatases not only play a role in counteracting the DDR related phosphorylation events but also play a role in initiating DNA repair via dephosphorylation of key proteins (Lee and Chowdhury, 2011). For the purpose of our study we will be reviewing ATM kinase and protein phosphatase 2A in detail.

ATM kinase

Ataxia-Telangietasia mutated (ATM) is a Ser/Thr kinase. The gene encoding ATM is mutated in the autosomal recessive disorder Ataxia-Telangietasia (A-T), which is

characterized by increased genomic instability, neurodegeneration, predisposition to cancer and immunodeficiency (Kastan et al., 2001). Cells from patients with A-T display extreme radiosensitivity (Taylor et al., 1975). Ectopic expression of ATM in these cells were able to rescue the A-T features (Zhang et al., 1998; Ziv et al., 1997). ATM, a PI3KK family member, shares sequence similarity in their C-terminal region with other members, ATR and DNA-PKcs. The C-terminal consists of kinase, FAT (conserved in FRAP, ATM and TRRAP) and the FATC (FAT-C terminal domain). The N-terminal is among the PIKK members consists of variable number of HEAT (Huntington/Elongation/A-subunit/Tor) repeats.

ATM kinase is a master regulator of the cellular response to DSBs and relays a wide spread signal to downstream proteins to initiate the DDR. ATM exists as an inactive dimer in the cell. In the event of a DSB, ATM is recruited to the DSB by the MRN complex (Lee and Paull, 2005), which acts as a damage sensor. The interaction between Nbs1 and ATM is crucial for ATM's recruitment and retention at the DSBs (Difilippantonio and Nussenzweig, 2007; Lee and Paull, 2005). The ATM dimer undergoes trans auto-phosphorylation at the S1981 residue in the FAT domain and dissociates into the active monomeric form (Bakkenist and Kastan, 2003). ATM also undergoes TIP60 mediated acetylation on K3016 residue, which is essential for its activation (Sun et al., 2005, 2007). Three other autophosphorylation sites have also been identified on ATM; however, abrogation of those affect DDR in humans but not in mice (Kozlov et al., 2006, 2011). Phosphorylation at S1981 of ATM is regulated by PP2A,

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which binds to ATM and prevents its activation in the absence of DNA damage (Goodarzi et al., 2004).

At the site of DNA damage ATM phosphorylates the histone variant H2AX at S139 also referred to as γ-H2AX (Rogakou et al., 1998). γ-H2AX starts accumulating at the damage site as early as one minute after damage and appear as discrete foci. This is followed by recruitment of MDC1, which interacts with γ-H2AX and MRN and mediates retention of ATM at the damage sites (Stucki et al., 2005). H2AX and MDC-1 act as scaffold proteins and recruit downstream repair proteins like 53BP1 and BRCA1 complex (Shiloh and Ziv, 2013). In response to DNA damage, ATM mediates checkpoint activation via phosphorylation-dependent activation and inhibition of several effector proteins. We have discussed some of the aspects of the same in the previous section of this chapter. ATM-mediated DDR is an example of multilayered regulation of several pathways. For example, ATM modulates the activity of protein kinases like Chk2, which in turn phosphorylate their own substrates. Thus, ATM lies at the apex of a very extensive network. One of the well-studied targets of ATM is p53. ATM activation stabilizes p53 and drives the expression of many genes that promote programmed cell death. ATM can also phosphorylate the IKKγ subunit of IKK. IKK mediated phosphorylation degrades the NF-κB inhibitor, IκBα. This releases NF-κB from IκBα in the cytoplasm and imports it to the nucleus where it drives the expression of anti-apoptotic genes. Thus, ATM-mediated phosphorylation on its substrates aids in making a decision between cell survival and activation of programmed cell death. However, there are several more players involved

whose action is dependent on specific post translational modifications (Shiloh and Ziv, 2013).

Although traditionally considered as a nuclear protein, studies have revealed cytoplasmic function of ATM, especially in insulin-mediated signaling. Insulin stimulation can activate ATM kinase, which then phosphorylates 4E-BP1, thus implicating ATM directly in the insulin signaling pathway (Yang and Kastan, 2000). ATM being a kinase with the capacity of modulating a vast number of proteins, ongoing studies are looking at the role of ATM exclusive to DDR.

Protein Phosphatase 2A

Ser/Thr phosphatases control a number of cell signaling pathways and crucial processes like DNA replication and gene transcription. Protein phosphatase 2A (PP2A) and Protein phosphatase 1 (PP1) are two major Ser/Thr phosphatases and together are responsible for almost 90% Ser/Thr phosphatase activity in a cell. Both PP1 and PP2A are sensitive to okadaic acid (OA). OA exhibits much greater affinity and inhibition efficacy towards the PP2A (IC_{50}=0.1–0.3 nM for PP2A vs 15–30 nM for PP1) (Cohen et al., 1989; Favre and Turowski, 1997; Swingle et al., 2007). PP2A is ubiquitously expressed in eukaryotic cells and regulates cell cycle, cell proliferation, cell survival and apoptosis (Janssens and Goris, 2001; Perrotti and Neviani, 2013; Schönthal, 2001; Seshacharyulu et al., 2013).

PP2A regulates many cellular processes including DNA replication, cell cycle progression and cell differentiation. The PP2A holoenzyme is a heterotrimer comprised of a scaffolding subunit (A), a catalytic subunit (C), and a regulatory subunit (B). In mammalian cells it exists either as a heterodimer (AC) or a heterotrimer (ABC) (Janssens et al., 2008) (**Fig. 1-4**). The A-subunit has two isoforms Aα and Aβ (Encoded by PPP2R1A or PPP2R1B respectively), which are 86% identical. The Aα isoform is expressed at much higher levels relative to the Aβ isoform (Hemmings et al., 1990) and 90% of the holoenzymes are comprised of the Aα isoform (Yang and Phiel, 2010). The PP2A-Aα and the Aβ isoforms have distinct functions, which has been documented by their differential B-subunit binding (Zhou et al., 2003). Also, in a *Xenopus* system, Aα and Aβ are involved at different developmental stages (Hendrix et al., 1993) and in mice, the Aβ subunit is not able to compensate for Aα (Eichhorn et al., 2009). Each isoform is shaped like a horseshoe composed of 15 non-identical internal repeats of a 39 amino acid sequence, termed as HEAT (Huntington/Elongation/A-subunit/Tor) motif. Each repeat is composed of two α helices connected by an intra-repeat loop (Walter et al., 1989; Hemmings et al., 1990; Ruediger et al., 1992).

The C-subunit also exists in two isoforms Cα and Cβ (encoded by PPP2R1C and PPP2R1C respectively) that share 97% sequence identity. The Cα isoform is more abundant than the Cβ isoform (Khew-Goodall and Hemmings, 1988). Previous studies indicate that the diversity of PP2A holoenzymes is mainly controlled by its regulatory B-
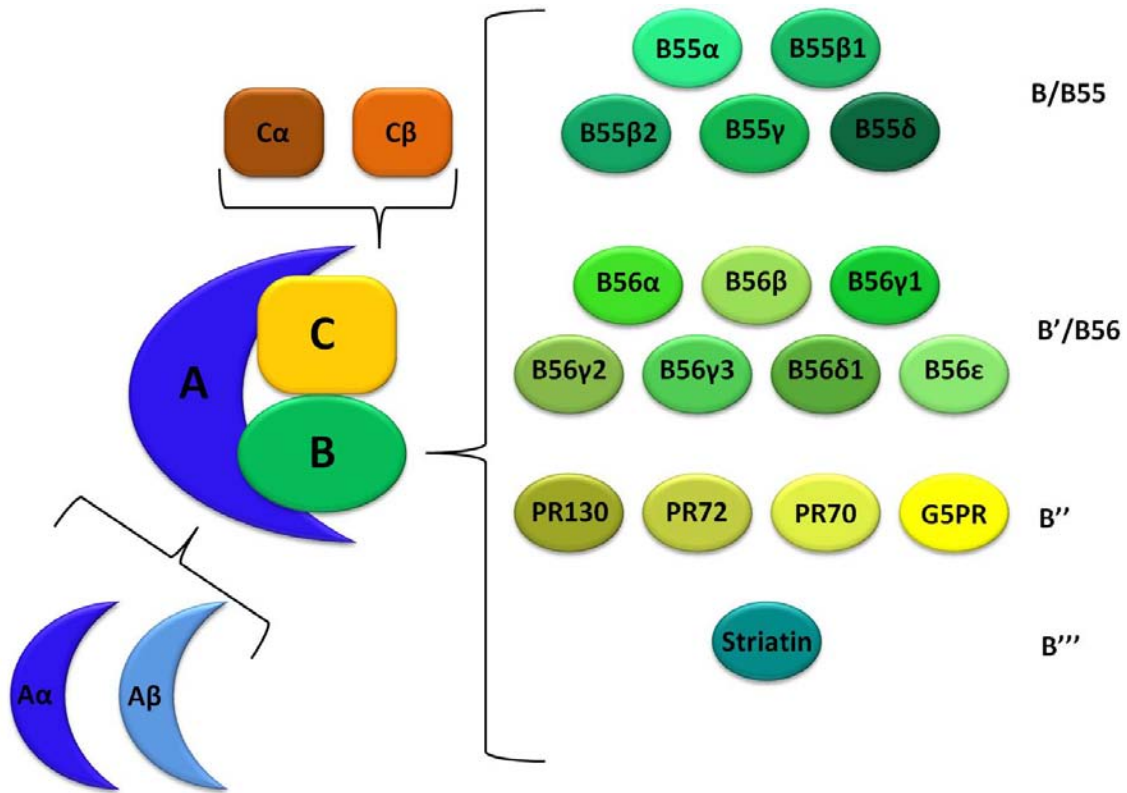
Figure 1-4: Complexity of the PP2A holoenzyme

Schematic of PP2A holoenzyme with the different isoforms of scaffolding (blue), regulatory (green) and the catalytic subunit (yellow). Adapted from Vishrup and Shenolikar (2009).

subunit. The B subunits of PP2A are classified into four distinct families, B/B55 (PPP2R2), B'/B56 (PPP2R5), B''(PPP2R3) and B'''/Striatin (PPP2R4) (Janssens et al., 2008; Mayer-Jaekel and Hemmings, 1994). So far, at least 26 sub types of the B subunit have been identified (Seshacharyulu et al., 2013; Zolnierowicz et al., 1994). For complete activation, the AC core dimer binds to a specific B-subunit to form a fully functional heterotrimeric protein, which determines its substrate specificity and subcellular localization.

Before being assembled into a holoenzyme, activation of PP2A is mediated by phosphotyrosyl phosphatase activator or PP2A specific phosphatase activator (PTPA) (Guo et al., 2014). PTPA mediates the loading of catalytic metal ions essential for p-Ser/Thr phosphatase activity (Guo et al., 2014). Post-translational modification on distinct subunits of PP2A also modulates the activity of the holoenzyme (**Fig. 1-5**). Methylation on the C terminal region of PP2A-C subunit by leucine carboxyl methyltransfeRASe-1 (LCMT1) is required for its holoenzyme assembly (De baere et al., 1999; Lee et al., 1996). Conversely, PP2A-specific methylesteRASe (PME-1), binds to the C-subunit active site and removes the methyl group to inactivate it (Xing et al., 2008). Studies have suggested that methylation on the C-subunit can affect the composition and substrate specificity of the holoenzyme (Janssens et al., 2008). Tyr-307 phosphorylation on the C-subunit by src-kinase or in response to growth signaling, also causes its inactivation (Chen et al., 1992). Three sites (S303, T268, S314) were identified on the A-subunit, which when phosphorylated, affect the C-subunit association and signaling in
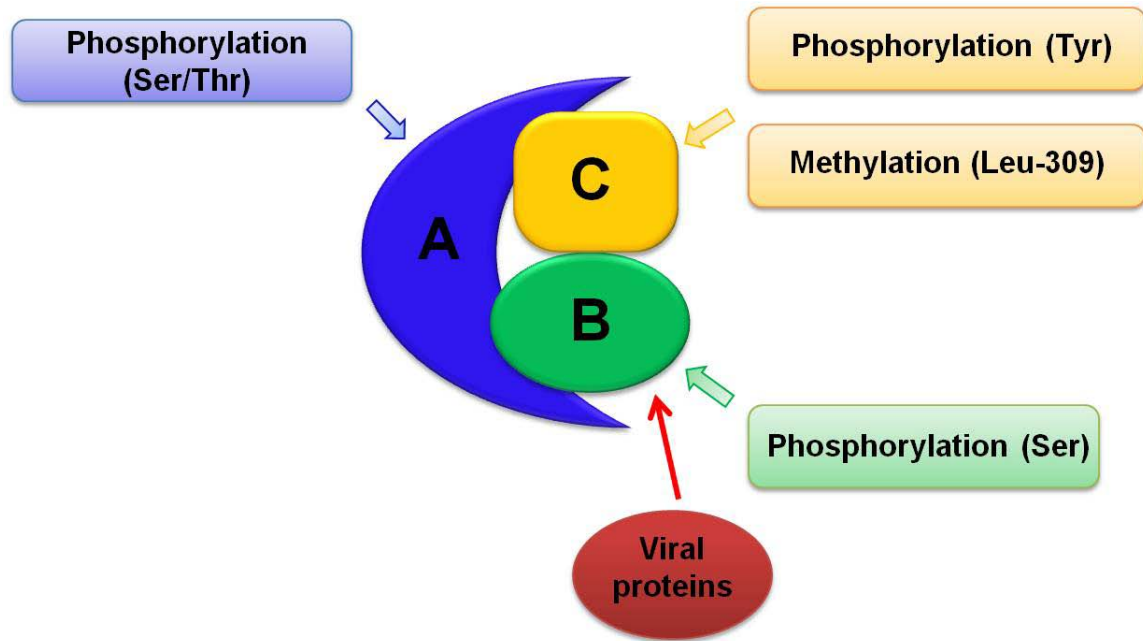
Figure 1- 5: Post-translational modifications on PP2A that affect its activity

Methylation on the C-subunit is essential for activation of PP2A, whereas Y307 phosphorylation cause its inactivation. There exist an array of phosphorylation sites on different B-subunits that regulate its activity. Post-translational modifications on the A subunit in form of Ser/Thr phosphorylation can affect the C-subunit binding.

the heart (Kotlo et al., 2014).

Also, viral protein from certain DNA tumor viruses can bind to the PP2A holoenzyme by replacing the B-subunit and thereby altering its activity and subcellular localization. The viral proteins; Polyoma small t (Pyst), Polyoma middle T (PyMT) and simian virus small t (ST) can form a complex with the PP2A core and alter its activity (Chen et al., 2004; Pallas et al., 1990; Walter et al., 1990). These interactions mainly facilitate cellular transformation via alteration of growth signaling pathways (Campbell et al., 1995). Studies are being carried out to identify additional post-translational modifications on PP2A subunits, which will give a perspective about its role in different cellular processes.

Given the complexity of PP2A composition, it has been suggested that PP2A can dephosphorylate over 300 substrates (Peng and Maller, 2010). Most of these substrates are involved in cell cycle regulation and cell proliferation pathways (Peng and Maller, 2010). PP2A regulates the Wnt, mTOR and MAP kinase pathways which play an important role in cell cycle initiation, cell proliferation and growth arrest (Wlodarchak and Xing, 2016). It regulates the Wnt signaling pathway both positively and negatively. PP2A-Bα dephosphorylates β-catenin to promote expression of Wnt responsive genes (Zhang et al., 2009). Moreover, in the absence of Wnt signaling, PP2A-B56α can also remove the inhibitory phosphorylation on GSK3β and promote degradation of β-catenin (Mitra et al., 2012). PP2A negatively regulates mTOR pathway by inhibiting IRS1 of insulin (Carlson et al., 2004) signaling or by inhibition of AKT (Kuo et al., 2008). MAP

kinase pathway is both positively and negatively regulated by PP2A. In presence of growth factors, the growth signal is relayed from the growth receptor to RAS, RAF, MEK and ERK. PP2A-mediated dephosphorylation releases RAF and KSR1 from 14-3-3, thereby activating RAF. KSR 1 is required for activation of downstream MEK (Ory et al., 2003). PP2A negatively regulates the MAP kinase pathway upstream by activating Sprouty which prevents RAS complex formation (Lao et al., 2007).

PP2A profoundly impacts DDR by modulating the activity of the PIKKs (ATM, ATR and DNA-PK) as well as Chk1 and Chk2 kinases (Lee and Chowdhury, 2011). Dephosphorylation of γ-H2AX at DSBs by PP2A is essential for efficient repair (Chowdhury et al., 2005). It promotes NHEJ by dephosphorylating DNA-PKcs, KU70/80 (Wang et al., 2009). Depletion or inhibition of PP2A results in impaired DDR, e.g., deficiency in IR-induced G2/M arrest (Yan et al., 2010). PP2A, by associating with its specific B-subunit, tightly regulates cell cycle at various stages and is vital in DDR, the details of which are discussed in the chapters to follow.

As it is clear from the various studies reviewed here that DDR response is an orchestrated effort of many proteins, with kinases and phosphatases being the chief modulators. It is therefore crucial to understand the intricate regulation among these proteins. The study herein focuses on ATM kinase and PP2A, as extensively discussed above. Our study aims to address how ATM and PP2A modulate each other's activity and control DSB repair and pro survival via spatio-metric regulation.

CHAPTER 2:

Generation and characterization of PP2A-Aα KO Mouse Embryonic Fibroblasts

Introduction

PP2A has been implicated in a variety of human cancers playing a role as a tumor suppressor, where it acts by negatively regulating some cancerous signaling pathways. It is a negative regulator of key signaling pathways like RAF/MEK/ERK and PI3K/AKT pathways, NF-KB, c-MYC, and WNT signaling (Eichhorn et al., 2009). Aberrant activation of these signaling cascades can lead to cancer development. PP2A is mutated in a variety of human cancers. Point mutations, deletions as well as mutations that can generate alternate transcripts are found in different PP2A subunits (Perrotti and Neviani, 2013; Strack et al., 1999). Many of these mutations result in disruption of the PP2A holoenzyme thereby affecting its enzymatic activity.

Studying transformation in human cell culture models over time has elucidated role of various signaling pathways in cancer. Studies by the Weinberg group and others demonstrated that expression of telomerase catalytic subunit (hTERT) in combination with the simian virus small t (SV40-ST) antigen and oncogenic allele of H-RAS caused transformation of several human cell lines. This transformation requires the interaction of the ST with PP2A (Hahn et al., 1999; Mumby, 1995; Yu et al., 2001b) consequently suppressing PP2A activity by displacing the regulatory B subunit (B56α, B56γ and PR72/PR130 have been identified) (Sablina et al., 2010). ST bound to the PP2A –AC heterodimer directly regulates AKT dephosphorylation at Serine 473 (Rodriguez-Viciana et al., 2006). Cells in which PP2A-AC is bound to ST show increased proliferation as

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well as upregulation of the MAP kinase pathway (Sontag et al., 1993a; Ugi et al., 2002). Polyoma small (PyST) and polyoma middle T (PyMT) also forms a complex with the PP2A dimer and this interaction is essential for cellular transformation (Pallas et al., 1990; Walter et al., 1990). Association of PyMT with PP2A-AC preferentially activates the MAP kinase pathway, which suggests that PyMT possibly displaces the B55α or B56 subunit and promotes unregulated growth (Rodriguez-Viciana et al., 2006). Loss of B56γ has been shown to activate the PI3K/AKT pathway (Chen et al., 2004). A previous study from our lab has suggested that ATM mediates the phosphorylation of AKT at S473 via an OA sensitive phosphatase (Golding et al., 2009). Also, OA at lower concentrations (PP2A IC_{50}) reduces p-AKT (S473) levels relative to higher concentration (PP1 IC_{50}) (Golding et.al. unpublished). We think that PP2A, an OA sensitive phosphatase, is regulated downstream of ATM, which could further regulate AKT dephosphorylation. We looked at AKT phosphorylation in presence and absence of ATM inhibitor (KU-60019) in the glioma cell lines U87 and U1242 as well as HEK293 and HEK293T cells (Golding et al., unpublished). Inhibition of ATM reduced p-AKT levels in U87, U1242 and HEK293 cells, but it failed to alleviate AKT phosphorylation in the 293T cells, that express the SV40 virus. Likewise, when PyMT was overexpressed in the glioma cells lines, AKT phosphorylation was not affected in cells treated with KU-60019 in presence and absence of IR (Golding et al., unpublished). This suggests that ATM regulates phosphorylation of AKT via inhibition of PP2A activity.

Both MAP kinase and PI3K/AKT pathways are governed by intracellular and extracellular stimuli to control cell proliferation, cell motility as well as metabolism. Traditionally, it has been suggested that these cascades function as two independent parallel pathways. However, studies have demonstrated that there are multiple points in these pathways where crosstalk exists (Fey et al., 2012). Coordinated actions of these pathways determine the cellular fate. However, dysregulation can often lead to deleterious effects. Both MAP Kinase and PI3K/AKT pathways have a mechanism to negatively feed into the other. Such cross inhibition has been revealed by chemical inhibition of specific players of these pathways. AKT negatively regulates ERK activation via inhibitory phosphorylation of RAF in response to IGF1 (Dhillon et al., 2002; Guan et al., 2000; Zimmermann and Moelling, 1999). Similarly, EGF induced ERK activation downregulates AKT phosphorylation via GAB1 (Yu et al., 2001a, 2002). Phosphatases like PP1 and PP2A act at several levels to activate or inhibit these pathways. PP2A can inhibit MAP kinase pathway by dephosphorylating both MEK1/2 and ERK1/2 proteins. Conversely, it can activate MAP kinase pathway by taking off the inhibitory phosphorylation on RAF. Crosstalk among pro-survival signaling pathways can often serve as a compensatory mechanism for cancer cells to dodge cell death.

MAP kinase and AKT pathways are also affected by DNA damage. We have previously demonstrated that in response to minor DNA damage, ATM mediates activation of the MAP kinase family member ERK via AKT, whereas extensive DNA damage abrogates phosphorylation of ERK, an important pro-survival molecule (Khalil et al., 2011).

Consequently, minor damage will promote DNA repair and cell survival, whereas in case of excessive damage, pro-survival signaling is shut off, which could ultimately lead to apoptosis or cell death. ATM also controls the activation of AKT, a major pro-survival molecule in response to IR and insulin, in an indirect manner (Golding et al., 2009; Viniegra et al., 2005a). Using a small molecule inhibitor KU-60019, we have previously reported that inhibition of ATM kinase blocks pro-survival signaling in glioma cell lines by reducing AKT (S473) phosphorylation (Golding et al., 2009). In this study we observed that OA treatment reduced the effect of KU-60019 on p-AKT levels by 50% relative to KU-60019 alone. This study revealed that ATM regulates AKT (S473) phosphorylation via an OA-sensitive phosphatase. OA acid inhibits Ser/Thr protein phosphatases; with PP1 and PP2A exhibiting much greater affinity and inhibition efficacy towards the PP2A (Cohen et al., 1989; Favre and Turowski, 1997; Swingle et al., 2007). Following DNA damage, ATM activity increases two-to three-fold, which is attributed to intermolecular autophosphorylation at the S1981 residue of ATM followed by its dissociation to the active monomeric form (Bakkenist and Kastan, 2003). This autophosphorylation on ATM is regulated by PP2A in absence of DNA damage. Following DNA damage, there is increased phosphorylation on ATM and subsequent dissociation of PP2A (Goodarzi et al., 2004). Goodarzi et al. also showed that PP2A exerts this regulation on ATM by interacting via the A subunit. However, this association was lost in cells, that were irradiated (Goodarzi et al., 2004).

The purpose of this study is to elucidate how ATM regulates AKT phosphorylation in response to DNA damage as well as in growth factor signaling. ATM which is Ser/Thr kinase phosphorylates its substrates on SQ /TQ motifs. In a study involving high-throughput substrate analysis of ATM and ATR in response to DNA damage, a vast network of over 700 phosphorylated substrates was discovered. This study revealed that the S401 site on PP2A-Aα was a highly probable target for ATM (Matsuoka et al., 2007), following which Li et al showed that ATM possibly phosphorylates the PP2A-Aα subunit on S401 and suppresses its activity (Li et al., 2012).

On the basis of our work and literature, we hypothesized that, on activation, ATM will phosphorylate the S401 residue of PP2A- Aα and suppress its activity, thereby increasing p-AKT (S373) levels. To study how this is regulated, we generated site-specific mutants at S401; the S401D mutant will act as a phophomimetic and the S401A will not be phosphorylated. For the purpose of this study we have also generated PP2A- Aα knock out MEFs and complemented them with human WT, S401A and S401D PP2A-Aα isoforms.
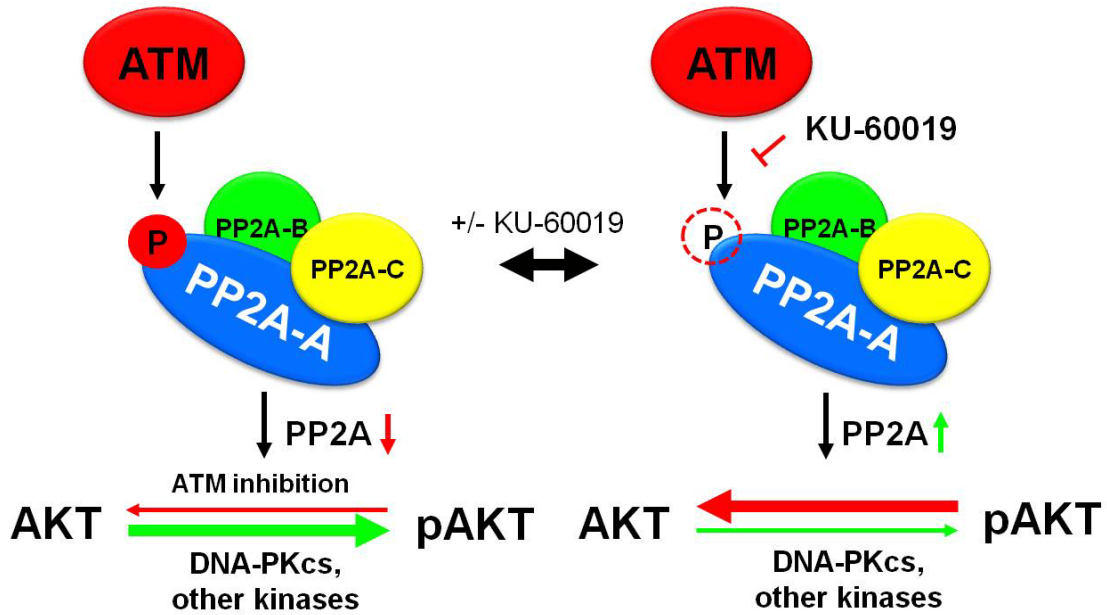
Figure 2-1: Hypothesis

ATM when activated can phosphorylate PP2A-Aα, inhibit its activity and thereby increase p-AKT level. Inhibition of ATM prevents ATM mediated phosphor-inhibition of PP2A and levels of p-AKT are decreased. The phosphorylation on AKT is also controlled by kinases like DNA-PKcs.

Materials and methods

Mouse information used to create PP2A-Aα conditional knock out (CKO) mouse embryonic fibroblasts

Fvb.129s-*ppp2r1ATM^{1wltr}*/j mice were purchased from Jackson laboratories as stock number 017441. These mutant mice possess *loxp* sites flanking exons 5-6 of the Aα gene (*PPPP2R1A*) of PP2A (Ruediger et al., 2011). All animal breeding and experiments have been approved by Virginia Commonwealth University, IACUC. Mouse embryonic fibroblasts (MEFs) were generated from 14 day FVB.129S-*PPP2R1ATM^{1wltr}/^{tm1wltr}* mouse embryos using standard methods (Lei, 2013). All the mouse generation and breeding was performed at the VCU Massey Cancer Center Transgenic/Knockout Mouse Core, under the direction of Dr. Jolene Windle.

Generation of PP2A-Aα KO MEFs expressing PP2A-Aα WT, S401A and S401D mutants

Mig-Aα-WT retroviral vector, which has a Flag epitope at the NH$_2$ terminus and an internal GFP (Chen et al., 2005), was obtained from (Addgene #10884). The S401 and S401D mutants were generated using QuikChange site-directed mutagenesis (Stratagene) using primers WT to S401A and WT to S401D (**Table 1).** The vectors were sequenced to verify the site-specific mutation at S401. The CKO PP2A-Aα MEFs were subjected to spontaneous immortalization via continuous passaging**.** We then infected them with the retrovirus: MIG-Aα-WT, S401A or S401D. The endogenous PP2A-Aα was floxed out by

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expression of Cre combinase (Ad-Cre). GFP positive cells were sorted before being

characterized.

Genotyping

High pure PCR template preparation kit (Roche Diagnostics Corporation) was used for

purification of DNA from the MEFs. The PP2A-Aα primers, to verify the CKO and KO

alleles were used (**Table 1**). PCR was performed using AmpliTaq Gold® master mix

(ThermoFisher Scientific) in an Eppendorf thermocycler. Amplified samples were run on

agarose gel and stained with ethidium bromide. DNA gels were visualized using the Gel

Doc^{TM} XR +Gel documentation system (Bio-Rad).

Plasmids

Mig-Aα WT (Addgene #10884), pGEX-2T PP2A-Aα : Mig-Aα WT was cut with

BamH1 and EcoR1, 1776 bp fragment was cloned in pGEX2T (GE Healthcare Life

Sciences #28-9546-53), pGEX-2T-p53 (first 100 amino acids of p53 were cloned on

pGEX2T Selivanova et al.,1999), pRSF-ATM-FAT KD: (1903 bp fragment of codon

optimized ATM- FAT and Kinase Domain (KD) were cloned in the pRSF-Duet-1

(EMD Millipore #71341) at EcoRI and XhoI sites using Gibson assembly), Ad-Cre

recombinase (Vectorlabs #1045)

Antibodies

Antibodies used in western blots were anti-AKT (1:1000) (Cell Signaling Technology), anti-p (S473) –AKT (1:1000) (Cell Signaling Technology), anti-PP2A-A (1:1000) (Cell Signaling Technology), anti-Flag (Sigma Aldrich), anti-PP2A-C (Millipore), anti-ATM (1:1000) (Cell Signaling Technology), anti-ERK (1:1000) (Santa Cruz Biotechnology) and anti-p (T202/Y204)-ERK1/2 (Santa Cruz Biotechnology).

Reagents

Insulin was purchased from Sigma Aldrich and used at a concentration of 350 nM. KU-60019 was provided by KuDOS pharmaceuticals and dissolved in DMSO and used at a concentration of 3 μM.

Cell culture

Mouse Embryonic Fibroblasts and HEK 293 cells expressing PP2A-Aα WT/S401A and S401D were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin antibiotic solution.

Irradiation

Irradiation was performed using MDS Nordion Gammacell 40 irradiator with Cs-137 source at a dose of roughly 1.05 Gy/min.

Western Blotting

Western blotting was performed as previously described (Adams et al., 2010a) with

additional modifications. Cells were lysed in 2X Laemmli buffer +β-mercaptoethanol and boiled for 10 minutes. Proteins were separated on CriterionTM TGX gels (Bio-Rad Laboratories) and transferred to PVDF membranes. Membranes were blocked in casein blocking buffer (Sigma Aldrich), following which they were exposed to primary antibodies (Dilution 1:1000 in Casein buffer or 5% BSA in TBST) overnight. Protein bands were detected and quantified using infrared-emitting conjugated secondary antibodies, either anti-rabbit or anti-mouse DyeLight 800 (Rockland Immunochemicals, Gilbertsville PA) or anti-rabbit or anti-mouse Alexa 680 (Invitrogen) using the Odyssey infrared imaging system from Li-Cor Biosciences (Lincoln, NE). Densitometry was performed using LiCor Odyssey Software V3.0.

Crystal Violet staining

Cells were grown to confluence; media was aspirated off the cells. Cells were stained with 0.5% crystal violet in 25% methanol for 15 minutes and the residual stain was washed with deionized water.

Growth Assay

Cell growth was determined by CellTiter-Glo® Luminescent Cell Viability Assay (Promega). PP2A-Aα KO MEFs were serially diluted and seeded on a 96-well plate. At days 2, 5 and 7 after seeding CellTiter-Glo® reagent was added to the medium at the recommended final concentration. Plates incubated for 10 minutes at room temperature

(RT). Luminescence was determined EnVision Multilabel reader (PerkinElmer) and the values were taken as the measure of cell growth.

Immunoprecipitation

Flag-PP2A-Aα was immunoprecipitated from HEK-293 cells stably expressing Mig-Flag-Aα-WT, S401A and S401D. Cells were lysed in EMSA buffer (10mM Tris-HCL pH 8.0), 60 mM KCL,1 mM EDTA, 1 mM dithiothreitol 0.1% NP-40, HALT protease and phosphatase inhibitors). Lysates were incubated with anti-Flag M2 beads (Sigma) 3-4 hours. The beads were washed three times in ice-cold PBS, mixed with Laemmli buffer +β-mercaptoethanol and boiled for 10 minutes. The lysates were then loaded onto a CriterionTM TGX gel and run as earlier described.

Bacterial protein expression

Fusion proteins (GST-PP2A-Aα, GST-p53 and His-ATM-FAT-KD) were expressed in *Escherichia coli* BL21 cells. Overnight grown starter culture was added to 200 ml LB media and allowed to grow for 1 hour at 37°C. Protein expression was induced by adding 0.1 mM IPTG. Cells were grown for further 4-5 hours. Cells were spun down and lysed in 50 mM tris pH 7.5, 150 mM NaCl and 0.05% NP-40 with 1 mM PMSF and bacterial protease inhibitors (Sigma Aldrich).

GST-Pull down

For GST-pulldown assay, GST fusion proteins were bound to Glutathione-Sepharose (GE healthcare) for 30 minutes at RT. This was followed by washing of beads (3X) with binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 8 mM KH_2PO_4, pH 7.3; PBS). The protein was eluted in 10 mM reduced glutathione in 50mM Tris-HCl (pH 8.0). Eluted protein was analyzed by SDS-PAGE followed by western blotting.

His-Pull down

HA-tagged proteins were bound to Ni-NTA Agarose (Molecular cloning Laboratories). Protein purification was performed according to manufacturer's instructions.

GST-PP2A-Aα and His-ATM FAT KD binding assay

Purified PP2A-Aα and ATM-FAT-KD were mixed together for 30 minutes on ice. A GST pull down assay was done on this mix. GST pull-down assay was also done on purified ATM-Fat-KD to eliminate non-specific binding if any.

In vitro kinase assay

YFP-ATM was immunoprecipitated from HEK 293 cells by GFP TRAP (ChromoTek). The immunoprecipitates were suspended in kinase assay buffer containing 2 uCi of [γ-^{32}P] ATP, 20 μM unlabeld ATP and GST-substrates (GST-PP2Aα or GST-P53). The kinase reaction was conducted at 30°C for 20 minutes and stopped by adding 2X Laemmli loading buffer. Samples were run on 10% polyacrylamide gels, following which

the gel was dried and exposed to a autoradiogram and quantified by a phosphoimager

(Typhoon) (Canman et al., 1998).

Results

Generation of PP2A-Aα KO MEFs complemented with mutant allele

The S401 site on PP2A-Aα is an SQ site and has been shown to be phosphorylated by ATM following which the activity of PP2A is inhibited (Matsuoka et al., 2007; Li et al., 2012). The S401 site of PP2A-Aα is highly conserved among mammalian species including human and mouse. We generated site-specific mutants at this site, a S401A mutant that cannot be phosphorylated and S401D, a phospho-mimetic mutant. Reudigar *et al*. have demonstrated that PP2A-Aα is an essential gene, as knocking out PP2A-Aα caused embryonic lethality (Ruediger et al., 2011). To prevent interference from endogenous PP2A-Aα, we generated PP2A-Aα Knock out (KO) mouse embryonic fibroblasts (MEFs) expressing the respective S401 mutants. The targeting strategy for generation PP2A-Aα Conditional Knockout (CKO) mice is outlined in **Fig. 2-2A**. Heterozygous 129S-*PPP2R1ATM^{1wltr}/j* (CKO/WT) (Ruediger et al., 2011) were bred to obtain 129S-*PPP2R1ATM^{1wltr}/^{tm1wltr}* (CKO/CKO) mice. MEFs generated from 129S-*PPP2R1ATM^{1wltr}/^{tm1wltr}* mouse embryos were immortalized. These immortalized MEFs were infected with retroviral vectors expressing GFP/Flag- tagged PP2A-Aα WT, S401A or a S401D mutant. The endogenous alleles were floxed out by infecting the cells with an adenovirus expressing Cre recombinase (**Fig. 2-2B)**. MEFs that were not complemented with the GFP/Flag-PP2A-Aα vectors did not survive post floxing of the endogenous
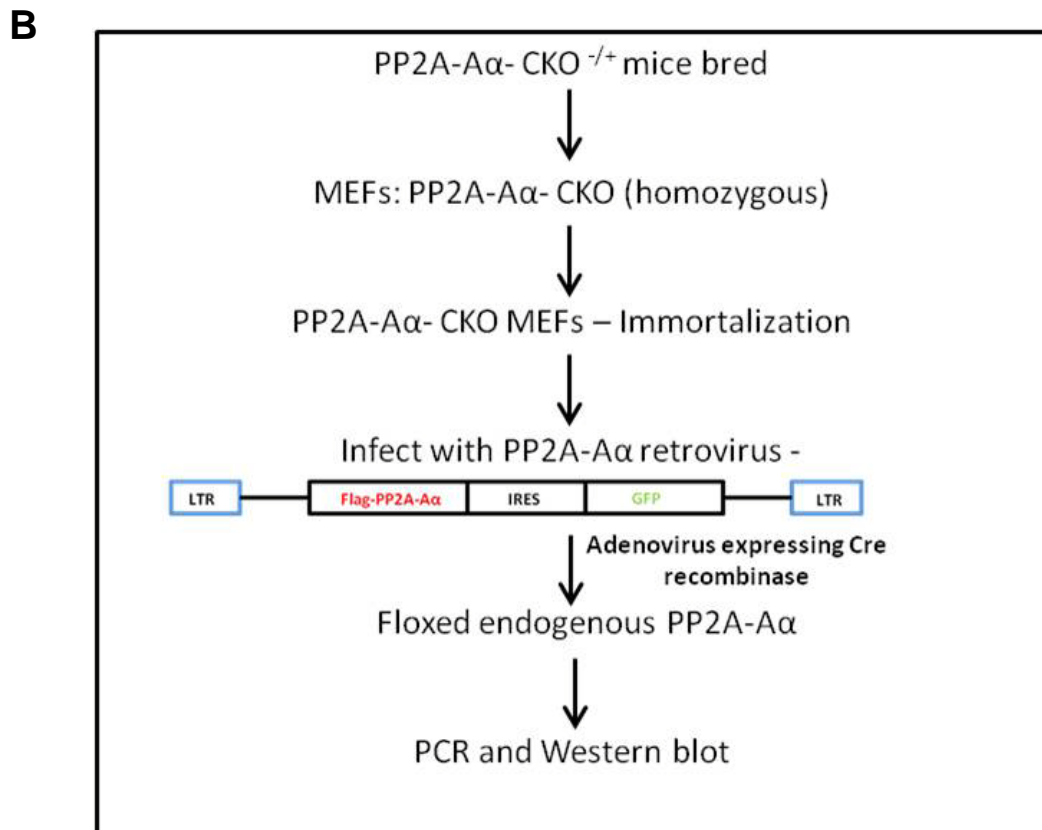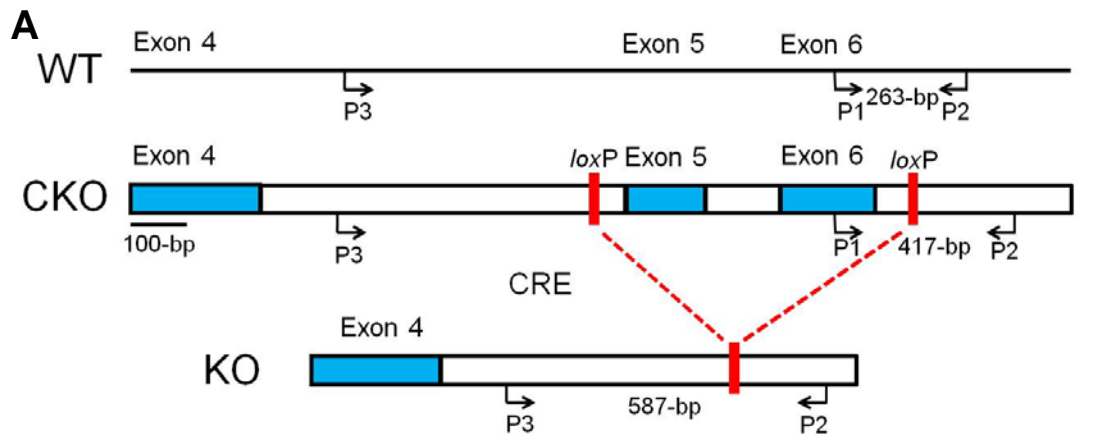
Figure 2-2: Scheme for PP2A-Aα knock out

(**A**) Representation of the WT, CKO and KO alleles of PP2A-Aα before and after Cre-mediated recombination at loxP (red bars). (**B**) Scheme for generation of PP2A-Aα CKO MEFs.
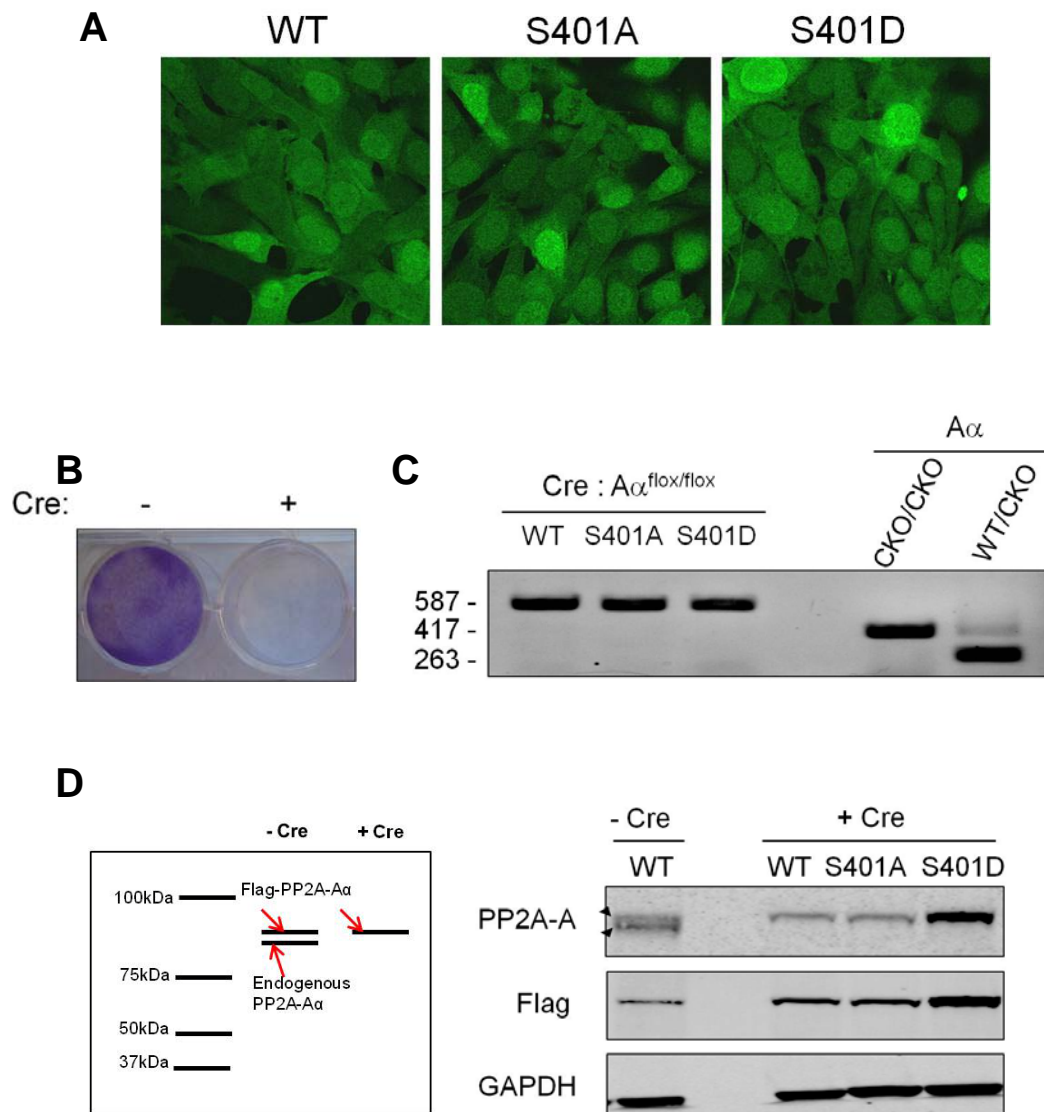
Figure 2-3: Verification of PP2A-Aα knock-out in MEFs

(**A**) MEFs expressing the Mig-PP2A-Aα-GFP (WT/S401A/S401D. (**B**) Crystal violet staining showing no cells in post expression of Cre-recombinase in MEFs not complemented with PP2A-Aα.(**C**) Genotyping: The PP2A-Aα primers generated a 263-bp product for WT allele and a 417-bp product for the CKO allele. In cells where Cre has been expressed, there would be a 587-bp product for the PP2A-Aα allele. (**F**) PP2A-Aα was detected by western blot in MEFs complemented with the site-specific mutants before and after Cre mediated recombination. Endogenous PP2A-Aα was lost post Cre expression. GAPDH serves as a loading control.

PP2A-Aα (**Fig. 2-3B).** The knocking out of endogenous PP2A-Aα in the complemented MEFs was verified by genotyping and evaluating the protein levels (**Fig. 2-3C and 3D).**

Phosphorylation of PP2A-Aα at S401 increases pro-survival

We and others have previously reported that ATM stimulates insulin- and radiation-induced phosphorylation of AKT at S473 (Golding et al., 2009; Viniegra et al., 2005a). Moreover, our studies and literature have indicated that ATM-mediated phosphorylation at S401 on PP2A inhibits its activity, thereby downregulating AKT signaling (Golding et al., 2009; Li et al., 2012). To validate this, serum-starved PP2A-Aα KO MEFs expressing Flag-tagged PP2A-Aα WT, S401A or S401D mutant were treated with insulin. We observed that S401D-expressing cells show elevated p-AKT (S473) levels post insulin treatment **(Fig. 2-4A and 4B)**. This may well be due to reduced phosphatase activity of the phophomimetic S401D mutant form as hypothesized. Consistently with the up regulation of p-AKT, a pro-survival molecule post insulin treatment, MEFs expressing S401D mutant also have increased growth rate **(Fig. 2-4C)**. However, the basal levels of p-AKT (S373) did not show any difference amongst the mutants in unstarved or starved cells (**Fig. 2-4D)**. Since PP2A also regulates the MAP kinase pathway (Silverstein et al., 2002), we looked at the pERK1/2 basal levels in these cells. The cells expressing S401D showed elevated pERK1/2 levels relative to the cells expressing WT and S401A mutant **(Fig. 2-5)**.
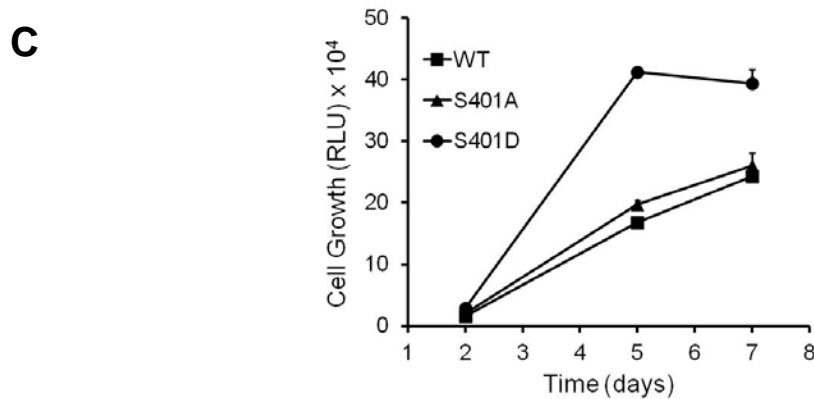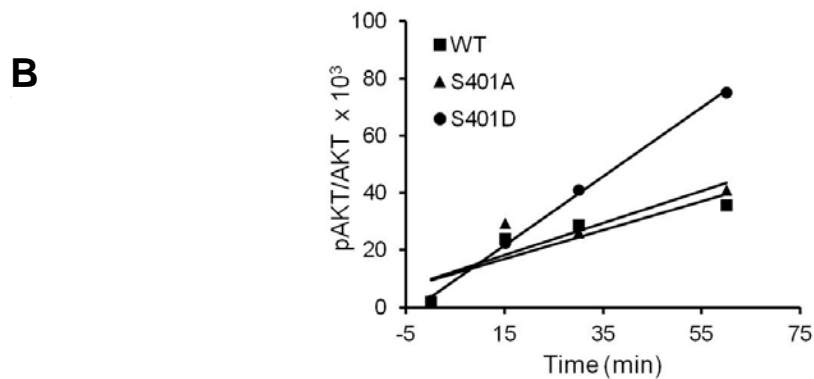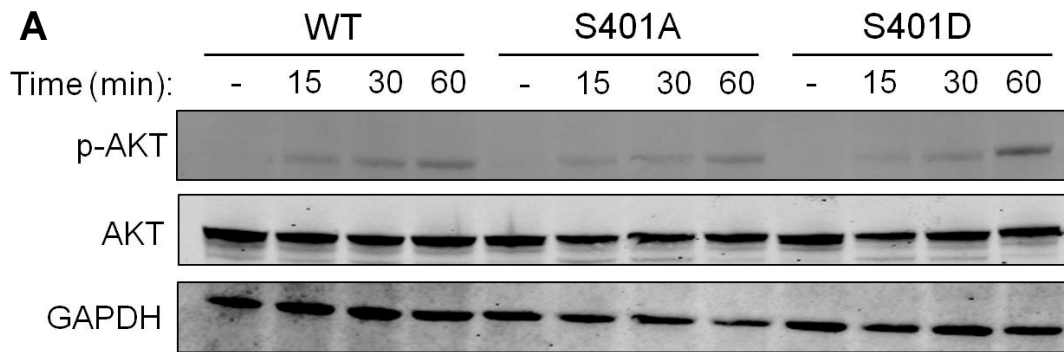
Figure 2-4: Pro-survival signaling is elevated in S401D expressing MEFs

(**A** & **B**) PP2A-Aα KO MEFs (WT, S401A OR S401D) were serum starved for 16 hours and treated with 350 nm insulin for 15, 30 or 60 min and cells were harvested for western blotting analysis. p-AKT (S473) levels were normalized to total AKT protein levels. GAPDH serves as a loading control. (**C**) PP2A-Aα KO MEFs (WT, S401A OR S401D) were grown at different dilutions. Growth was determined Celltiter-glo® after 2, 5, and 7 days. Data points; growth, Relative Luminescence Units (RLU). Error bars; SEM n = 3.
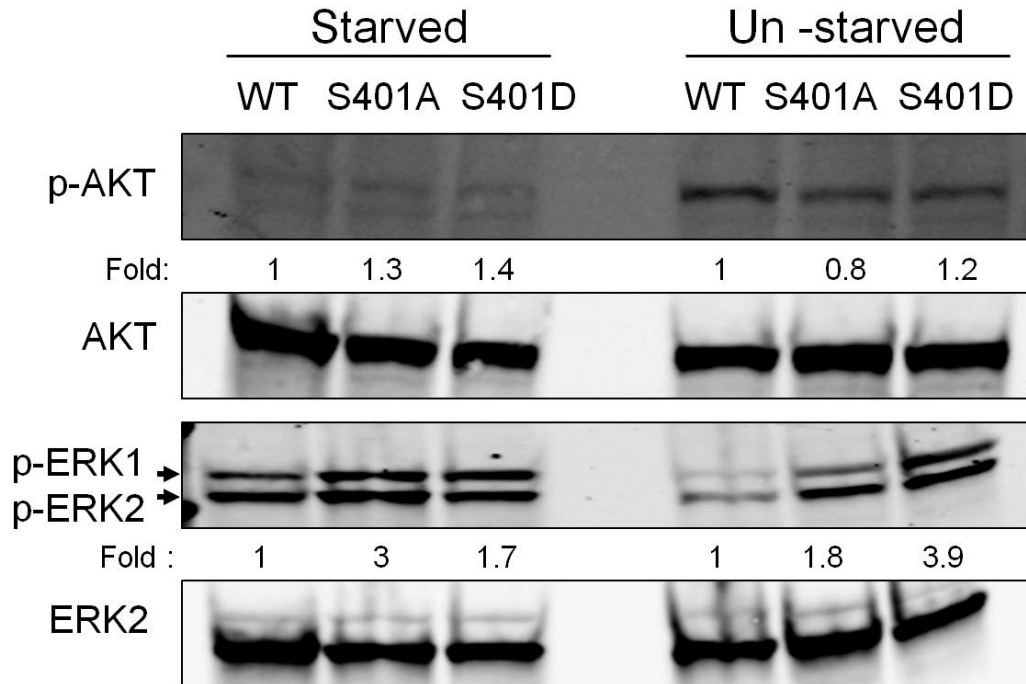
Figure 2-5: p-ERK basal levels are increased in S401D expressing MEFs

PP2A-Aα KO MEFs (WT, S401A OR S401D were treated with or without serum-free media for 16h and harvested for western blot analysis. P-AKT (S473) levels were normalized to total AKT protein levels. P-ERK (T202/Y204) levels were normalized to total ERK levels.

Phosphorylation of PP2A-Aα at S401 causes dissociation of the holoenzyme

PP2A-A interacts with ATM kinase at amino acids (250-522, 1245-1435 and 2427-2841) (Goodarzi et al., 2004) (**Fig. 2-6A).** To determine if phosphorylation at S401 of PP2A-Aα affects binding with ATM, we analyzed the Flag-PP2A-Aα immunoprecipitates for the presence of endogenous ATM, AKT and the catalytic subunit of PP2A (PP2A-C) in HEK293 cells. We found a strong association of ATM, AKT and PP2A-C with the WT and the non-phosphorylatable (S401A) PP2A-Aα, but not with the phosphomimetic PP2A-Aα (S401D) (**Fig. 2-6B**). Lack of association of PP2A-C with the S401D implies that phosphorylation at the S401A site of PP2A-Aα results in inhibition phosphatase activity. We also pursued molecular modeling studies in collaboration with Dr. Kellogg from Department of Medicinal chemistry in School of Pharmacy. Dr. Mosatafa Ahmed from his lab helped us with these studies A 6 ns molecular dynamics run using implicit solvent was done on the S401-phosphorylated PP2A-Aα chain alone as well as the unphosphorylated structure as a control. The phosphorylation of S401 induced a local movement in the helices around it and ultimately caused a slight conformational change of the whole protein especially at its N-terminus distorting the horseshoe shape of the subunit. This did not happen in the control simulation and might potentially cause the dissociation of the PP2A complex (**Data not shown**). Altogether this suggests that phosphorylation at S401 of PP2A-Aα could cause dissociation of the holoenzyme which further increases pro-survival signaling.

A

B
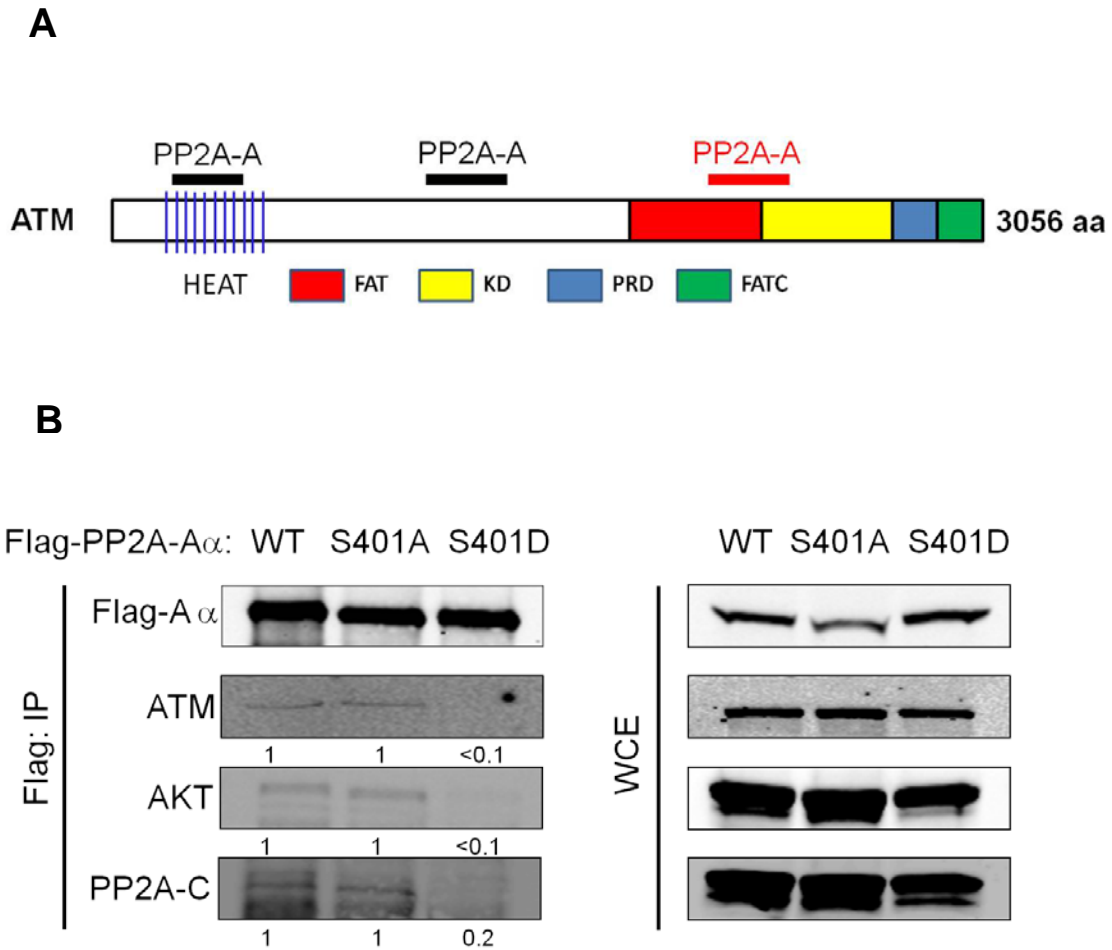
Figure 2-6: Phosphorylation at S401 of PP2A-Aα prevents binding of ATM, AKT and the C-subunit

(**A**) Schematic of regions of the ATM protein with which PP2A-A interacts. (**B**) HEK 293 cells stably expressing Flag- tagged WT, S401A and S401D PP2A-Aα were harvested and cell lysates were immuno-precipitated with anti-Flag beads and examined for ATM, AKT and PP2A-C binding by western blot analysis.

PP2A-Aα interacts with FAT kinase domain of ATM and is phosphorylated by ATM *in vitro*

In a yeast system, Goodarzi and colleagues have demonstrated that PP2A-A interacts with ATM. Also, in an *in vitro* study, fragments of ATM (covering the entire protein sequence) were incubated in irradiated and un-irradiated cell extracts. PP2A-A from the un-irradiated lysates bound to 250-522, 1245-1435 and 2427-2841 amino acids of ATM kinase (Goodarzi et al., 2004). The 2427-2841 amino acid residues overlap with the FAT-KD of ATM (**Fig. 2-6A**). We expressed the codon-optimized FAT - kinase domain (FAT-KD) of ATM with a His tag and the GST tagged PP2A-Aα in bacterial cells. The two proteins were purified and GST pull-down was performed on the mixture of the two recombinant proteins. In line with the data from Goodarzi and colleagues, we saw that GST-PP2A-Aα interacted with the FAT-KD of ATM (**Fig. 2-7**). ATM kinase phosphorylates its substrates on an SQ/TQ motif. YFP-ATM was immunoprecipitated from HEK-293 cells using the GFP trap (Chromtek) (**Fig. 2-8A**). We performed an *in vitro* kinase assay to show that full length ATM phosphorylates PP2A-Aα *in vitro*. This phosphorylation was reduced in presence of ATM inhibitor (KU-60019). Phosphorylation or the kinase activity of ATM towards the substrates was measured by looking at the incorporation of [γ-^{32}P] ATP on the substrate proteins. GST–p53, which is a known substrate of ATM, was used as a control (**Fig. 2-8B**).
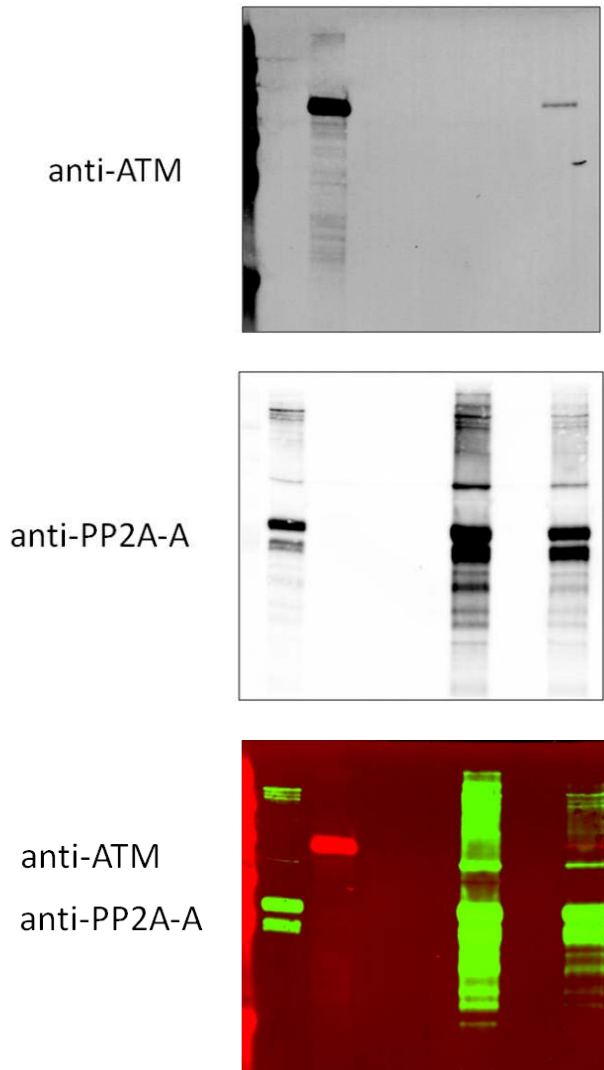
Figure 2-7: PP2A-Aα binds to the FAT kinase domain of ATM *in vitro*

Lane 1 and 2: Affinity purification of GST-PP2A-Aα and His- ATM –FAT-Kinase Domain. Lane 3,4 and 5: Controls for non-specific binding. Lane 6: PP2A-Aα binds to His-ATM –FAT-KD *in vitro.* Proteins were examined for PP2A-A and ATM-FAT-KD by western blot analysis. Bottom Panel: Merged western blot of ATM (Red-Alexa680) and PP2A-Aα (Green- Dylight 800).
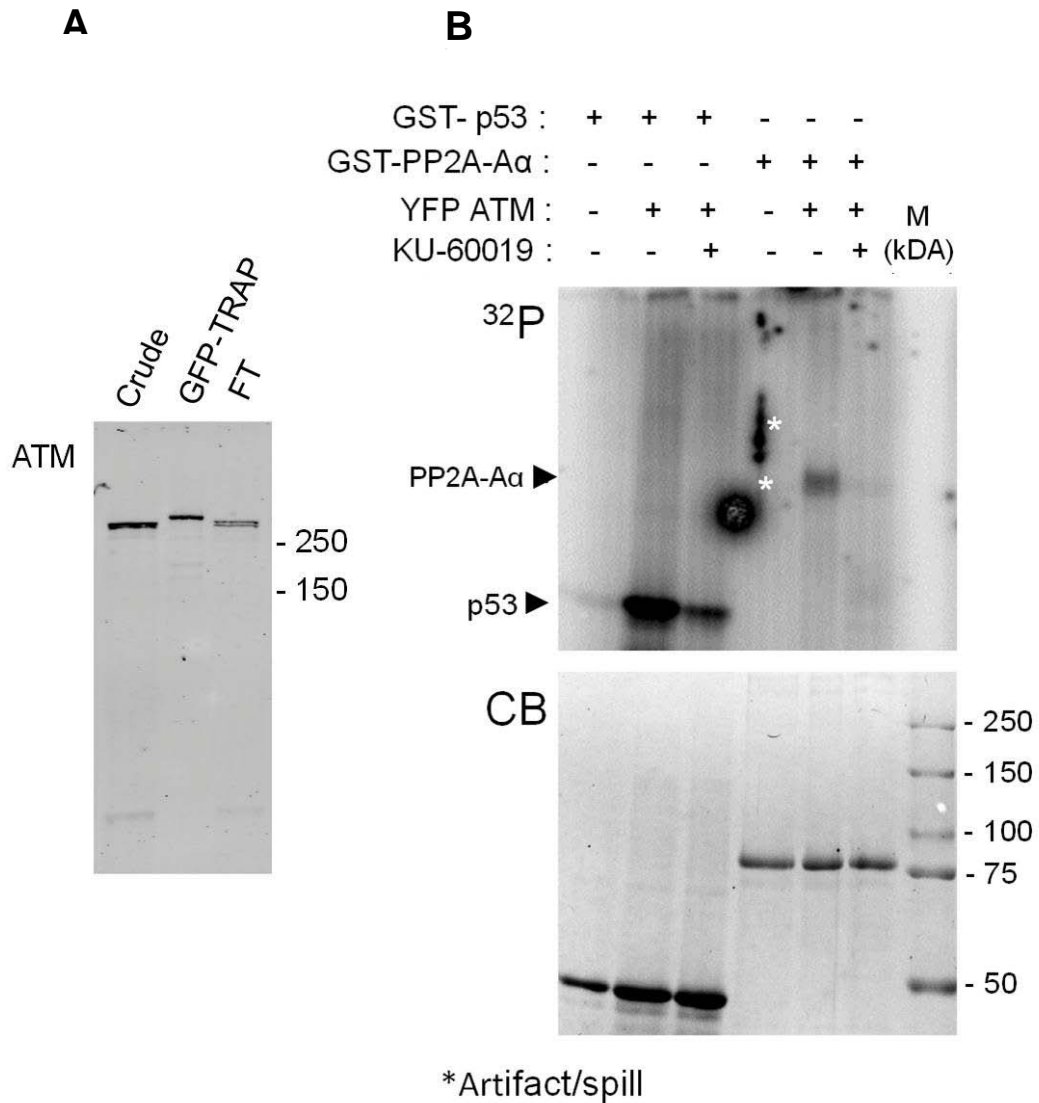
Figure 2-8: ATM kinase phosphorylates PP2A-Aα *in vitro*

(**A**) Western blot showing the immuoprecipitated YFP-ATM (middle lane). (**B**) Top panel: YFP-ATM phosphorylates GST-p53 (lane 2) and GST-PP2A-Aα (lane 5). Phosphorylation is reduced in presence of KU-60019 (lane 3 and lane 6). Bottom panel: Coomassie stained image of the gel showing equal loading of the GST-p53and GST PP2A-Aα in all the lanes.

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Chapter summary

Existing literature from various groups and our lab have put forth evidence that ATM regulates AKT phosphorylation via an OA-sensitive phosphatase. On exposure to IR, p-AKT levels decreased in U87, U1242 as well as HEK 293 cells in presence of ATM inhibitor KU-60019, but not in HEK 293T cells. The HEK 293T cells express a stably integrated copy of SV40 DNA. They express the large-T (LT) antigen as well as an unspliced SV40 early mRNA that encodes the small-T (sT) antigen (Fu and Manley, 1987; Pear et al., 1993). The ineffectiveness of KU-60019 in these cells could be because of downregulation of PP2A by its association with the SV40 ST, thereby reducing its dephosphorylation function towards p-AKT. Likewise, in U87 and U1242 cells overexpressing PyMT, ATM inhibition did not lower p-AKT levels as seen in the control cells. Current understanding of the role of viral oncoproteins in cellular transformation via PP2A as well as our data supports our hypothesis that PP2A functions downstream of ATM kinase and further regulates the dephosphorylation of AKT.

Herein we have established a genetic model for studying the site-specific modification at S401 of PP2A-Aα without interference from the endogenous protein. We effectively floxed out the endogenous PP2A-Aα alleles in MEFs and complemented it with the human PP2A-Aα WT, S401A or S401D. Cells floxed by Cre recombinase but not complemented with the WT and mutant copies failed to survive, which recapitulated the

study performed by Ruediger et al. that the PP2A-Aα is an essential protein (Ruediger et al., 2011).

Being the master regulator of DNA damage response, ATM has been traditionally considered as a nuclear protein. However, recently studies have elucidated the role of ATM kinase in the cytoplasm, particularly in insulin signaling. ATM kinase activity increases on insulin stimulation, which further brings about complete activation of AKT on S473 as well as phosphorylates 4E-BP1(repressor of mRNA translation) (Boehrs et al., 2007; Viniegra et al., 2005b; Yang and Kastan, 2000). On stimulating the MEFs expressing PP2A-Aα (WT, S401A or S401D) with insulin, the phosphomimetic mutant displayed elevated p-AKT (S473) levels. This observation was in line with our hypothesis: insulin stimulation would increase ATM kinase activity followed by inhibitory phosphorylation of PP2A-Aα, resulting in increased p-AKT levels (**Fig. 2-2**). The phosphomimetic PP2A-Aα here represents the inhibited or inactive form of PP2A.

AKT is a key player in some of the major signaling pathways, which are activated in response to insulin or by growth factors. Being known as a pro-survival molecule, one of its major functions is to control cell survival and cell growth (Brazil and Hemmings, 2001). Also, we have seen that ATM inhibition by KU-60016 reduces glioma cell growth *in vitro* (Golding et al., 2009). In our study, the MEFs expressing PP2A-Aα S401D grow at an increased rate relative to WT and S401A expressing cells. Interestingly, instead of increased basal p-AKT levels, the S401D cells expressed much higher basal p-ERK1/2

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levels. This observation was not in line with our initial thought process, that increased growth signaling in MEFs would correspond to the increased basal p-AKT levels.

One possible reason for increased p-ERK1/2 levels in S401D cells could be negative regulation of MAP kinase pathway by PP2A. PP2A can both negatively as well as positively regulate MAP kinase pathway by acting at different levels and on variety of substrates. Studies have shown that PP2A can dephosphorylate MEK1/2 and ERK1/2 and downregulate MAP kinase signaling (Sontag et al., 1993a; Westermarck et al., 1998). PP2A also acts on upstream regulators of the pathway such as, Shc, to which it binds and prevents signal progression (Ugi et al., 2002). Another possibility may be that the elevated pERK can negatively feed into PI3K pathway. Activated ERK can phosphorylate GAB1 and inhibit GAB1/PI3K association, which will inhibit the downstream AKT activation (Lehr et al., 2004) (**Fig. 2-9**).

So far we have established that ATM phosphorylates PP2A at S401. This single site modification can further regulate growth signaling via PI3K or MAP kinase pathway. We have yet to look at other growth signaling pathways like mTOR. More thorough studies are required to identify specific substrates of PP2A in these signaling pathways.

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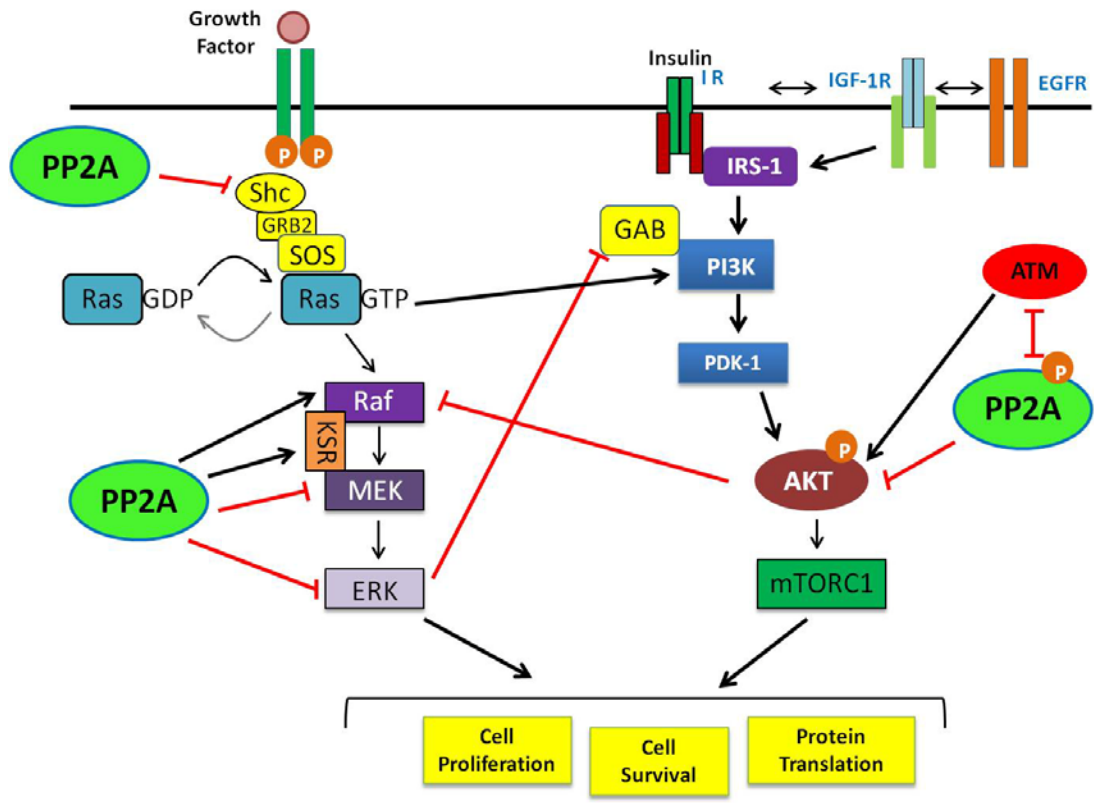
Figure 2-9: Crosstalk between AKT/PI3K and MAP kinase pathway

The figure shows cascade of activation of PI3K kinases via IRIS-1 post insulin stimulation as well as activation of the MAP kinases ERK1/ERK2 mediated by growth factor binding to receptor tyrosine kinases leading to activation of AKT and ERK respectively. PP2A exerts negative as well as positive control on both the pathways. Cross talk between these pathways exist via AKT mediated RAF inhibition as well as ERK mediated GAB-PI3K inhibition.

CHAPTER 3:

Role of p-PP2A-Aα (S401) in DNA damage response and DSB repair

Introduction

Cells are constantly under stress due to DNA damage caused by endogenous or exogenous sources. In response the cell elicits a signaling cascade, commonly referred to as DNA damage response (DDR) to maintain genomic integrity, which is facilitated by regulation of DNA repair processes, cell cycle arrest, pro-survival signaling, apoptosis as well as senescence. The response is initiated by sensor or mediator proteins like ATM kinase, which form large complexes at the sites of double strand breaks (DSBs). Alongside, a plethora of posttranslational modifications is induced on proteins at the sites of damage to set the stage for checkpoint activation and DNA repair. The signal is then transduced to effector proteins which regulate downstream pathways like cell survival and apoptosis (Shiloh and Ziv, 2013).

The phosphorylation network initiated, as a part of DDR by primary kinases like ATM, ATR and DNA-PK, is further amplified by checkpoint kinases Chk1 and Chk2. This phosphorylation cascade has to be counteracted to set the cell to normalcy and phosphatases play a crucial role in this process. For example, PP2A regulates both the primary kinases (ATM, ATR and DNA-PK) (Douglas et al., 2001; Goodarzi et al., 2004; Li et al., 2007; Petersen et al., 2006) as well as the secondary kinases (Chk1 and Chk2) (Dozier et al., 2004; Freeman et al., 2010; Leung-Pineda et al., 2006; Li et al., 2007). PP2A can enhances DNA-PK activity (Douglas et al., 2001) whereas it maintains ATM in inactive form (Dozier et al., 2004; Goodarzi et al., 2004).

ATM kinase is the master regulator of DDR activated post DSBs and is localized at sites of DNA damage. One of the first proteins to be modified following DSB induced response is H2AX, one of the histone variants in eukaryotic cells. H2AX is phosphorylated by ATM and /or DNA-PK at S139 (Rogakou et al., 1998, 1999). This event happens within minutes of the damage. Tanya Paull's group and others demonstrated that γ-H2AX localized at the sites of DNA damage form discrete foci and comprise repair factors like MRN repair complex, 53BP1, BRCA1 and MDC1 (Goldberg et al., 2003; Paull et al., 2000). Once the repair has been initiated γ-H2AX has to be eliminated from the foci. Chowdhury et al., 2005 identified PP2A as an enzyme that regulates the kinetics of γ-H2AX at the damage foci. They demonstrated that dephosphorylation of γ-H2AX facilitates efficient DSB repair.

PP2A also regulates the cell cycle in various stages. It plays an important role in the G1/S transition by regulating the Retinoblastoma tumor suppressor protein (Rb) pocket family proteins. Rb is major regulator of the G1/S checkpoint. The Rb pocket proteins when phosphorylated by the cyclin/CDK enzymes, release the E2F transcription factors, which cause the expression of E2F response genes. This S phase induction by Rb proteins commits the cells to DNA synthesis. (Wlodarchak and Xing, 2016). Hence, regulation by phosphatases is essential at this stage. PP1 and PP2A both regulate Rb proteins; PP1 after mitosis whereas PP2A throughout the cell cycle. PP2A-B55 can bind and

dephosphorylate Rb p107 and cause cell cycle arrest (Kolupaeva et al., 2013), whereas PP2A-PR70 regulates p130 (Purev et al., 2011; Soprano et al., 2006).

Once the cell has committed to begin DNA synthesis in the S phase, the origin replication complex (ORC) assembles at the origin of replication. The DNA only needs to be replicated once; hence, regulation of S phase proteins is extremely crucial. Cell division control 6 protein (Cdc6) binds to the ORC proteins and sets a stage for recruitment of replication machinery. PP2A ensures that a single origin is fired only once by dephosphorylating Cdc6 on its N-terminal region and marking it for ubiquitin mediated proteasomal degradation (Wlodarchak and Xing, 2016).

PP2A acts at mitotic entry as well as mitotic exit. Prior to mitotic initiation, PP2A-B56δ dephosphorylates CDC25 at T138 and keeps it inactive and sequestered in the cytoplasm (Margolis et al., 2006a). At the end of G2 phase, CDK2-mediated phosphorylation activates CDC25, which subsequently activates CDK1 leading to mitotic progression (Margolis et al., 2006b). Also, several studies have pointed out that Greatwall kinase mediated inhibition of PP2A facilitates CDK1 activation, which subsequently promotes mitotic entry (Burgess et al., 2010; Castilho et al., 2009; Lorca et al., 2010; Vigneron et al., 2009). Endosulfine-α (ENSA) and c-AMP-regulated phosphoprotein -19 (ARP-19) are phosphorylated by Greatwall kinase, following which ENSA and ARP-19 bind B56δ subunit of PP2A resulting inhibition of PP2A-B56δ (Gharbi-Ayachi et al., 2010; Mochida et al., 2010).

During mitosis PP2A is critical in regulation of various cellular organization events. Condensin I and Condensin II have an important function in mitotic chromosomal condensation (reviewed in Hagstrom and Meyer, 2003). PP2A targets condensins to the chromosomes and for this process its phosphatase activity is not essential (Takemoto et al., 2009). PP2A also counteracts the Aurora B-mediated phosphorylation on kinetochore-associated substrates, which causes kinetochore-microtublule destabilization (Foley et al., 2011; Welburn et al., 2010). Plk1-mediated phosphorylation on BUBR1, facilitates the recruitment of PP2A-B56δ to the kinetochores, possibly resulting in stabilization of the kinetochore-microtuble interactions (Suijkerbuijk et al., 2012). In mammalian cells, a shugoshin 1 (Sgo-1) protects the centromeric cohesin. Cohesins are present on the centromeres during mitosis for correct mitotic spindle assembly (Salic et al., 2004; Tang et al., 2004). Previous studies have suggested that PP2A holoenzymes are recruited to the centromeres by Sgo1, where they counteract the Plk1 and Aurora B mediated phosphorylation on cohesins (reviebed in Barr et al., 2011). This prevents premature separation of sister chromatids. PP2A-mediated dephosphorylation of the Golgi matrix protein induces Golgi reassembly during mitotic exit (Lowe et al., 2000; Schmitz et al., 2010). Both PP1 and PP2A are implicated in controlling mitotic exit (Forester et al., 2007; Grallert et al., 2015; Mochida et al., 2010; Wu et al., 2009). Furthermore, PP2A-B55 dephosphorylates CDC25, leading to CDK1 inactivation and eventually exit from mitosis (Forester et al., 2007). In retrospect, PP2A acts as a mitotic gatekeeper.

Given this extensive role of PP2A in regulation of DDR proteins as well as in mitosis, it is evident that any dysregulation can lead to genomic instability. The DDR proteins have to be regulated efficiently for DNA repair proficiency. Failure to do so can cause improper DNA repair, leading to increased radiosensitization and other deleterious effects like mitotic catastrophe.

Herein, we investigated how does ATM-mediated phosphorylation on the S401 site of PP2A-Aα affect DNA damage response, mitosis and DNA repair. We have shown that there are severe defects in DDR proteins as well as mitotic regulation in the constitutively phosphorylated mutant, S401D, as well as the S401A mutant, which cannot be phosphorylated. Phosphorylation of the S401 site of PP2A- Aα also controls DNA repair, as we observed that DSB repair was significantly impaired in these two mutants.

Materials and methods

Plasmids

For end-joining repair assay: pcsCMV:tdTomato (Addgene #30530) plasmid was linearized with BamHI, and a linker (5′-GATCTCCCGGGA-3′) was added at this site to generate a SmaI restriction site. This modified pcsCMV: tdtomato was linearized with BamHI and SmaI to create incompatible DNA ends.

For DR-GFP assay: DR-GFP plasmid (Pierce et al., 1999) was used.

Antibodies

Antibodies used in western blots were anti-AKT (1:1000) (Cell Signaling Technology), anti-p (S473) –AKT (1:1000) (Cell Signaling Technology), anti-p(S139)- H2AX (1:1000) (EMD Millipore), anti-53BP1(1:1000) (Novus Biologicals) and anti-PP2A-A (1:1000) (Cell Signaling Technology).

Cell Culture

MEFs expressing PP2A-Aα were maintained as described in Chapter 2.

Generation of MEFs with DR-GFP cassette:

The FVB MEFs were infected with the DR-GFP plasmid (Pierce et al., 1999) and then, cell selected for puromycin resistance. The stable clonal cell lines were infected with the retrovirus: Mig-Aα-WT, S401A or S401D. The endogenous PP2A-Aα was floxed out by expression of Cre combinase (Ad-Cre).

Irradiation

Irradiation was performed using MDS Nordion Gammacell 40 irradiator with the Cs-137 source at a dose of roughly 1.05 Gy/min.

Western blotting

Western Blotting was performed as described in Chapter 2.

Clonogenic survival assay

MEFs expressing PP2A-Aα (WT/S401A/S401D) grown to 80% confluency in a 6 cm dish were trypsinized to generate single cell suspensions and cells were seeded in 60 mm dishes at cloning densities. Cells were allowed to attach and were either left untreated or irradiated at 1, 2 and 5 Gy. Cells were then incubated at 37°C and 5% CO_2. After 10 days, the media was removed and the cells were stained with 0.5% crystal violet in 25% methanol and colonies were counted.

Transfection

Transfections for the end-joining repair assays were done using Amaxa Nucleofector. (Program: N-024). MEFs expressing PP2A-Aα (WT/S401A/S401D) were transfected with linearized modified pcsCMV-td-tomato. (Transfection buffer recipe: Solution I: 20% ATP-disodium salt, 12% $MgCl_2$ (filter sterilize). Solution II: 2% KH_2PO_4, 2% $NaHCO_3$, 0.06% glucose (filter sterilize and adjust pH to 7.4). Just before use, solution I and solution II (1:50) were mixed. Use 100 μM for each transfection (Vriend et al. 2014).

End-joining repair assay

A 6 cm dish (almost confluent) was trypsinized. 1 µg of DNA (linearized pcsCMV: tdTomato plasmid) was transfected in these cells and the cells were split into a 12 well dish. Cells were collected (at 4, 8 and 16 hours). Unlinearized plasmid was used as a control and harvested at 24 hours. Red cells were counted using the Nexecelom Cellometer. For the PCR assay, post transfection as described above, cells were harvested at 1 and 4 hours. The media was collected from the wells, and then spun down. The remaining cells in the well were trypsinized and pooled with the pellet from previous step. The pellet was then re-suspended in re-suspension buffer (15% sucrose, 50 mM Tris, 25 mM EDTA) with 0.1% triton X-100 and treated with RNAse. Theses tubes were incubated at 50°C for 3-4 hours. Equal amount of re-suspension buffer was added with 0.1% SDS and 50 µg/ml Proteinase K. Samples were incubated at 60°C overnight. This was followed by phenol chloroform treatment and DNA was precipitated with 1: 1 iso-propanol. Repaired DNA was assessed by quantitative Real-time PCR, using an ABI 7900HT (Applied Biosystems) and SYBR green detection.

Flow cytometry

MEFs expressing PP2A-Aα (WT, S401A or S401D) and the DR-GFP cassette were fixed with 100% methanol and permeabilized in 1% Triton X. Cell cycle distribution were analyzed by 7-aminoactnomycin D (7-AAD). Flow cytometry was done on a BD Biosciences FACS Canto flow cytometer at the VCU Flow cytometry. Data was analyzed using FCS Express software.

Confocal Microscopy

Cells were grown in chamber slides (Lab-Tek -Naperville, IL) to 80-90% confluence.

Cells were fixed with 4% paRAFormaldehyde for 15 minutes, washed with PBS 4 times

for 5 minutes. Cells were permeabilized with 0.5% Triton X-100 in PBS and blocked

with Casein/2% goat serum. Primary antibody incubation was performed at 4°C

overnight. Slides were then washed with PBS 4 times for 5 minutes and incubated with

secondary antibody (dilution 1:500) at room temperature for 2.5 hours. The nuclei were

counterstained with DAPI (1 mg/ml) and were mounted in Vectashield mounting media

(Vector Laboratories). Imaging was performed using Zeiss LSM 710 Meta Imaging

system in the VCU microscopy Facility and analyzed using the Zeiss Zen software as

well as PERKinElmer's Volocity software.

Live – Cell Imaging

MEFs expressing PP2A-Aα (WT/S401A/S401D)/H2B-mCherry were seeded on a 4

chamber glass bottom CELL view tissue culture dishes (Greiner Bio-One) and allowed to

grow until 80% confluency. Treatment group was irradiated at 5 Gy. Still images were

taken at every 7 minutes for a total of 16 hours beginning 1 hour after irradiation. Cells

were kept on an incubated stage at 37°C and 5% CO_2 throughout the imaging duration.

Live cell imaging was performed on Spinning Disc Confocal Microscope at VCU

Microscopy Facility. Videos were analyzed using Zeiss Zen software.

Statistics

Unpaired two-tailed t tests were performed on triplicate or more data sets using Graphpad

Prism 3.0 (Graphpad Software, inc.). *P* values are indicated as *, <0.05; **, <0.01; and

***, <0.001.

Results

MEFs expressing PP2A-Aα S401D show sustained γ-H2AX phosphorylation but no significant effect on p-AKT

ATM kinase is a key molecule that facilitates DDR. In response to DSBs caused by ionizing radiation, ATM phosphorylates a large number or targets at specific positions as a cellular response to DSBs. ATM phosphorylates histone H2AX at S139 (γ-H2AX) in response to DNA damage at the sites of DSBs where they form discrete foci. Furthermore, PP2A mediates DSB repair via dephosphorylation of γ-H2AX to reverse chromatin accessibility (Chowdhury et al., 2005). To test if phosphorylation of PP2A-A at S401 would affect γ-H2AX levels, we looked at the γ-H2AX levels in response to low dose radiation. PP2A-Aα KO MEFs, expressing PP2A-Aα WT, S401A or S401D mutants, were irradiated at 2 Gy. In the WT cells, γ-H2AX peaked at 15 minutes before declining at around one hour. γ-H2AX phosphorylation was partially inhibited in the cells expressing the S401A mutant, whereas it continued to increase in the S401D expressing cells at around 60 minutes **(Fig. 3-1A and 1B).** We looked at γ-H2AX foci formation in these cells post DNA damage by immunocytochemistry. The S401D cells exhibited significantly higher number of foci per cell after 5 hours relative to the WT and S401A cells (**Fig. 3-2A and 1B**). This data is indicative of the S401D mutant form having less activity towards γ-H2AX dephosphorylation. Since ATM also regulates the
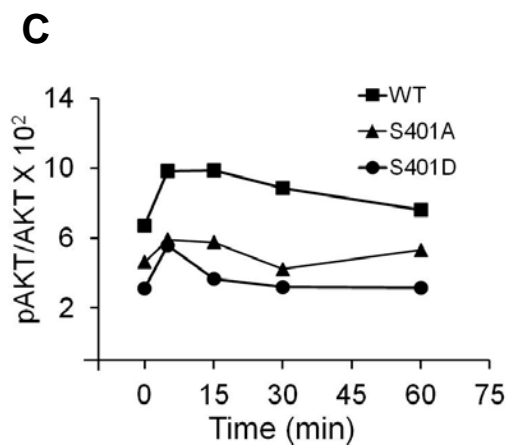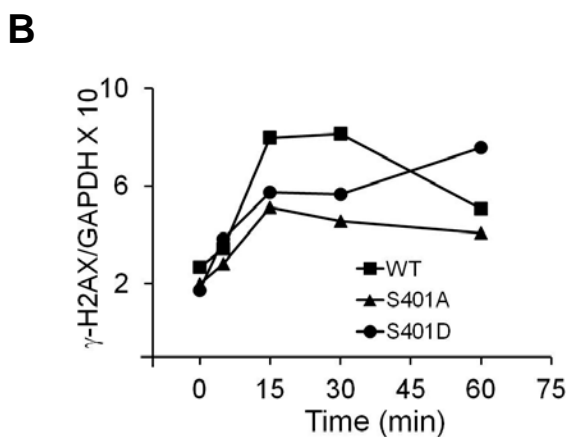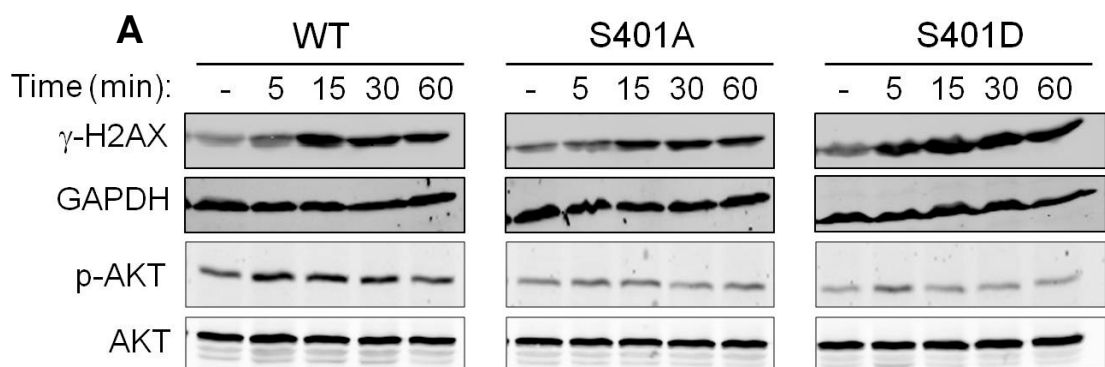
Figure 3-1: Sustained γ-H2AX but not p-AKT were seen in the PP2A-Aα S401D MEFs post IR

(A) PP2A-Aα KO MEFs (WT, S401A or S401D) were exposed to 2 Gy of ionizing radiation and harvested after 5, 15, 30 and 60 minutes. p-AKT (S473), total AKT and γ-H2AX levels were detected by western blotting. (B & C) p-AKT levels were normalized to total AKT protein levels. γ-H2AX levels were normalized to GAPDH.
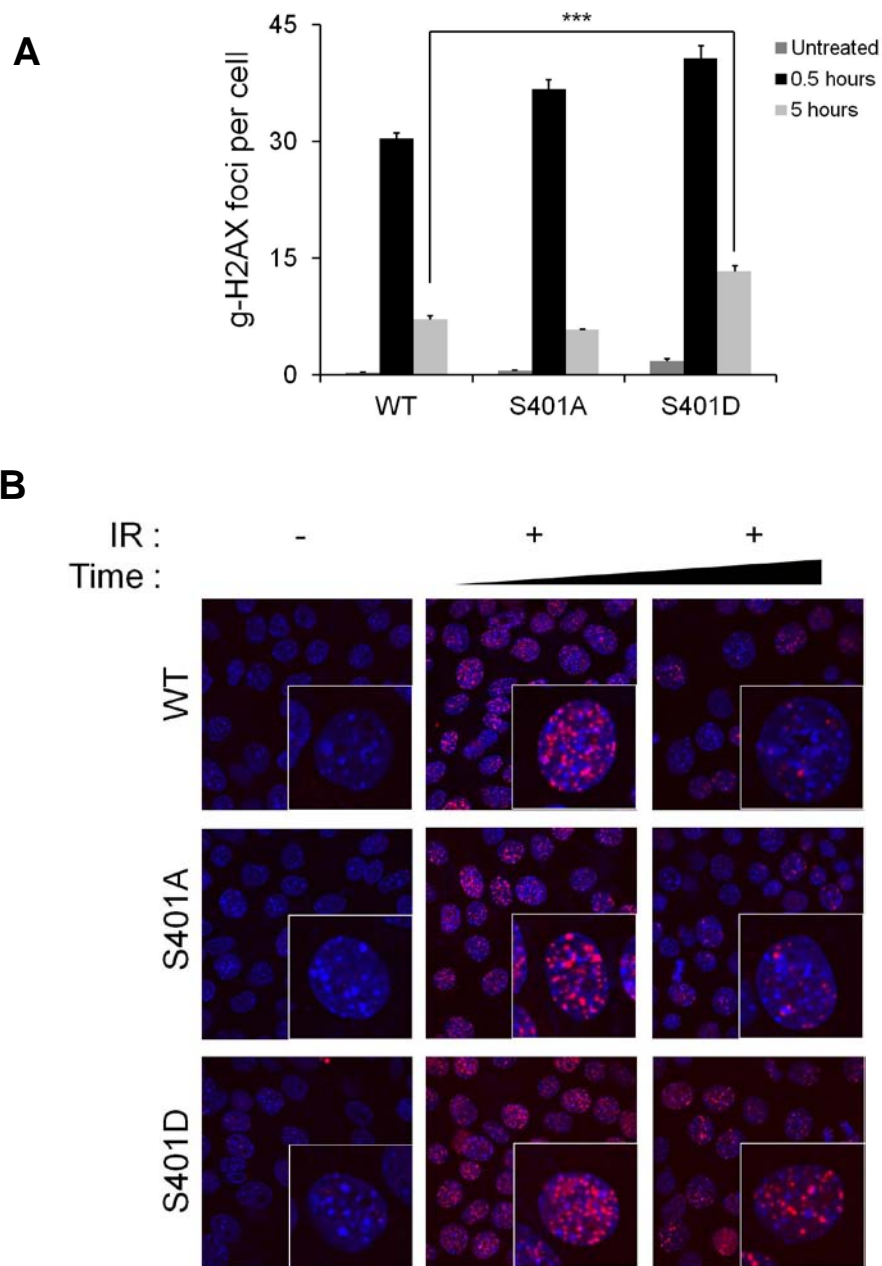
Figure 3-2: S401D MEFs had sustained γ-H2AX levels post IR

(A) PP2A-Aα KO MEFs (WT, S401A and S401D) were exposed to 5 Gy of ionizing radiation or left un-irradiated and fixed after 0.5 and 5 hours. γ-H2AX foci in at least 30 cells were counted for each group. Error bars SEM $n = 3$, ***$p < 0.001$ relative to WT **(B)** Representative images of cells immunolabeled with γ-H2AX antibody (Red) and counterstained with DAPI (Blue) to label nuclei, Images were acquired at 63x power.

phosphorylation of AKT (S473) in response to IR (Golding et al., 2009; Viniegra et al., 2005a), we assessed the levels of p-AKT (S473) post IR treatment. Time-dependent increase in AKT phosphorylation was seen as early as 5 minutes in the WT-PP2A-Aα KO MEFs (**Fig. 3-1C**). However, this increase was abrogated in the cells expressing S401A and S401D, suggesting that PP2A-Aα phosphorylation at S401 may not regulate p-AKT (S473) in response to IR.

Improper phospho-regulation at S401 site of PP2A-Aα leads to abnormal mitoses

PP2A holoenzyme has a distinct function in different phases of mitosis by associating with the respective B subunits. Mitotic entry is promoted by inhibition of PP2A via Greatwall kinase (Vigneron et al., 2009; Lorca et al., 2010). During mitosis PP2A holoenzyme with specific B' subunits are recruited at the centrosomes along with Sgo-1, where they play a critical role in protecting centromeric cohesins by inhibiting Plk1 and Aurora A kinases (Hauf et al., 2005; Kitajima et al., 2006; Riedel et al., 2006). PP2A also controls mitotic exit along with PP1 (Forester et al., 2007; Grallert et al., 2015; Mochida et al., 2010; Wu et al., 2009). Taking into account the extensive role of PP2A in mitosis, we examined mitosis in PP2A-Aα KO-MEFs (WT/S401A/S401D). We performed live cell imaging in these cells labeled with histone H2B-mCherry construct that aided us in visualizing dividing nuclei. The WT cells underwent normal mitosis in the absence of IR-induced DNA damage. The S401A and S401D cells exhibited abnormal mitoses
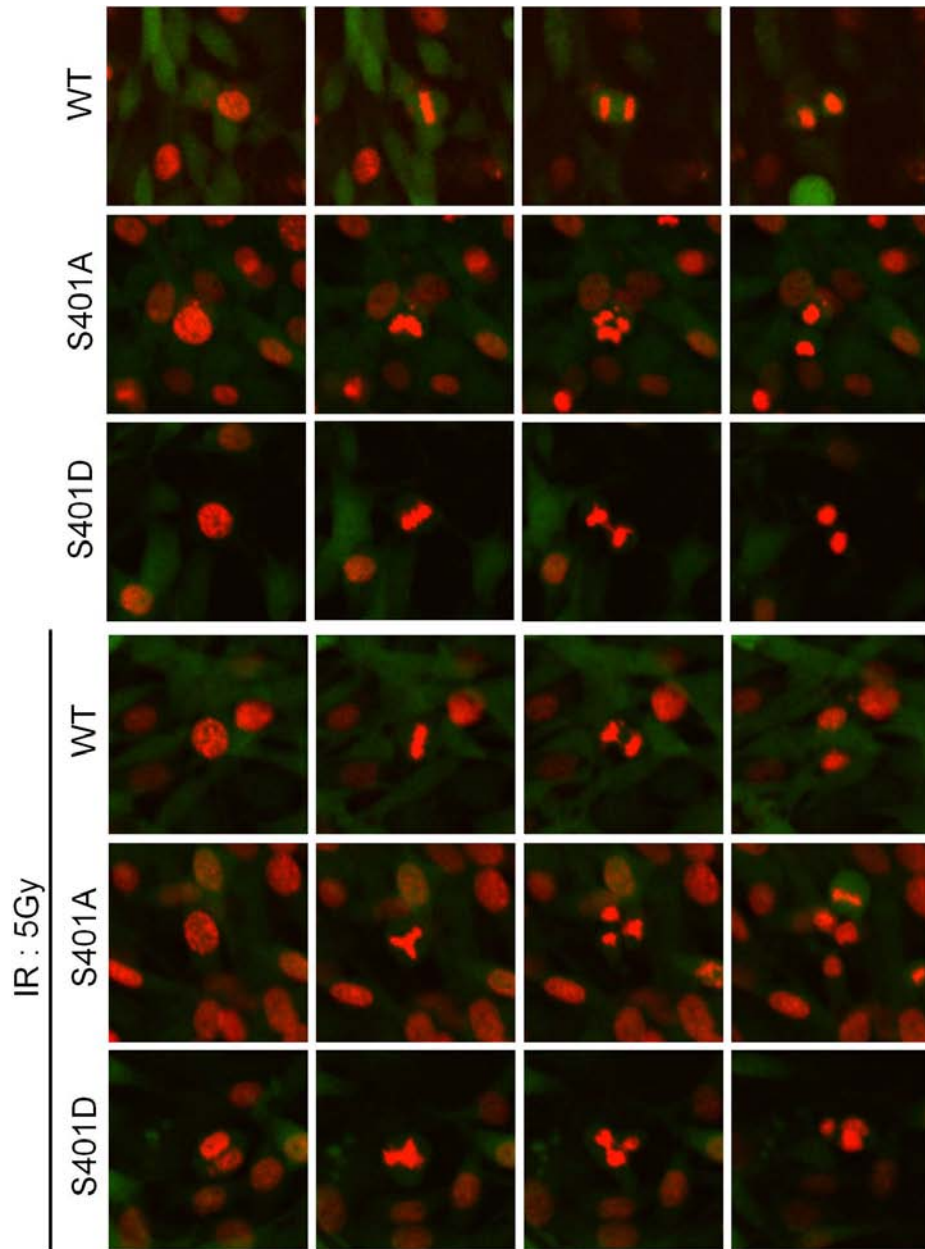
Figure 3-3: S401A and S401D cells under go aberrant mitosis

Time series of mitosis in PP2A-Aα KO MEFs (WT, S401A and S401D) /H2B-mCherry cells. Cells were exposed to 5Gy of IR and subjected to live-cell video imaging. Representative still images of cells undergoing aberrant mitosis are shown.
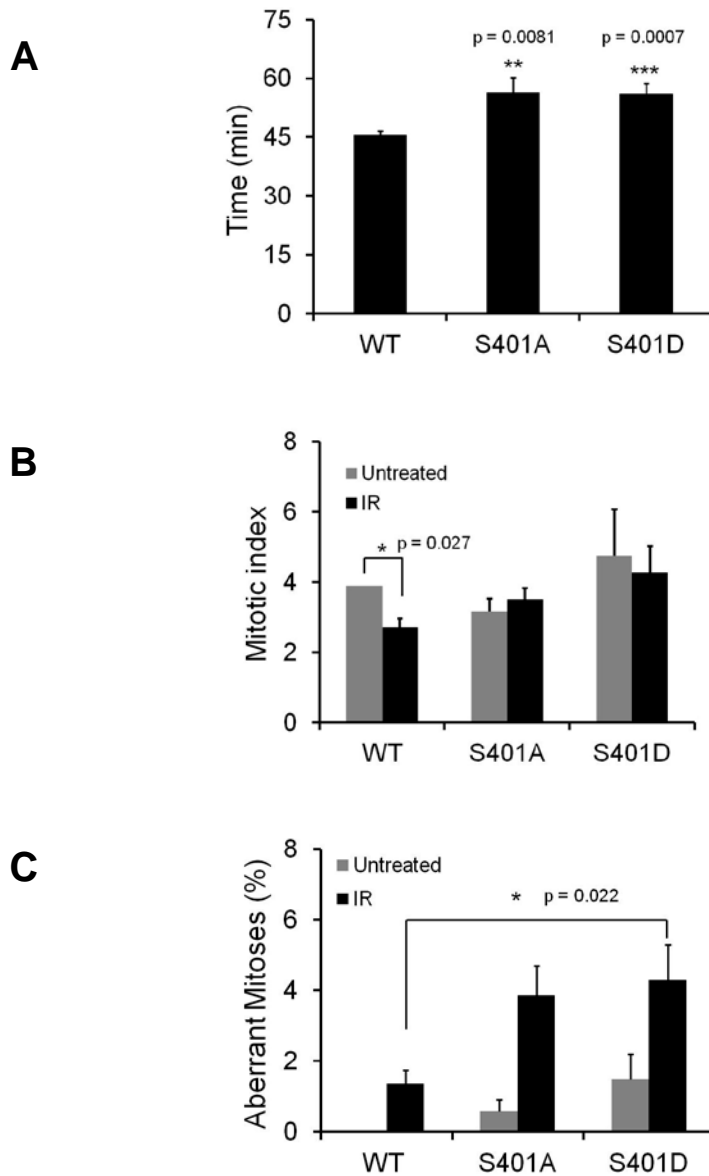
Figure 3-4: S401A and S401D MEFs undergo prolonged mitosis and accumulate increased chromosomal aberrations

(A) Length of mitosis was measured in untreated PP2A-Aα KO MEFs (WT, S401A and S401D)/H2BmCherry cells. **(B&C)** PP2A-Aα KO MEFs (WT, S401A and S401D) were irradiated at 5Gy, fixed after 24h. Mitotic index was calculated as the ratio of mitotic cells to the total number of cells. Aberrations were scored as total number of micronuclei, bridges and abnormal metaphase plates relative to the total number of nuclei. At least 200 cells were counted for each group. SEM; n = 3, ***p < 0.001, **p< 0.01 and *p<0.05 relative to WT.

including formation of micronuclei and tetraploidy whose frequency increased on exposure to IR. Most of the mutant cells suffered mitotic catastrophe (**Fig. 3-3**). In undamaged cells, the length of mitoses in the S401A and S401D cells was significantly longer thanthe WT (**Fig. 3-4A**). Additionally, we measured the mitotic index (ratio of cells undergoing mitoses to the total number of cells) and chromosomal abnormalities after exposure to IR. As predicted, the mitotic index in the WT cells decreased but not in the S401A and S401D cells (**Fig. 3-4A**), suggesting a deficiency in checkpoint activation in the mutants. Furthermore, relative to WT cells the S401A and S401D had significantly increased numbers of chromosomal aberrations (**Fig. 3-4B and 4C**). All these data suggest that the S401 site of PP2A-Aα plays an important role in mitotic regulation.

MEFs expressing PP2A-Aα S401D and S401A displayed increased radiosensitization

DDR activity is protective towards the cell in an event of DNA damage. It upregulates DNA repair in order to protect the cell from genomic instability. Dysregulation of DDR and genomic instability can often lead to radiosensitization of cells. ATM kinase regulates DSB repair as well as controls cell cycle checkpoints; therefore inhibition or loss of ATM kinase increases radiosensitivity (Lavin, 2008; Valerie and Povirk, 2003). We are studying the regulation of PP2A by ATM. Our mitosis data suggested a possible role of the S401 site of PP2A-Aα in cell cycle and mitotic regulation. Therefore we
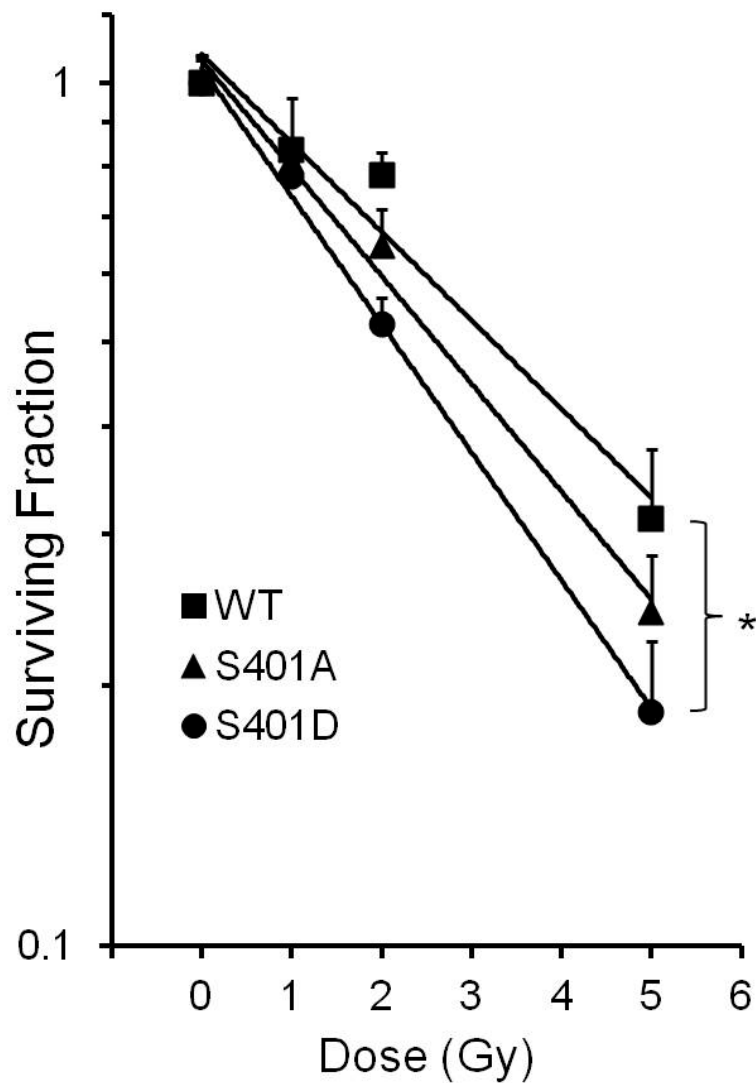
Figure 3-5: S401D MEFs display increased radiosensitization

PP2A-Aα KO MEFs (WT, S401A and S401D) were irradiated at 1, 2 and 5Gy and radiosurvival was determined after 10days. Survival fractions were determined by crystal violet staining and colony counting. Data points, surviving cells plotted as fraction of control (- irradiation). Error bars SEM; n=4. S401D is significantly different from WT ,p< 0.05.

performed a clonogenic survival assay in PP2A-Aα KO MEFs (WT, S401A and S401D).

We observed that the S401A and the S401D cells were more radiosensitive relative to

WT, the S401D being significantly impaired in radiosurvival. (**Fig. 3-5**). This suggests

that the irregular phosphorylation at S401 of PP2A-Aα can result in checkpoint

deficiencies causing build up of genomic instability, which can result in increased

radiosensitivity.

Inappropriate phosphorylation at S401 of PP2Aα leads to inefficiency in DSB repair

PP2A has known function in maintaining genomic stability by modulating proteins

involved in DNA repair. PP2A is involved in the NHEJ pathway of DSB repair, where it

facilitates DNA end joining by dephosphorylation and subsequent activation of KU and

DNA-PKcs. These proteins are major players of NHEJ (Wang et al., 2009). Increased

chromosomal aberrations in the MEFs expressing S401A and S401D mutants suggest

inefficient DNA repair post damage, the accumulation of which could lead to mitotic

catastrophe. To determine how the phosphorylation of PP2A-Aα at S401 might affect

DSB repair, we performed an *in vivo* as well as *in vitro* end-joining assay. PP2A-Aα KO

MEFs expressing WT, S401A and S401D mutants were transfected with pCSCMV:

tdTomato plasmid linearized with BamHI and SmaI to create a 5'-overhang and blunt end

that cannot easily be ligated without DNA resection and DSB repair. Once the plasmid is

repaired and circularized; red fluorescence protein (RFP) will be expressed (**Fig. 3-6**).

Cells were collected at various time points and RFP+ cells were quantified. At
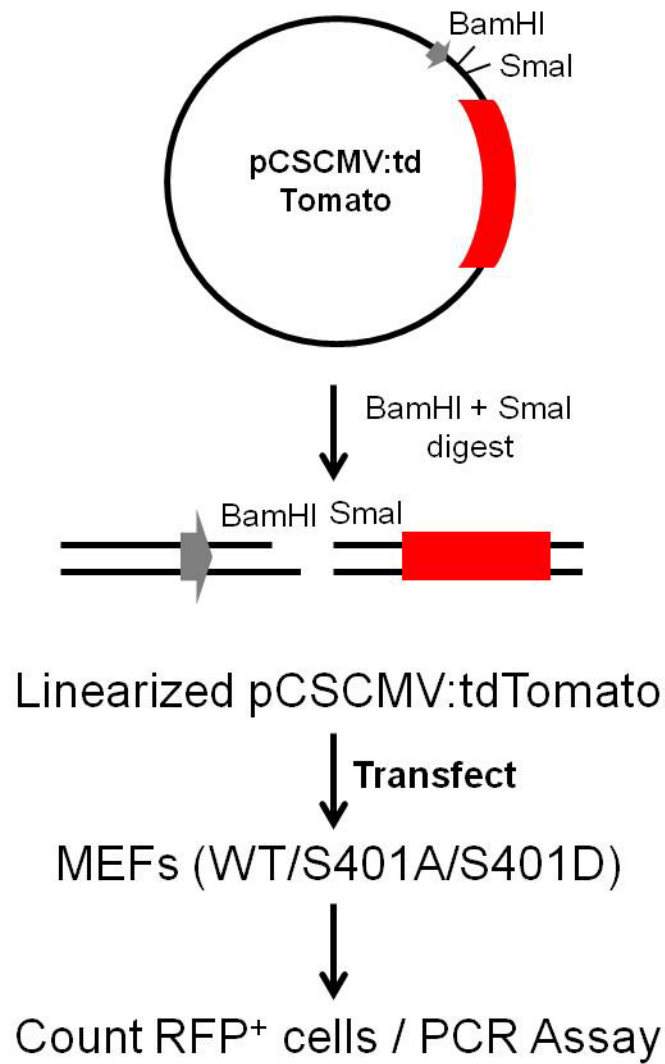
Figure 3-6: **Scheme for *in vitro* end joining assay**

PP2A-Aα KO MEFs (WT, S401A and S401D) were transfected with BamHI/SmaI linearized pCSCMV: tdTomato.
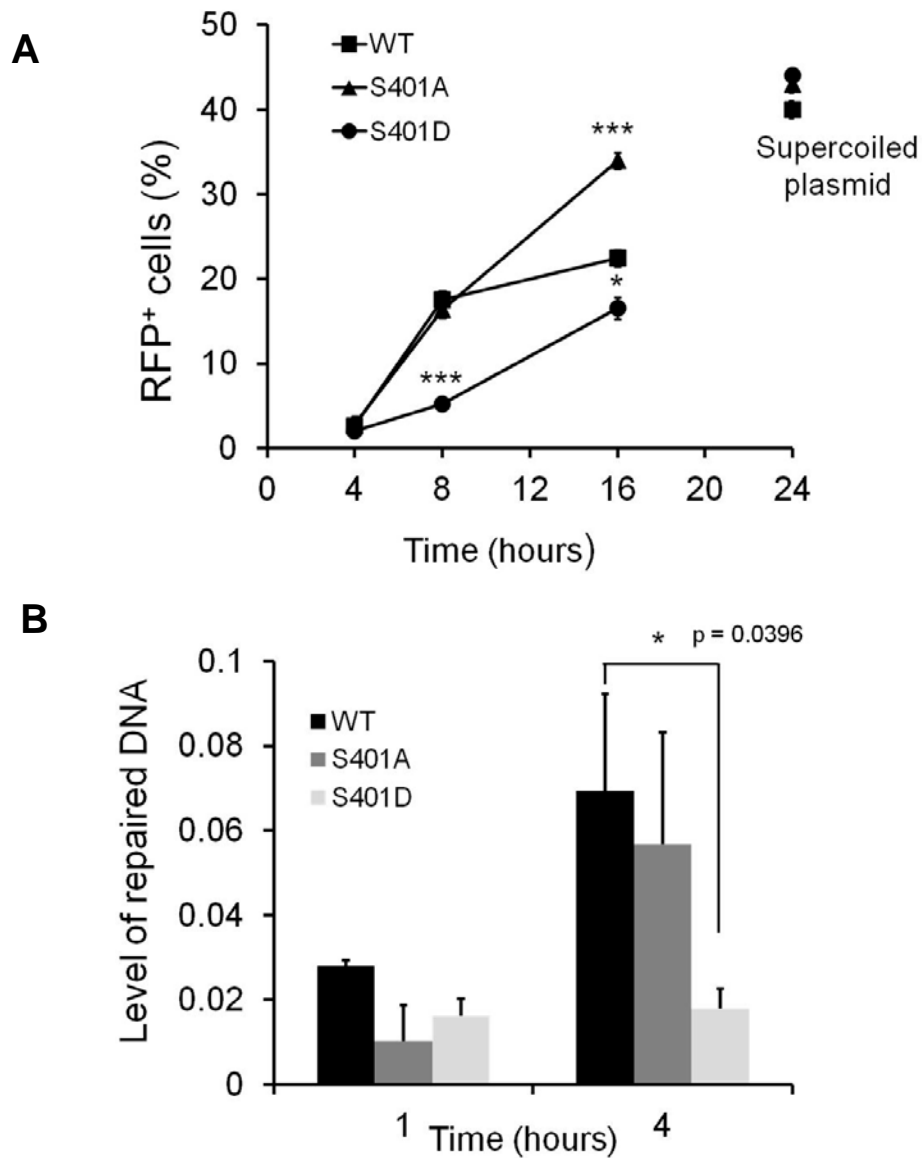
Figure 3-7: S401D MEFs were inefficient in end-joining mediated repair

(**A**) Cells were collected after 4, 8 and 16 hours and RFP positive cells were quantified. Supercoiled pCSCMV: tdTomato was used as transfection control. (**B**) PCR assay: Cells were collected after 1 and 4 hours. Plasmid DNA was isolated and the repaired DNA was quantified by Real time PCR. SEM; n = 3, ***p < 0.001, **p< 0.01 and *p<0.05 relative to WT.

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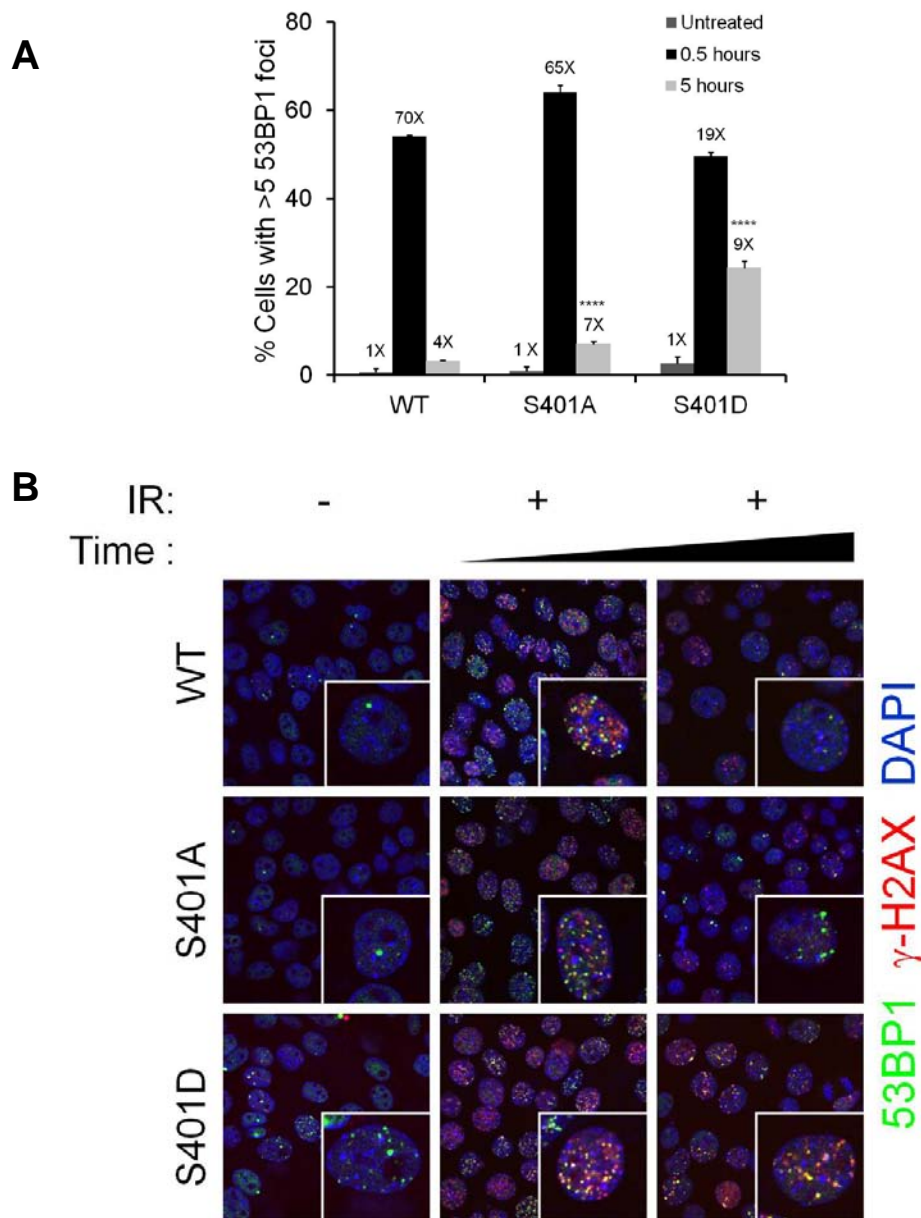
Figure 3-8: S401A and S401D cells show sustained 53BP1 foci

(A) PP2A-Aα KO MEFs (WT, S401A and S401D) were exposed to 5 Gy of ionizing radiation or left un-irradiated and fixed after 0.5 and 5 hours. Cells with more than 5 53BP1 foci were counted for each group. Error bars SEM $n = 3$, ***$p < 0.001$ relative to WT **(B)** Representative images of cells immunolabeled with 53BP1 antibody (Green)γ-H2AX antibody (Red) and counterstained with DAPI (Blue) to label nuclei, Images were acquired at 63x power.

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16 hours the S401A-PP2A-Aα KO MEFs had almost twice the number of red cells relative to the WT cells, whereas the S401D cells had less than the WT cells (**Fig. 3-7A**). In a different experiment with the same set up, cells were harvested after 1 and 4 hours, plasmid DNA was isolated, and end-joining was quantitated by real-time PCR. Consistent with the previous assay, the S401D cells were significantly deficient in end joining **(Fig. 3-7B).** We also looked at the DDR factor tumor suppressor 53BP1,which promotes c-NHEJ and suppresses repair via HRR. We saw that the S401D mutant had sustained 53BP1 foci, which co-localized with γ-H2AX foci (**Fig. 3-8A & B**). The S401A MEFs also had few bigger foci at 5 hours (**Fig. 3-8B**).

Since multiple pathways repair DSBs, we looked at HRR efficiency in these cells. PP2A holoenzyme associated with B55 subunit has been implicated in regulation of homologous recombination repair (HRR) via modulation of ATM phosphorylation and the G1/S check point (Kalev et al., 2012). To determine the importance of ATM mediated phosphorylation of PP2A-Aα in HRR, we expressed DR-GFP repair reporter (Beckta et al., 2015) in the PP2A-Aα CKO/CKO MEFs. These cells were infected with Mig-Flag-Aα-WT, S401A and S401D retrovirus and the endogenous PP2A-Aα was floxed out with an Adenovirus expressing Cre recombinase. To measure HRR, DSBs were induced by adenoviral-mediated expression of I-SceI enzyme, which cleaves the DR-GFP gene; HRR at this break will restore WT-GFP expression. 72 hours post infection with Ad-SceI, the cells were processed and analyzed to count GFP expressing cells by flow cytometry. We observed that the number of GFP cells in S401A and S401D were

A DR-GFP assay

B
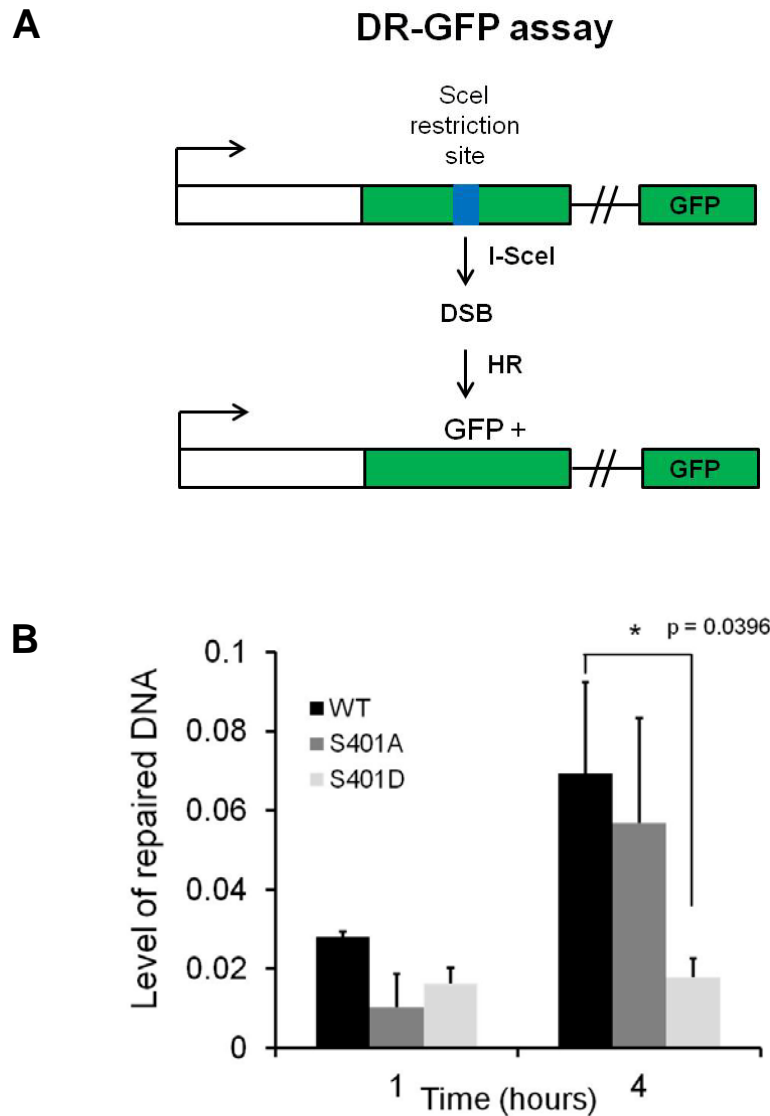
Figure 3-9: S401A and S401D MEFs are impaired in HRR

(**A**) Scheme for DR-GFP assay. (**B**) PP2A-Aα KO MEFs (WT, S401A and S401D) expressing the DR-GFP cassette were infected with Ad-SceI, 72 hours post Ad-SceI infection, 30000 cells were analyzed for GFP (HRR) events using flow cytometry. SEM; n = 3, *p<0.05 relative to WT.
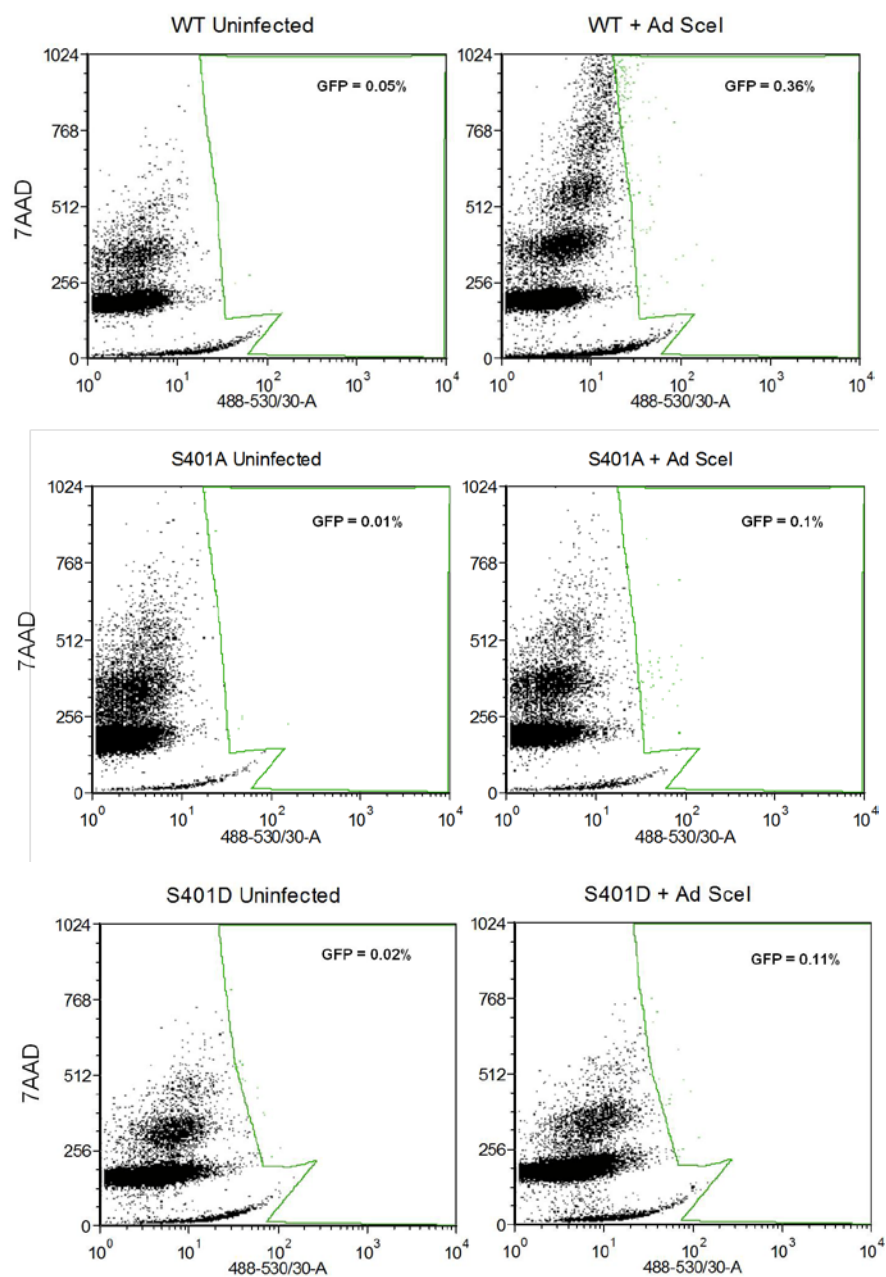
Figure 3-10: Representative histograms for DR-GFP assay

Representative histograms from uninfected control and infected (Ad-SceI) for PP2A-Aα KO MEFs (WT, S401A and S401D) from one single experiment are shown.

significantly less than the WT. This data suggests that in the cells expressing the mutant

proteins, there was significant reduction in HRR (**Fig 3-9 and 3-10**).

Chapter summary

Of all the DNA lesions, DSBs are the most lethal ones. Failure to repair DSBs correctly can lead to increased genomic instability like chromosomal breaks and translocations that have the potential to cause oncogenic transformations (Goldberg et al., 2003; Stucki et al., 2005). Reversible phosphorylation is an extremely important posttranslational modification of proteins, and plays a crucial role in many cellular events including DDR. Kinases and phosphatases work together to regulate this signaling cascade in response to specific molecular cues.

As discussed earlier, it is well established that ATM is a master regulator of DDR, and PP2A is required for IR-induced activation of ATM. Activated ATM phosphorylates and recruits γ-H2AX at the sites of DSBs, which further recruits DNA repair proteins on chromatin adjacent to the DSBs (Xu et al., 2012; Melander et al., 2008; Stucki et al., 2005; Fernandez-Capetillo et al., 2002; Paull et al., 2000; Rogakou et al., 1998). The dissociation of the repair proteins from the DSBs requires elimination of γ-H2AX from the DSBS. This is important for checkpoint recovery (Keogh et al., 2006). One of the mechanisms by which this is achieved is by PP2A-mediated dephosphorylation of γ-H2AX (Chowdhury et al., 2005). In our study we assessed γ-H2AX induction kinetics in PP2A-Aα KO MEFs expressing the WT, S401A and S401D PP2A-A in response to low dose of IR. In WT cells, γ-H2AX protein levels as well as foci peaked around 30 minutes, which diminished over a period of 5 hours. However, in the S401D cells,

sustained γ-H2AX foci were seen at 5 hours. The γ-H2AX kinetics in S401A mutant cells were not significantly distinct than the WT cells, though protein levels seemed to be lower at earlier time points (**Fig. 3-1A, B and 3-2)**. Recently, the Li group demonstrated that suppression of PP2A- B56ε leads to a delay in elimination of γ-H2AX, and presented a possibility that PP2A holoenzyme with the B56ε mediates the dephosphorylation of γ-H2AX at DSBs (Li et al., 2015). This is in line with our hypothesis that ATM-mediated phosphorylation at S401 of PP2A (S401D phosphomimetic) would inhibit PP2A's activity leading to increased γ-H2AX levels.

Also, ATM regulates AKT phosphorylation (S473) in response to irradiation (Golding et al., 2009; Viniegra et al., 2005b). When we looked at p-AKT (S473) levels in MEFs post IR, we did not see any significant difference between our mutants and the WT (**Fig. 3-1A**). Given the number of distinct regulatory subunits of PP2A, which directs it towards respective substrates, it is possible that ATM mediates AKT activation via PP2A by a mechanism different from what we have hypothesized. AKT, which is regulated by phosphorylation at T308 and S473, can promote cell growth or apoptosis depending on the signals received from the cellular environment (Andrabi et al., 2007).

Impaired DDR can often lead to genomic instability and abrogated checkpoints. In the absence of DNA damage, the S401A and S401D PP2A-Aα spent more time in mitosis relative to the WT cells (**Fig. 3-4A**). Also, they harbored increased chromosomal

aberrations relative to WT in response to IR (**Fig. 3-3 and 3-4C**). As reviewed earlier, PP2A plays a wide range of roles in mitosis. It regulates mitotic entry as well mitotic exit. In Xenopus egg extracts, depletion of PP2A-B55δ allowed entry into mitosis but the cells were unable to exit mitosis (Mochida et al., 2009). This could explain the reason of prolonged mitosis. There are other phosphatases like PP1 that can often complement and allow cell cycle progression eventually. Proteins like Plk-1 and Sgo1, regulated by PP2A, are responsible for mitotic checkpoints. Failure of these checkpoints in presence of DNA damage can affect microtubule and spindle assembly leading to aberrant mitosis (Hauf et al., 2005; Kitajima et al., 2006). Also, deficient cell cycle checkpoints and abnormal chromosomes can cause mitotic catastrophe, consequently resulting in increased radiosensitization. PP2A is essential for IR-induced G2/M check point (Yan et al., 2010) and PP2A-Aα depletion via siRNA radio-sensitized pancreatic cells (Wei et al., 2013). The PP2A-Aα S401D mutant cells can go through G2/M transition without efficient DNA repair thereby resulting in increased radiosensitivity.

DDR appeared to be dysregulated in the S401D cells due to sustained γ-H2AX levels. This suggested that DNA repair factors are still present at the foci and checkpoint recovery is possibly delayed. To validate if DSB repair is affected in these cells, we looked at NHEJ efficiency and HRR efficiency in PP2A-Aα KO MEFs (WT, S401A and S401D). From our end-joining assay, we can conclude that the S401D mutant cells are extremely inept at repairing the DSBs via non-homologous end joining (NHEJ) (**Fig 3-7A and B**). The DDR factor 53BP1 promotes c-NHEJ by blocking 5' resection at DSBs,

which is required for HRR (reviewed in Zimmermann and Lange, 2014). 53BP1 forms IR induced foci that co-localize with γ-H2AX (Anderson et al., 2001; Rappold et al., 2001; Schultz et al., 2000). We saw that in the S401A and S401D mutants there was a delay in disappearance of 53BP1 foci at 5 hours. One possible reason could be slow repair kinetics in these mutants. The S401A mutant shows few but bigger 53BP1 foci (**Fig 3-8**), which could represent a site of complex DNA damage (Marková et al., 2007). PP2A elicits control on NHEJ via positively regulating Ku/DNA-PK activity required for end joining. PP2A-mediated dephosphorylation of Ku70/Ku86 and DNA-PKcs elevated DNA-PK activity *in vitro* and *in vivo* (Douglas et al., 2001; Wang et al., 2009). Consequently, the inefficiency of DSB end joining we observe in our study could be attributed to reduced activity of DNA-PK, which could slow down DNA repair via c-NHEJ in the S401D cells.

We also looked at HRR pathway, which is the other major and more efficient DSB repair pathway. There was significant reduction in HRR+ (GFP) cells post I-SceI infection in MEFs expressing the S401A and S401D mutant (**Fig 3-9 and 3-10**). A study by the Sablina group has implicated the B55 subunit in HRR where it acts via ATM and G1/S check point regulation (Kalev et al., 2012). Also, pharmacological inhibition of PP2A also inhibited HRR and accumulated IR induced DNA damage in pancreatic cancer cells (Wei et al., 2013). The S401A cells looked relatively proficient in NHEJ but inefficient in HRR. Perhaps it is possible that in the S401A cells the repair is directed from HRR to NHEJ. The S401D MEFs, which are deficient in both the NHEJ and HRR pathway, still

survive due to a very inefficient DNA repair mechanism, which leads to increase in genomic instability as seen in our study.

The data presented in this chapter imply that ATM-mediated S401 phosphorylation of PP2A-Aα is an essential post-translational modification. ATM-mediated phosphorylation can regulate the repair kinetics in the cell. Our studies show that cells expressing S401 mutants are inefficient in DNA repair. We have seen a significant amount of mitotic aberrations in these cells. Accumulation of unrepaired DNA can have deleterious effects on the cells, like the mitotic catastrophe seen in the S401 mutants.

Interactions of PP2A with its substrates depend on specific B subunits, which further regulate DDR proteins. We have observed that phosphorylation at S401 causes the holoenzyme to dissociate from its substrates as well as the C-subunit. The enzyme could undergo structural change post S401 phosphorylation thus causing this dissociation and thereby affecting its interaction with its substrates and regulatory subunits. Studies in the next chapter have helped us to deduce a possible mechanism by which the DDR could be regulated by ATM-mediated phosphorylation on PP2A-Aα.

CHAPTER 4:

Phosphorylation of PP2A-Aα at S401 causes its nuclear export via CRM1

Introduction

Studies have shown that phosphatases respond to DNA damage primarily by altering subcellular localization, which can govern substrate association and subsequently downstream cellular signaling. In our study, we observed that the S401D PP2A-Aα was cytoplasmic and the S401A was mostly nuclear, whereas the WT was both cytoplasmic and nuclear. This brought us to the next question. Does ATM mediated phosphorylation on S401 cause its translocation to the cytoplasm?

There are a number of pathways by which nuclear export of a protein can take place (reviewed in Ossareh-Nazari et al., 2001). One of the karoypherins, chromosome region maintenance 1/exportin/Exp1/Xpo1 (CRM1), has been studied extensively. CRM1 binds to the leucine-rich nuclear export signal (NES) in many organisms (Fornerod et al., 1997; Fukuda et al., 1997; Haasen et al., 1999; Neville and Rosbash, 1999; Ossareh-Nazari et al., 2001; Stade et al., 1997). Efficient binding of CRM1 to the NES sequence, requires the NES to have five consensus hydrophobic residues that can be spatially coordinated with the NES docking site on CRM1 (Engelsma et al., 2004; Güttler and Görlich, 2011; Kudo et al., 1998). CRM1-mediated nuclear export can be effectively inhibited by a *Streptomyces* metabolite, Leptomycin B (LMB) (Hamamoto et al., 1983a, 1983b, 1985). LMB has been shown to interact directly with CRM1, where it binds covalently to the cysteine residue in CRM1 (Kudo et al., 1999). This disrupts the interaction between

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CRM1 and the NES on its target, thereby inhibiting the subsequent CRM1-mediated export of the target protein (Fornerod et al., 1997; Kudo et al., 1998; Nishi et al., 1994).

The translocation of CRM1-mediated transport takes places across a RanGTP gradient. The binding of CRM1 to RanGTP in the nucleus is essential, as it provides metabolic energy for the directional transport (Görlich et al., 1996, 1997; Rexach and Blobel, 1995). The protein containing the NES forms an export complex with CRM1 and RanGTP in the nucleus (Fornerod et al., 1997; Fukuda et al., 1997; Görlich and Kutay, 1999; Mattaj and Englmeier, 1998; Stade et al., 1997). On translocation through the nuclear pore complex (NPC), RanGTP is hydrolyzed to RanGDP, causing irreversible dissociation of the export complex (Englmeier et al., 1999; Görlich and Kutay, 1999; Izaurralde et al., 1997; Kehlenbach et al., 1999; Mattaj and Englmeier, 1998). The export cycle is completed by reimport of RanGDP to the nucleus, where GDP is exchanged to GTP (Ribbeck et al., 1998; Yi et al., 2002).

The diversity of the PP2A holoenzyme is imparted to it due to the association of the AC core dimer with the distinct variety of B subunits (Janssens and Goris, 2001). The substrate specificity and the subcellular localization of the holoenzyme are governed by specific B-subunits. Tsuchiya et al. identified a functional NES in the PP2A-Cα subunit, whose disruption inhibited nuclear export of PP2A-Cα (Tsuchiya et al., 2006). Also, LMB treatment caused accumulation of PP2A-Cα in the nucleus in this study. In a following study conducted by Flegg et al., a functional NES in PP2A-B56α subunit was

identified (Flegg et al., 2010). LMB treatment blocked the cytoplasmic translocation of the PP2A-B56α. They also reported that disruption of the PP2A-B56α NES prevents the PP2A-Cα's translocation to the cytoplasm, thereby concluding that PP2A-B56α association is important for the nuclear export of PP2A-Cα (Flegg et al., 2010). Another B-subunit that is also subjected to CRM1-mediated nuclear export is PP2A-B56ε (Jin et al., 2009). In their study, one of the PP2A-B56ε isoforms that lacked the N-terminus was cytoplasmic (Jin et al., 2009). Flegg et al. verified this by identifying a highly potent NES on the C-terminus, which clarified this observation (Flegg et al., 2010).

Depending on the function of the specific PP2A holoenzyme, it possibly gets displaced in order to respond to cellular cues. The cytoplasmic localization of the PP2A-Aα S401D mutant led us to pursue localization studies in PP2A-Aα KO MEFs. We hypothesized that, in response to DNA damage, activated ATM kinase phosphorylates S401 of PP2A-Aα and causes export to the cytoplasm via CRM1. Our studies indicated that PP2A-Aα and CRM1 indeed interact and nuclear retention of PP2A-Aα increased post LMB treatment. We also identified a putative NES sequence in PP2A-Aα.

Materials and methods

Plasmids

pFlag-hCRM1 (Addgene #17647), pmCherry-C1 Ran Q69L (Addgene #30309),

mRuby2-PP2A-Aα : pGEX2T- PP2A-Aα (Chapter 2) was cut with BamHI and EcoRI,

1776 bp fragment cloned into mRuby2-C1 (Addgene#54768), pPAmcherry-PP2A-Aα :

(**1**. pGEX2T- PP2A-Aα was cut with BamHI and EcoRI. The 1776 bp fragment was

cloned in pEGFP-C3-hYAP1 (Addgene #17843), linearized with BglII and EcoRI. **2**.

pPAmCherry-α-tubulin (Addgene31930) was cut with NheI and BglII, the 739 bp

fragment was cloned in pEGFP-C3- PP2A-Aα digested with NheI and BamHI.)

Antibodies

Antibodies used in western blots were anti- Flag (1:1000) (Sigma Aldrich), anti-p(S139)-

gH2AX (1:1000) (EMD Millipore) and anti-PP2A-A (1:1000) (Cell Signaling

Technology), Hoeschs stain 333242 (Sigma Aldrich).

Cell Culture

MEFs expressing PP2A-Aα (WT, S401A and S401D) and HEK293 cells were

maintained as described in Chapter 2.

Irradiation

Irradiation was performed using MDS Nordion Gammacell 40 irradiator with Cs-137

source at a dose of roughly 1.05 Gy/min.

Western Blotting

Western Blotting was performed as described in Chapter 2.

Immunoprecipitation

HEK 293 cells were co-transfected with Flag-CRM1, untagged RanQ69L and mRuby2 PP2A-Aα plasmids. 72 hours post transfections cells were lysed in NP40 buffer (50 mM Tris-HCl, pH 8.0, 150 nM NaCl, 1% NP40, protease inhibitor cocktail) containing 2 mM GTP-γ-S for 15 minutes on ice and lysates were cleared by centrifugation. The supernatants were incubated with anti-Flag beads in NP40 lysis buffer supplemented with 10 mM GTP for 30-60 minutes rotating at 4°C. Beads were washed in ice-cold PBS containing 10Mm GTP. Proteins were eluted in Laemmli buffer and processed for western blotting (Maiuri et al., 2013).

Confocal microscopy and live cell imaging

Confocal imaging (described in Chapter 3) and activation of photoactivatable mCherry – PP2A-Aα was done with a Zeiss LSM 710 Meta Imaging system in the VCU microscopy Facility. A 35 mW blue diode 405 nm laser was used at fairly high output (25–50% transmission) to target small regions in the nucleus using the photobleaching function of the Zeiss software in time-series mode. Around 180 pulses of the 405 nm laser were required to activate PAmCherry PP2A-Aα for fluorescence emission that was detected by excitation at 561nm. The cells were followed for 10 minutes and images and ROI intensities were acquired every 5 seconds.

Results

Phosphomimetic mutant, S4101D of PP2A-Aα is localized to the cytoplasm in MEFs whereas the S401A mutant is nuclear

Karl Herrup's group observed that, in neurons, endogenous PP2A-A was largely cytoplasmic as opposed to ATM-deficient neurons, where it was primarily nuclear. They also demonstrated that ATM-mediated phosphorylation is responsible for altered localization of PP2A-A (Li et al., 2012). According to their study in neurons the S401A mutant of PP2A-A localized in the nucleus and the S401D mutant is localized in the cytoplasm. Since, we are also studying the ATM regulation on S401 site of PP2A-Aα; we looked at the localization of these mutants in our system, i.e., PP2A-Aα KO MEFs expressing the WT, S401A and S401D mutant forms. The Flag-WT PP2A-Aα was present in the cytoplasmic as well as the nuclear compartments. The S401A isoform was predominantly nuclear whereas the S401D isoform was predominantly cytoplasmic (**Fig. 4-1**). Through this study we were able to recapitulate the observations made by Herrup's group in neurons in our genetic system.

PP2A-Aα interacts with CRM1

Observation from our previous study shows that ATM phsphorylation on S401 PP2A-Aα causes it to translocate to the cytoplasm (Li et al., 2012) (**Fig. 4-1**). To charaterize the

89
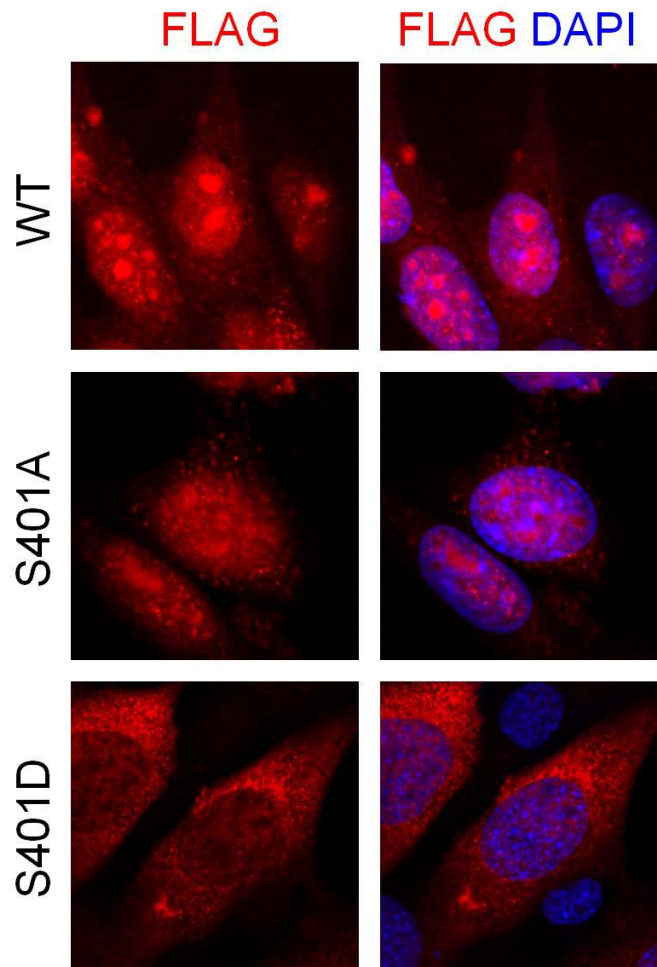
Figure 4-1: Cellular localization of PP2A-Aα

PP2A-Aα KO MEFs (WT, S401A and S401D) were immunolabeled with Flag antibody (Red) and counterstained with DAPI (Blue) to label nuclei, Images were acquired at 63x power.

mechanism of this nuclear export, we assesesd the protein sequence of PP2A-Aα. We

plugged the PP2A-Aα sequence in the NetNES 1.1 server, which predicts leucine-rich

nuclear export signals in eukaryotic proteins (Cour et al., 2004). We got a hit for PP2A

with a predictive sequence LLPLFL$_{370}$AQL$_{373}$ (**Fig. 4-2A**). The nuclear export signal

(NES) consensus sequence is LxxLxL (Cour et al., 2004). The export of proteins

harboring a functional NES sequence is facilitated by CRM1 (Fornerod et al., 1997;

Fukuda et al., 1997; Haasen et al., 1999; Ossareh-Nazari et al., 2001). We therefore

looked at the interaction of CRM1 with PP2A-Aα. We performed co-

immunoprecipitation in HEK293 cells expressing the Flag-CRM1, mRuby2-PP2A-Aα

and untagged RanQ69L. The CRM1 mediated export occurs across a Ran GTP gradient,

which is disrupted on cell lysis (Güttler and Görlich, 2011). Hence, we used the

RanQ69L mutant that is incapable to exchange GTP for GDP (Klebe et al., 1995), which

prevents the dissociation of the Ran-CRM1 complex in whole cell extracts. The

immunoprecipitation experiment showed that CRM1 interacted with endogenous PP2A-

A. Also, the predictive NES was in proximity to the S401 site of PP2A-Aα (**Fig. 4-2A**).

This leads us to hypothesize that binding of CRM1 to the PP2A-Aα NES could be

essentially influenced by the phosphorylation state at S401.

PP2A-Aα export to cytoplasm post IR is blocked by Leptomycin B

Leptomycin B is a metabolite from *Streptomyces* which inhibits the CRM1 nuclear export

A

NES : LxxxLxxLxL


```
  1  MAAADGDDSL YPIAVLIDEL RNEDVQLRLN SIKKLSTIAL ALGVERTRSE
 51  LLPFLTDTIY DEDEVLLALA EQLGTFTTLV GGPEYVHCLL PPLESLATVE
101  ETVVRDKAVE SLRAISHEHS PSDLEAHFVP LVKRLAGGDW FTSRTSACGL
151  FSVCYPRVSS AVKAELRQYF RNLCSDDTPM VRRAAASKLG EFAKVLELDN
201  VKSEIIPMFS NLASDEQDSV RLLAVEACVN IAQLLPQEDL EALVMPTLRQ
251  AAEDKSWRVR YMVADKFTELQKAVGPEITKTDLVPAFQNL MKDCEAEVRA
301  AASHKVKEFC ENLSADCREN VIMTQILPCI KELVSDANQH VKSALASVIM
351  GLSPILGKDNTIEHLLPLFLAQLKDECPEV RLNIISNLDC VNEVIGIRQL
401  SQSLLPAIVE LAEDAKWRVR LAIIEYMPLL AGQLGVEFFD EKLNSLCMAW
451  LVDHVYAIRE AATSNLKKLV EKFGKEWAHA TIIPKVLAMS GDPNYLHRMT
501  TLFCINVLSE VCGQDITTKH MLPTVLRMAG DPVANVRFNV AKSLQKIGPI
551  LDNSTLQSEV KPILEKLTQD QDVDVKYFAQ EALTVLSLA
```

B
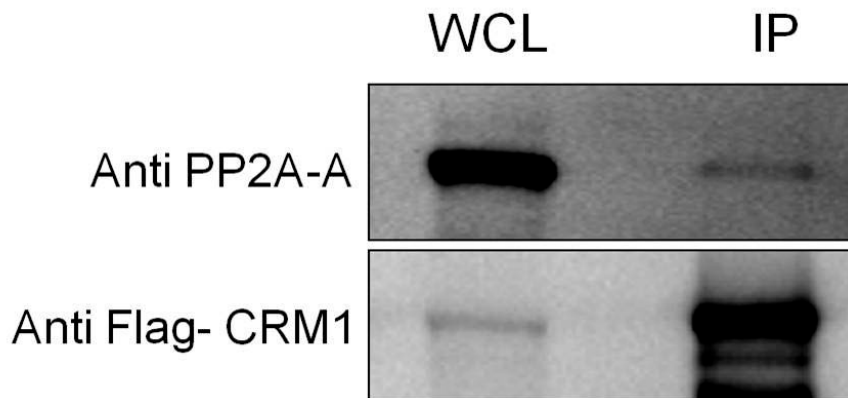
Figure 4-2: PP2A-Aα binds to CRM1

(A) Protein sequence of PP2A-Aα with the putative NES sequence highlighted in red. **(B)** HEK 293 cells were transfected with Flag-CRM1, mRuby2-PP2A-Aα and untagged RanQ69L. 72 hours post transfection, the cell extract was co-immunoprecipitated with Flag-CRM1 and examined for PP2A-A by western blot analysis.
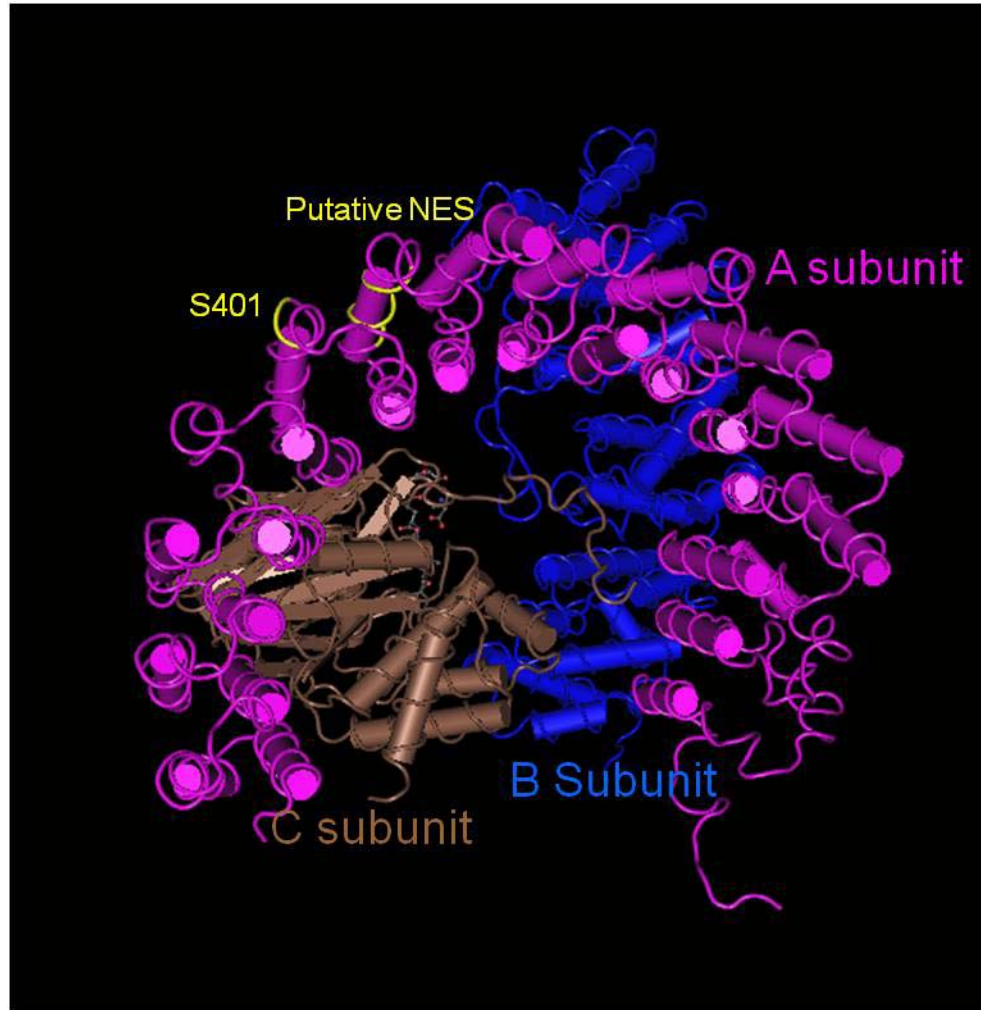
Figure 4-3: Structure of PP2A with the Aα S401 and NES sites

Crystal structure of PP2A holoenzyme (PDB: 2IAE_A), highlighting the different subunits, the S401 site and the putative NES sequence.

receptor and inhibits CRM1-dependent nuclear export (Kudo et al., 1998; Neville and

Rosbash, 1999). The presence of predictive NES in PP2A-Aα and its interaction with

CRM1 indicated that CRM1 could mediate PP2A-Aα nuclear export. To validate this

further, we treated PP2A-Aα KO MEFs expressing the WT form with Leptomycin B for

30 and 60 minutes. We observed that Flag- PP2A-Aα-WT started accumulating in the

nucleus post leptomycin B treatment (**Fig. 4-4A**). We also looked at the localization of

PP2A-Aα-WT post irradiation in presence and absence of leptomycin B. On exposure to

irradiation, Flag-PP2A-Aα was chiefly cytoplasmic after 60 minutes. When the cells were

treated with leptomycin B prior to irradiation, this translocation was inhibited. We saw

that treatment with leptomycin B prevented IR-induced translocation of PP2A-Aα to the

cytoplasm (**Fig. 4-4A**). These results suggest that, DNA damage caused by ionizing

radiation promotes the translocation of PP2A-Aα to the cytoplasm via CRM1.

Monitoring translocation of PP2A-Aα using photoactivatable fluorescent proteins

Our data so far suggests that DNA damage triggers trranslocation of PP2A-Aα to the

cytoplasm possibly after ATM-mediated phosphorylation at S401. To visualize the

translocation of PP2A-Aα in real time, we explored the photo activation system, which

has been extensively used to follow intracellular proteins over a period of time. We used

the PAmCherry vector. PAmCherry is originally non-fluorescent but when exposed to

350-400 nm light, emits red fluorescence (Subach et al., 2009) (**Fig. 4-5A**). We cloned
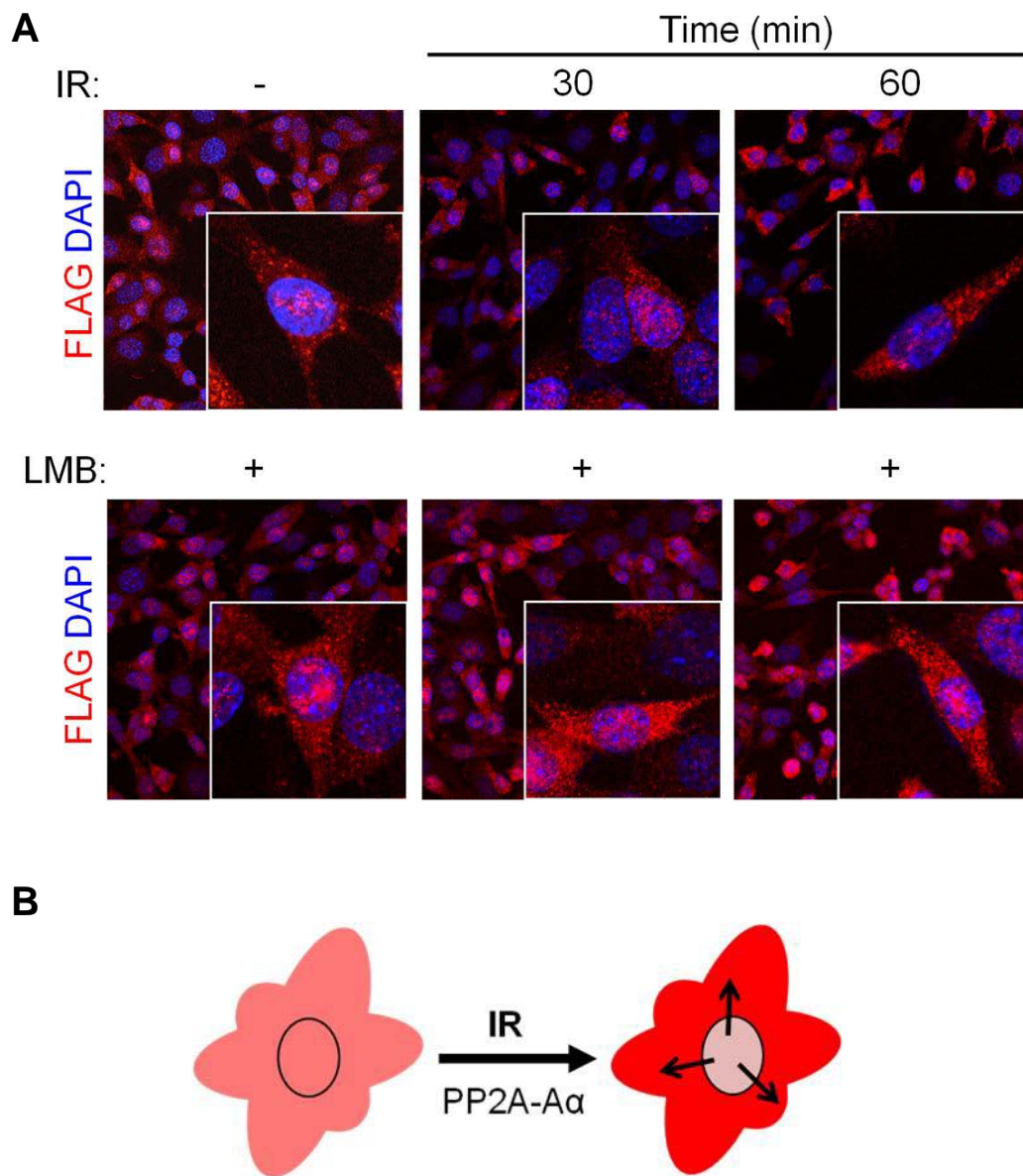
Figure 4-4: PP2A-Aα is exported to the cytoplasm post IR

(**A**) PP2A-Aα KO MEFs (WT, S401A and S401D) were treated with Leptomycin B (2ng/ml) alone for total of 6 hours. For irradiation treatment, cells were exposed to 10Gy and fixed after 30and 60minutes, The cells were immunolabeled with Flag antibody (Red) and counterstained with DAPI (Blue) to label nuclei, Images were acquired at 63x power. (**B**) Schematic representation of PP2A-Aα export post irradiation.

PP2A-Aα in the PAmCherry vector and transfected PAmcherry-PP2A-Aα in HEK293 cells. 48 hours post transfection the cells were set incubated stage at 37°C and 5% CO_2. A region in the nucleus was activated using the 405 nm laser line and fluorescence was detected at 561 nm. We also performed this experiment on cells pretreated with a DNA intercalating agent, Hoechst dye (Kong et al., 2009) which sensitizes cells to DNA damage. In our preliminary experiments we observed that PAmCherry-PP2A-Aα is activated in the nucleus. In the Hoechst treated cells there seems to be translocation of PP2A-Aα to the cytoplasm faster than the untreated (**Fig. 4-5B**). However, more experimental repeats are required to make any final remarks. We confirmed by γ-H2AX staining that Hoechst pretreatment caused DNA damage (**Fig. 4-6**).
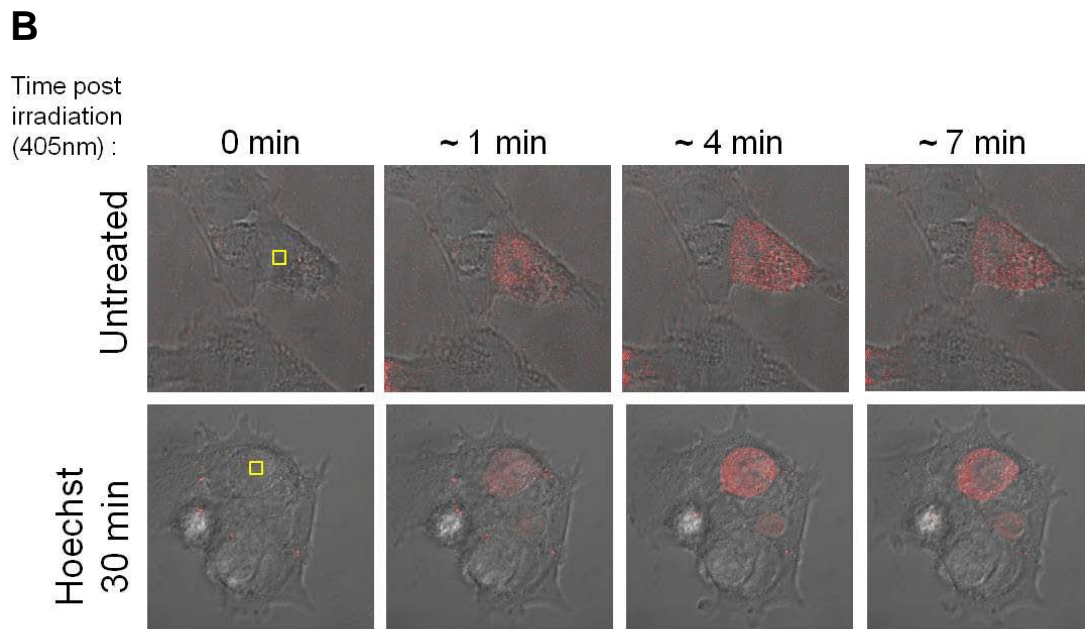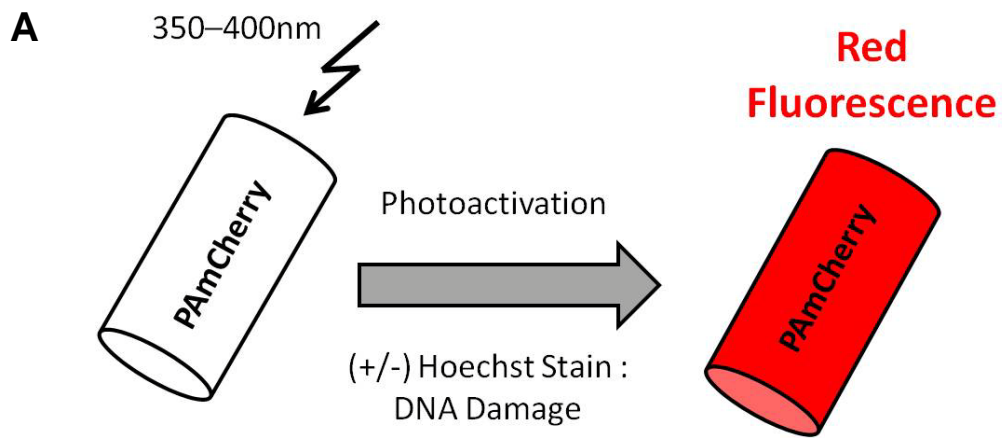
Figure 4-5: Photo activation of PAmCherry-PP2A-Aα

(**A**) Schematic representation for mechanism of photoactivation of PAmcherry-PP2A-Aα. (**B**) HEK 293 cells were transfected with PAmCherry-PP2A-Aα. 48 hours later PAmcherry-PP2A-Aα was activated by 405nm laser in the nuclear region (yellow boxes) and the red fluorescent PP2A-Aα was monitored over a time period.
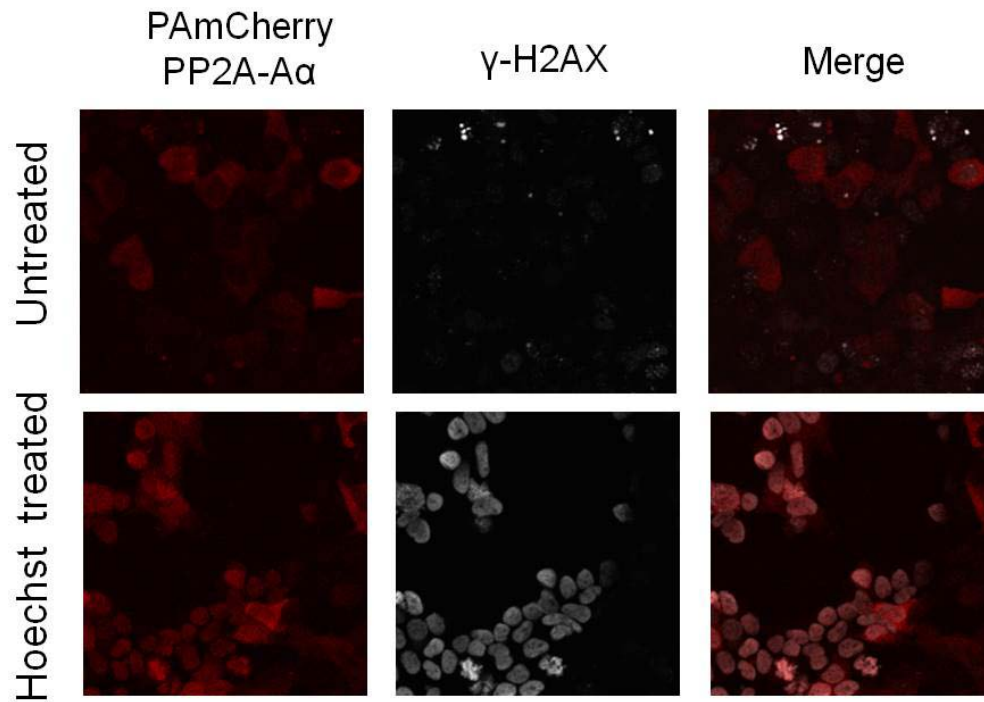
Figure 4-6: Hoechst treatment causes DNA damage

HEK 293 cells were transfected with PAmCherry-PP2A-Aα. 48 hours later PAmcherry-PP2A-Aα was activated by UV light and fixed after 30 minutes. The cells were imunolabeled with γ-H2AX antibody.

Chapter summary

Li et al looked at the localization of PP2A-Aα WT, S401A and the S401D mutant in neurons. According to their study, WT PP2A-Aα is nuclear and cytoplasmic and S401A was nuclear, whereas S401D was cytoplasmic (Li et al., 2012). In line with their observations, our genetic model system behaved similarly: the WT PP2A-Aα is nuclear and cytoplasmic; S401A was nuclear, whereas S401D was cytoplasmic in the PP2A-Aα KO MEFs.

At this point we know that B56α, B56ε and the Cα subunits harbor the NES. However, at this stage we are not aware which B subunit is associated with the PP2A holoenzyme, and acting downstream to ATM. Since the molecular modeling studies (**Chapter 2. data not shown**) suggest that the S401 phosphorylation causes distortion in the horseshoe shape of PP2A-Aα, we checked if PP2A-Aα has a NES. The NetNES 1.1 Server predicted the $LLPLFL_{370}AQL_{373}$ as a putative NES sequence (Cour et al., 2004). Classically, the NES is comprised of short peptides comprising four spaced hydrophobic residues (denoted $\Phi^1–\Phi^4$) and to follow the consensus Φ^1-(x)2–3-Φ^2-(x)2–3-Φ^3-x-Φ^4, where x is an charged or a polar amino acid (Kutay and Güttinger, 2005). Although the PP2A-Aα NES does not directly fit the classical NES consensus, Güttler et al. demonstrated that CRM1 can recognize diverse NESs that differ from the consensus (Güttler et al., 2010). Although the NES is referred to as leucine-rich, several proteomic screens have revealed that the leucine can be replaced by isoleucine, valine, methionine

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or phenylalanine at hydrophobic positions (Kosugi et al., 2008; Yi et al., 2002; Zhang and Dayton, 1998). Also, when we looked at the position of the putative NES relative to the S401 residue on PP2A-Aα, they looked proximal. Based on this we speculated that phosphorylation on S401 could affect the NES affinity to CRM1. We plan to mutate the Leucine$_{373}$ of this putative NES to Alanine and verify the functionality of this region with respect to nuclear export.

Our immunoprecipitation data shows that PP2A-A and CRM1 interact. Dr. Mostafa Ahmed, who helped us with modeling studies in Chapter 2, also helped us to study PP2A-Aα binding with CRM1 *in silico*. The modeling studies (**Data not shown**) revealed that the WT, S401A and the S401D have different affinities to CRM1. The HINT (Hydropathic INTeractions) score (Kellogg et al., 1991) which relates to the affinity between two proteins, was highest for the S401D mutant followed by the WT and the S401A. This was a preliminary run, and indicated that the S401D, phosphomimetic PP2A-Aα had a higher binding affinity followed by the WT and S401A mutant. High-resolution and more extensive modeling studies as well as in vivo binding studies need to be pursued to establish a relationship between CRM1 and the PP2A-Aα (WT, S401A and S401D) forms. More importantly, we wish to understand how this binding is affected in response to DNA damage.

We further looked at effect of LMB in the WT PP2A-Aα KO MEFs in the presence and absence of DNA damage. Our studies show that on treatment with Leptomycin B alone,

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the PP2A-Aα seemed to accumulate in the nucleus. With IR alone, the PP2A-Aα was cytoplasmic, whereas when the cells were treated with Leptomycin B prior to IR treatment, we could still see the PP2A-Aα accumulating in the nucleus. This observation aligned with our hypothesis that post DNA damage, PP2A-Aα is exported to the cytoplasm via CRM1.

Furthermore, to have a visual handle on the nuclear export of PP2A-Aα, we used a photoactivatable PP2A-Aα (PAmCherry- PP2A-Aα). We activated the PP2A-Aα in presence and absence of DNA damage (here pre-sensitization with Hoechst dye). In our preliminary experiment, we successfully activated the PAmCherry-PP2A-Aα. From our study, it looks like in cells pretreated with Hoechst, the exclusion of PAmCherry-PP2A-Aα is faster and it seems to accumulate in the periphery of the nucleus. We are pursuing more studies to look at dose response and nuclear export kinetics using this tool, which will give us a better picture real time.

The findings from this part of the studies are very exciting. The subcellular localization of the PP2A-Aα provides explanation for the extreme phenotypes we have seen in the S401 mutants. Therefore, based on studies from this chapter we speculate that post DNA damage, ATM kinase mediated phosphorylation on S401 of PP2A-Aα possible causing a conformational change in its structure, providing access to CRM1 binding. Subsequently CRM1 is exported from the nucleus (**Fig. 4-6**).
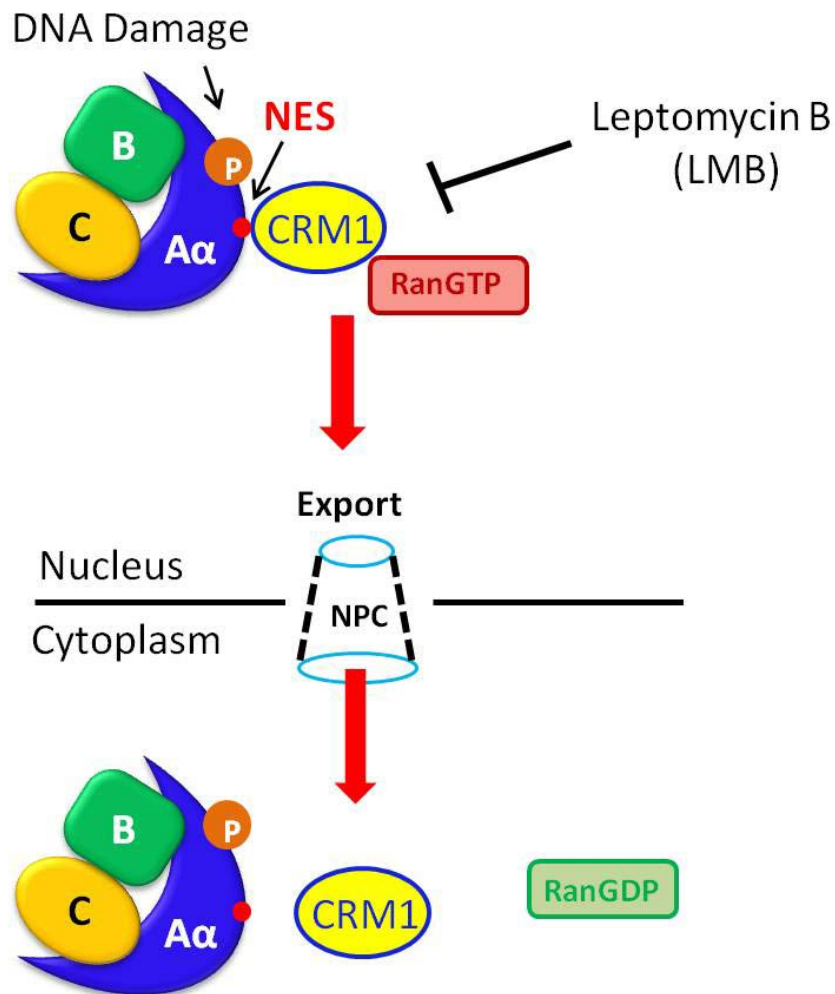
Figure 4-6: Model for CRM1 mediated PP2A-Aα nuclear export

On DNA damage, ATM phosphorylates PP2A- Aα. This increases PP2A-Aα-CRM1 binding and PP2A-Aα is exported to the cytoplasm. Inhibition on CRM1 with Leptomycin B will block this export.

CHAPTER 5:

Generation of mouse model to conditionally knock out PP2A-Aα in the brain

Introduction

Subcellular compartmentalization of PP2A is governed by the composition of the holoenzyme. Association with different subunits direct it to different locations and targets in the cells (Hiraga and Tamura, 2000; McCright et al., 1996; Sontag et al., 1995). Our studies show that ATM, the major regulator of DDR, phosphorylates PP2A-Aα and subsequently causes its translocation to the cytoplasm. One of the characteristics of Ataxia-Telangiectasia (A-T) is neurodegeneration. ATM deficiency, leading to upregulation of PP2A activity, caused nuclear accumulation of HDAC4 (Li et al., 2012). This cytoplasmic loss of HDAC4 is partially responsible for neurodegeneration in patients with A-T (Li and Jiang, 2015).

Strack et al. were the first to look at the subcellular localization of the different PP2A subunits in rat brain (Strack et al., 1998). They observed that certain B subunits are present in distinct neural populations and also they differ in their expression during brain development (Strack et al., 1998). There is a possibility that ATM-mediated regulation on PP2A-Aα can affect the formation of holoenzyme and alter its activity. PP2A-Aα is indispensible in adult mice (Ruediger et al., 2011). Ruediger et al. generated a mouse in which one PP2A-Aα was floxed and looked at the PP2A-Aα protein levels in different organs. PP2A-Aα levels in most of the organs had 50% PP2A-Aα protein compared to the WT mouse except in the cerebellum, cortex and the brain stem where it was around

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80% (Ruediger et al., 2011). This study showed that in spite of presence of only one allele, the mouse brain acquired a way of maintaining higher levels of PP2A-Aα.

We therefore set forth to investigate further the role PP2A-Aα in the brain. To study this we are generating a mouse model in which PP2A-Aα is specifically knocked out in the brain. It has been long known that the class IV intermediate filament nestin is a neuronal stem cell marker (Cattaneo and McKay, 1990; Johansson et al., 1999; Lendahl et al., 1990). In this study, we are working on generating two lines of nestin-Cre mice. Our goal is to knock out PP2A-Aα in the central and peripheral nervous system as well as the neuronal precursors and glial cell precursors. We plan to express floxable alleles of PP2A-Aα (loxp flanking exon 5 and 6) (Ruediger et al., 2011) with Cre-recombinase under the control of Nestin promoter. For deletion of DNA segment of a gene, a gene is targeted with two lox sites flanking the DNA segment to be deleted. The lox sites are 34 bp long and, in presence of Cre recombinase, recombination takes place and the region between the loxP sites is excised (Sauer, 1987).

Another line of nestin-Cre mice, which express Cre recombinase post tamoxifen administration, will help us to study the effect of PP2A-Aα loss in adult mice. These mice will express the transgene (CreER[T2]) triple mutant (G400V/M543A/L544A) of the estrogen receptor ligand-binding domain (Feil et al., 1997). Tamoxifen binds to the ER[T2] treatment causes translocation of CreER[T2] to the nucleus where Cre will cause the recombination between two lox sites and flox out the exons 5 and 6 of PP2A- Aα

(Ruediger et al., 2011), thereby preventing the gene expression. Here, the CreER^{T2} will be fused to the nestin promoter.

Apart from A-T, PP2A is also involved in Alzheimer's disease (AD). Increased tau phosphorylation is a hallmark of AD. Dysregulation of PP2A has been implicated in increased tau phosphorylation (Sontag et al., 2004). Given the importance of PP2A-Aα in brain and various neuropathologies, it is important to study its role. Not much is known about the ATM/PP2A regulation in the neurons post DDR. Our model will allow us to look at the ATM mediated PP2A-Aα regulation in brain and its effect on neuronal signaling.

Material and methods

Mouse information used to generate mice lines with brain specific KO of PP2A-Aα

Fvb.129s-*PPP2R1ATM^{1wltr}/*j mice were purchased from Jackson Laboratory as stock number 017441. These mutant mice possess *loxp* sites flanking exons 5-6 of the Aα gene (*PPPP2R1A*) of PP2A (Ruediger et al., 2011). We also purchased B6.Cg-Tg (Nestin-Cre)1Kln/J from Jackson Laboratory as stock number 003771. The Nestin-Cre transgenic mice express Cre recombinase in central and peripheral nervous system, including normal and glial cell precursors (Tronche et al., 1999). The Nestin –CreER^{T2} mice were a generous gift from R. Hen (Dranovsky et al., 2011). All animal breeding and experiments have been approved by Virginia Commonwealth University, IACUC.

Verification of nestin-GFP mice

One-day-old pups were imaged with IVIS imaging system (PERKin Elmer). The GFP positive pups were then kept in separate cages.

Genotyping

Mice were ear punched about a week post weaning. DNA from the ear punches was isolated using a High pure PCR template preparation kit (Roche Diagnostics Corporation). The PP2A-Aα and Cre alleles were verified using PCR (**described in Chapter 1.**).

Results

Generation of nestin-Cre – PP2A-Aα KO mice

Brain specific knock out of PP2A-Aα will help us understand the role of this protein in a number of neurological processes. To generate mice with PP2A-Aα in the brain, we employed the Cre-lox system. The targeting strategy is outlined in **Fig. 5-1**. To generate these mice we bred *PPP2R1ATM*^{1wltr}/^{tm1wltr} (see **Chapter 2**.) with B6.Cg-Tg(Nes-cre)1Kln/J (**Fig. 5-2A**). The F1 litter was a mix of different genotypes. We screened for mice that were positive for nestin-Cre nestin-GFP and *PPP2R1ATM*^{1wltr}/ j. This was verified by genotyping (**Fig. 5-2B**). Currently, we are in the process of breeding the F1 littermates. The F2 litters, which are fit for our experiments, will be the one which are homozygous for PP2A-Aα CKO alleles (*PPP2R1ATM*^{1wltr}/^{tm1wltr}) and have the nestin-Cre and nestin-GFP alleles. The F2 mice, which have the homozygous for PP2A-Aα CKO alleles but lack the nestin-Cre, will serve as control (**Fig. 5-2B**). Studies have shown that PP2A-Aα is indispensible for the whole mice. Also, in the presence of only one copy of PPP2R1A, the expression of Aα is more than 50% in the brain (Ruediger et al., 2011). Therefore, we are not very optimistic that our F2 litter will survive. However, if they do we will have a better insight on the effect of PP2A-Aα loss in the mouse brain at the time of development. By looking at markers of neuronal integrity we can understand if and how loss of PP2A-Aα affects neurodegeneration. If we don't obtain a F2 litter, we can
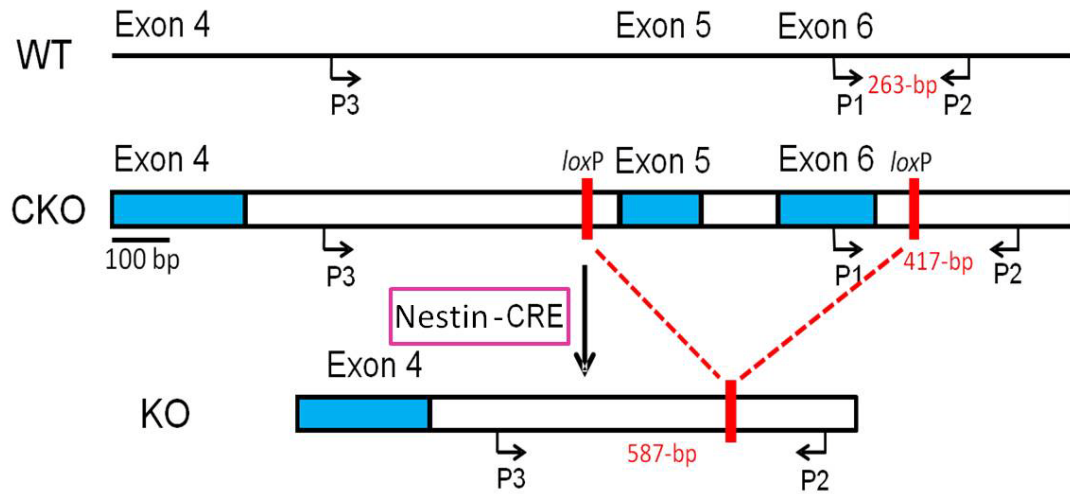
Figure 5-1: Strategy for knocking out PP2A-Aα in mouse brain using nestin-Cre recombinase

Representation of the WT, CKO and KO alleles of PP2A-Aα before and after Nestin-Cre-mediated knock out.
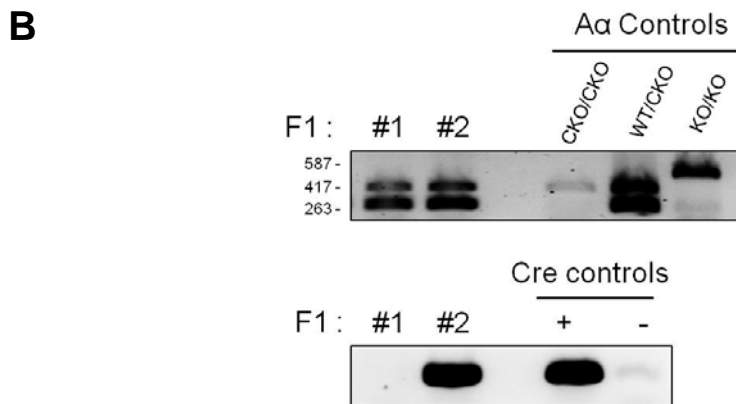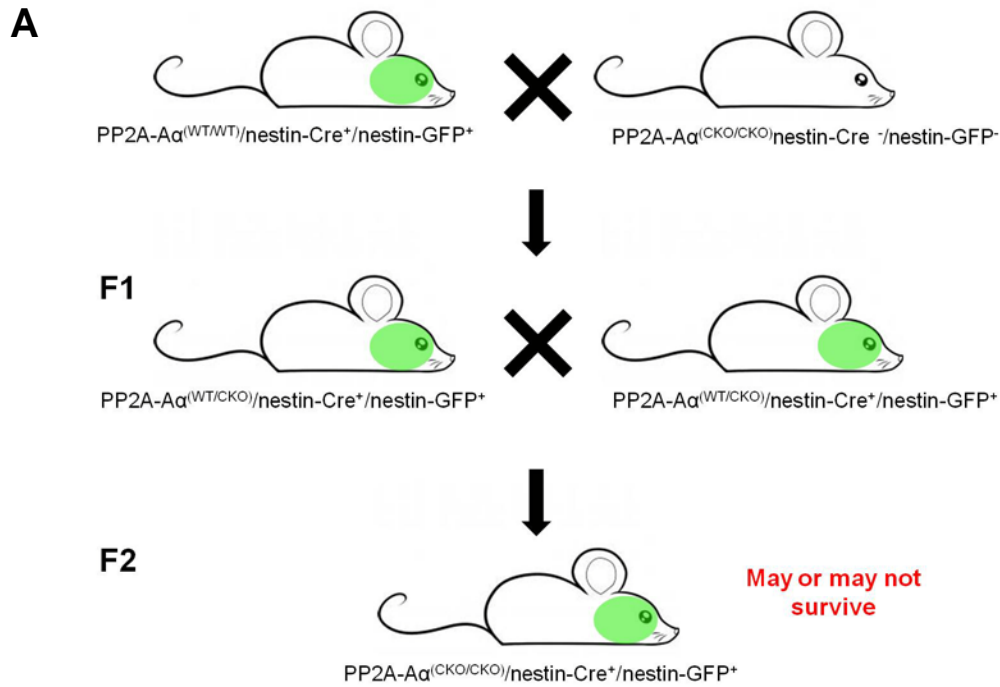
Figure 5-2: Mouse breeding scheme for generating Nestin–Cre/PP2A-Aα mice and verification by genotyping

(**A**) Strategy for nestin-Cre mediated knock out of PP2A-Aα in mouse brain. (**B**) Representative genotyping data. The PP2A-Aα primers generated a 263-bp product for WT allele and a 417-bp product for the CKO allele for the two heterozygous mice. The Cre primers verified that one mouse expressed a Cre-Recombinase and the other did not. study the heterozygous animals and monitor for any phenotypic changes in them over time.

Generation of Tamoxifen-inducible nestin-CreER – PP2A-Aα CKO mice

Since there is a possibility that nestin-Cre /PP2A-Aα (CKO/CKO) mice may not survive, we decided to generate another mouse line in parallel. We procured the tamoxifen-inducible nestin-CreER^{T2} mouse line from Dr. R Hen. These mice have Cre recombinase fused to triple mutant (G400V/M543A/L544A) of the estrogen receptor ligand-binding domain (Feil et al., 1997). Post tamoxifen administration, tamoxifen will bind to the ER^{T2} fused to Cre, and cause translocation of CreER^{T2} to the nucleus where it will mediate inactivation of the gene. The CreER^{T2} fusion is expressed under nestin promoter (Dranovsky et al., 2011). These nestin-CreER^{T2} mice were bred with F2 mice from the previously mentioned litter (nestin-CRE **X** 129S-*PPP2R1ATM^{1wltr}/^{tm1wltr}*), which were homozygous for PPP2R1A CKO alleles, were nestin-GFP but negative for nestin-Cre (**Fig. 5-4A**). The F1 litter was a mix of different genotypes. We screened for mice, which were positive for nestin- CreER^{T2}, nestin-GFP and *PPP2R1ATM^{1wltr}/*j. This was verified by genotyping (**Fig. 5-4B**). At present, we are breeding the F1 littermates. The F2 generation mice, suitable for our experiments, are the ones, that are homozygous PP2A-Aα CKO alleles (*PPP2R1ATM^{1wltr}/^{tm1wltr}*), nestin-GFP positive and have the nestin-CreER^{T2} allele. The F2 mice, which have the homozygous for PP2A-Aα CKO alleles, but lack the nestin-CreER^{T2} allele, will serve as controls. Once the animals are 8-10 weeks.
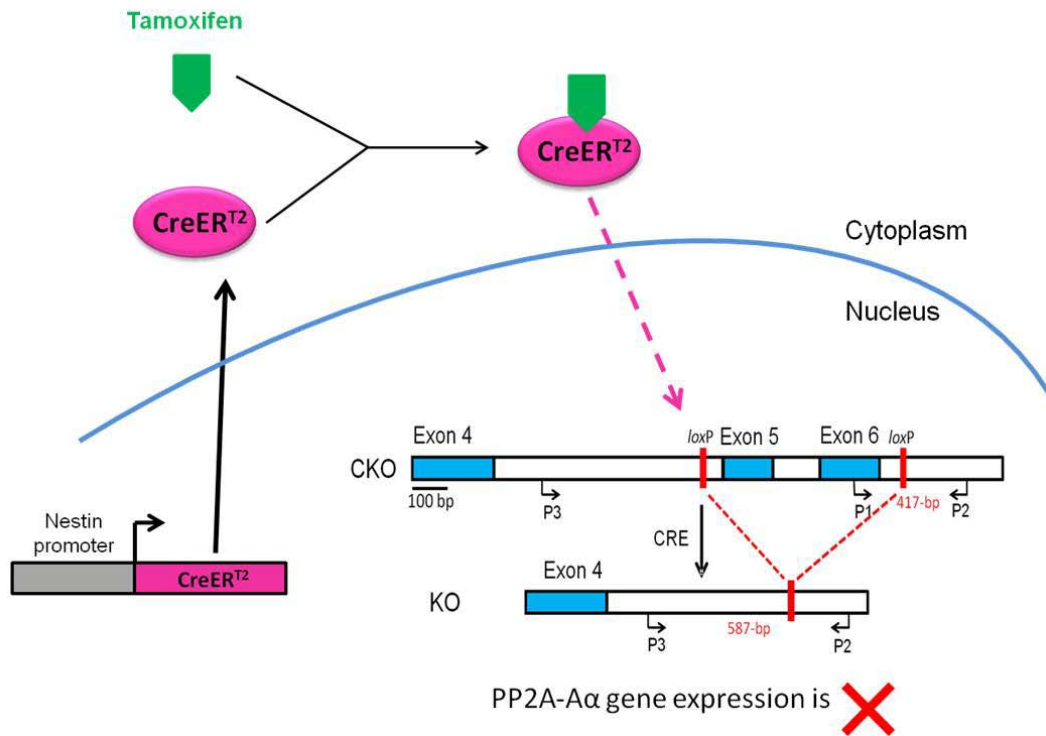
Figure 5-3: Schematic representation of tamoxifen inducible Nestin-CreER^{T2} system

CreER^{T2} is expressed under nestin promoter. Post tamoxifen administration, tamoxifen will bind to the ER^{T2} fused to the Cre, and cause the translocation of CreER^{T2} to the nucleus where it will inactivate the PP2A-A via Cre-mediated recombination at loxP.
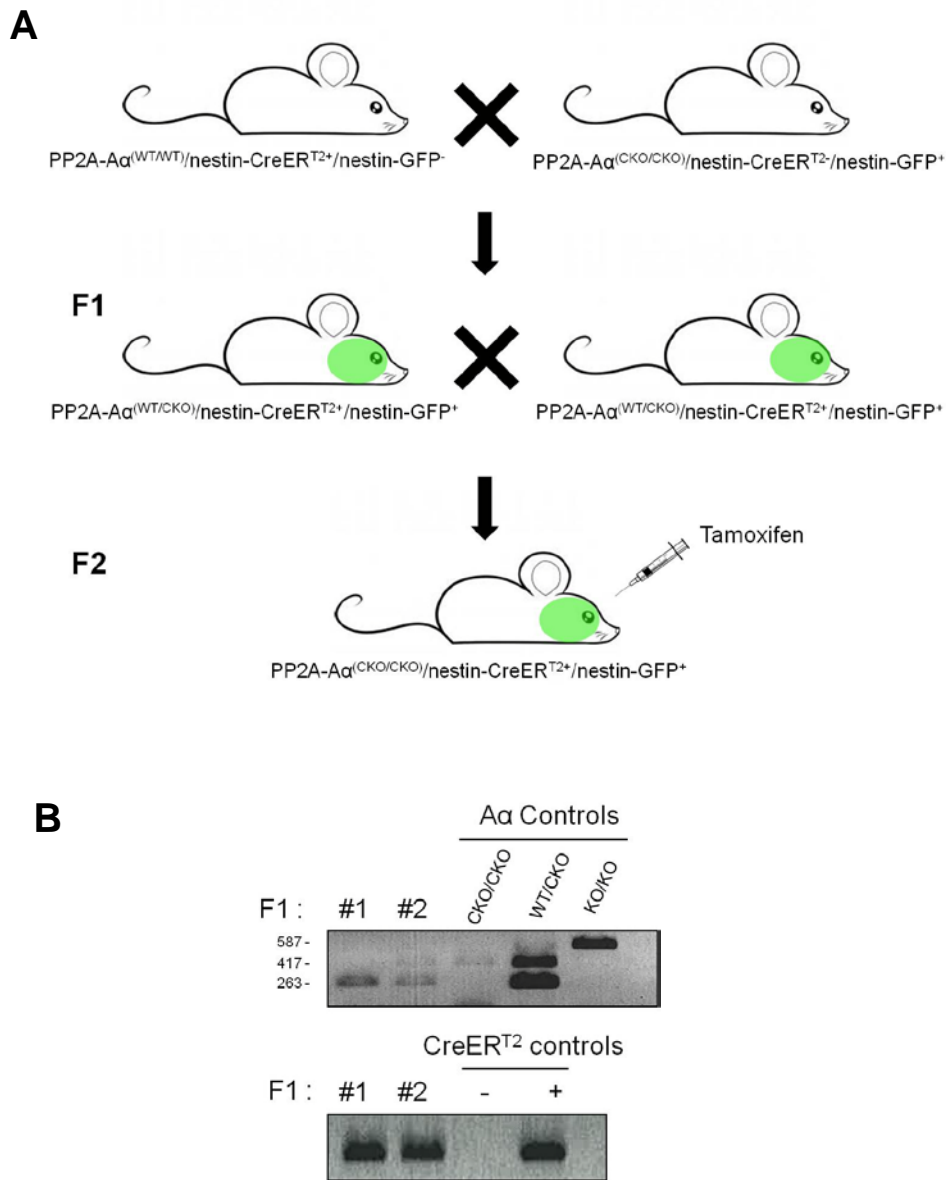
Figure 5-4: Mouse breeding scheme for generating tamoxifen inducibe Nestin – Cre/PP2A-Aα mice

(**A**) Strategy for tamoxifen inducible nestin- CreER^{T2} mediated knock out of PP2A-Aα in mouse brain. (**B**) Representative genotyping data. The PP2A-Aα primers generated a 263-bp product for WT allele and a 417-bp product for the CKO allele for the two heterozygous mice. The Cre primers verified that both the mouse expressed Cre-Recombinase.

old, tamoxifen will be administered to them once a day for 5 days. We will then take the

brains out; isolate DNA for genotyping as well as look at PP2A-Aα protein levels to

verify brain specific knock out

Chapter summary

We have reviewed in the previous chapter that PP2A holoenzyme is involved in regulation of complex signaling networks. The aim of this this study is to understand the role of PP2A-Aα in neuronal signaling in DDR.

PP2A has been implicated in AD. In brains of patients suffering from AD, the levels of PP2A-A, C and Bα subunits were substantially reduced, subsequently reducing the phosphatase activity in the brain and causing severe phosphorylation of tau (Sontag et al., 2004). Furthermore, increased T307 phosphorylation of the PP2A-C-subunit co-related with the rich phospho-tau neurons in the AD post moterm brains (Liu et al., 2008). There exits an overwhelming amount of evidence that associates dysregulated PP2A with the phospho-tau pathology in AD. Currently work is being done towards targeted therapy against PP2A to lower the phospho-tau pathology (reviewed in Sontag and Sontag, 2014).

The role of PP2A in other neurological disorders is still emerging. In a large-scale study of undiagnosed developmental disorders in 1133 children and their parents, 4 *de novo* PPP2R5D (B56δ) and 3 *de novo* PPP2R1A (Aα) mutations were identified (The Deciphering Developmental Disorders Study, 2015). In a follow up study (Houge et al., 2015), *de novo* mutations in the B56δ and Aα resulted in dysregulation of PP2A activity and was one of the causes for intellectual instability in the patients assessed. Functional studies revealed that cases with B56δ were impaired in binding the A and C subunit

whereas in cases with Aα mutations, subunit A bound the B56δ but not the C-subunit affecting the total phosphatase activity (Houge et al., 2015).

These studies indicate that mutations in the Aα or other regulatory subunits can affect the integrity of the enzyme, resulting in deleterious effects. Our mouse model system will help us study the role of PP2A-Aα in the brain. We have so far successfully generated Nestin-Cre as well as the nestin-CreER^{T2} F1 lines. We are currently breeding the F1 mice to generate the F2 mice with the required PP2A-Aα (CKO/CKO) genotype. Studies in these mice will enable us to study the brain specific role of PP2A-Aα in diverse processes.

CHAPTER 6:

Conclusions

Discussion

Understanding how the cell deals with genomic instability is of utmost importance. DSBs are the most lethal form of DNA damage, which can have deleterious consequences on the cell. The DDR elicited by the cell in response to genotoxic stress determines if the damage needs to be repaired or if the cell should proceed via the cell death pathway. Uncovering specific players and the mechanism by which they are regulated can open new avenues in therapeutics. The DDR is a coordinated response between phosphatases and kinases, which aids in determining the cell's fate. Phosphorylation and dephosphorylation acts as an ON/OFF switch for signaling proteins and directs them towards or away from their target.

In the previous chapters we have reviewed the extensive role of PP2A in diverse cellular functions. It has a crucial part in the specific cell cycle checkpoints activated by DDR as well as activation and inhibition of key players involved in DNA repair. ATM, the master regulator of DDR, is maintained in its native inactivated state by PP2A-mediated S1981 dephosphorylation. The PP2A-A and ATM interaction is disrupted post IR mediated DNA damage. Studies have shown that ATM can also exert control on PP2A by phosphorylating it on a S401 residue of the Aα subunit and inhibiting the holoenzyme's activity (Li et al., 2012). Furthermore, studies from our lab also indicate that ATM can regulate pro-survival molecules like AKT in response via a PP2A-like phosphatase (Golding et al., 2009). Also, viral antigens, like Polyomavirus middle t (PyMT) and SV40

small t-antigen, facilitate cell transformation by inhibiting PP2A activity. The antigens

bind to PP2A by replacing the B subunit (Mumby, 1995). Golding et al observed that

ATM inhibition in cells over-expressing PyMT had elevated p-AKT (S473) levels

(Golding et al, unpublished) suggesting a role of PP2A downstream of ATM. In this

study we have addressed how ATM-mediated phosphorylation on S401 of PP2A-Aα

modulated pro-survival signaling and DDR and also deciphered the mechanism

underlying the phenotype we observed.

We verified the ATM-mediated phosphorylation on PP2A-Aα by in vitro kinase assay.

Also, our in vitro studies show that the PP2A-Aα binds to the FAT kinase domain of

ATM. Using molecular modeling, Dr. Mostafa Ahmed (Kellogg lab), simulated

phosphorylation on the S401 of PP2A-Aα. These studies revealed that p-S401-PP2A-Aα

potentially undergoes a conformational change *in silico*. Immunoprecipitation in HEK293

cells expressing the WT, S401A and S401D PP2A-Aα form, reveal that the S401D

PP2A-Aα was unable to bind ATM, AKT as well as the catalytic subunit. This suggests

that phosphorylation at the S401 causes a structural change in the PP2A-Aα subunit,

which could result in the dissociation of the complex. Our observation of impaired

binding of the phosphomimetic S401D to the C-subunit could perhaps result in the loss of

its phosphatase activity.

Here, we have developed a genetic system to study how a posttranslational modification

on a single residue can affect the DDR signaling. Our initial experiments were aimed at

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generating PP2A-Aα knockout MEFs, which were complemented with the PP2A-WT, S401A or S401D forms. The cells were characterized for the knock out of the endogenous PP2A-Aα and expression of the WT and the S401A and S401D forms. We further examined these cells for any characteristic differences.

MEFs expressing the phosphomimetic S401D had increased growth rate and showed increased p-AKT (S473) levels post insulin treatment. When we assessed the basal p-AKT (S473) levels in the three PP2A-Aα KO MEFs, we saw no significant difference. However the p-ERK1/2 levels were elevated in the S401D-expressing MEFs. This suggests that ATM mediated phosphorylation on S401 of PP2A-Aα possibly upregulates pro-survival signaling via the MAP kinase pathway instead of PI3KK pathway. However, we need to assess other players like the upstream regulators in the MAP kinase pathway. PP2A can regulate MAP kinase pathway both positively and negatively; also, studies have shown that elevated p-ERK can negatively feed into PI3K pathway (Lehr et al., 2004; Sontag et al., 1993b; Westermarck et al., 1998).

PP2A regulates ATM and other DDR proteins involved in processes like cell cycle check points as well as key players that mediate DNA repair. Our studies show that DDR elicited by MEFs expressing S401A and S401D is highly misregulated. Both the mutant cells harbored aberrant chromosomes, the frequency of which increased post DNA damage. Perhaps, this accumulation of genomic instability caused increased radiosensitization in these cells, as seen in our clonogenic survival assay. The S401D

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cells displayed delayed γ-H2AX dephosphorylation kinetics implying unrepaired damage. The S401D-expressing cells were impaired in both HRR and c-NHEJ whereas the S401A cells were impaired in HRR but not in c-NHEJ where it could possibly function by upregulating the activity of DNA-PK.

The most exciting finding of our study was that the S401D mutant was localized in the cytoplasm whereas the S401A was more nuclear and the WT PP2A-Aα was both nuclear and cytoplasmic. We supposed that the phosphorylation on the S401 subunit causes its translocation to the cytoplasm. To validate this, we looked at nuclear export mechanisms. We found a nuclear export signal (NES) in the PP2A-Aα protein that is targeted by the CRM1 protein. Our immunoprecipitation studies show that CRM1 bound to PP2A-Aα *in vivo*. Also, on inhibiting CRM1 in the WT MEFs, the PP2A-Aα accumulated in the nucleus. DNA damage by irradiation in the WT cells caused the PP2A-Aα to translocate to the cytoplasm, which was prevented to some extent by CRM1 inhibition. The NES is located close to the S401 site on the HEAT repeats of PP2A-A; therefore, the phosphorylation at S401 could affect PP2A-Aα-CRM1 binding.

Many of our findings can be explained by the altered localization of these mutants in their phosphorylated and unphosphorylated form. The delayed γ-H2AX dephosphorylation kinetics in the S401D-expressing MEFs could be due to cytoplasmic sequestration of S401D PP2A-Aα. Since this cytoplasmic form of PP2A-Aα does not have access to its nuclear substrates, this can further affect the quality of repair and lead

into accumulation of genomic abnormalities. PP2A has vital functions in cell cycle checkpoints, especially mitotic entry and exit. Cytoplasmic sequestration (S401D) or nuclear retention (S401A) of PP2A-Aα can result in early or delayed mitotic entry or exit. PP2A also regulates the proteins like Sgo1 and Plk1, which are crucial for integrity of the kinetochores and in spindle assembly checkpoint. Dysregulation can result in deleterious effects like mitotic catastrophe.

Taken together our findings suggest that, in response DSBs induced by IR, ATM is activated and then phosphorylates PP2A-Aα on S401. This phosphorylation may result in a conformational change in the scaffolding subunit increasing its affinity to CRM1 and thereby causing its export to the cytoplasm. Meanwhile, ATM-mediated DDR is upregulated in the nucleus as seen by the increased γ-H2AX levels which then recruits DNA repair factor to initiate repair. Checkpoint activation follows as the cell assesses the damage in the nucleus, while in the cytoplasm. PP2A-Aα reassembles with C and B subunits and dephosphorylates pro-survival signaling proteins leading to growth arrest. During the damage recovery phase, PP2A-Aα is dephosphorylated in the cytoplasm and shuttles back to the nucleus where it can dephosphorylate γ-H2AX to ensure efficient DNA repair. A model is outlined in **Fig. 6**, depicting how we believe this occurs. Thus, S401 seems like a key residue which when phosphorylated, governs timely nuclear export as well as import of PP2A-Aα, which is essential for accomplishing a DDR.

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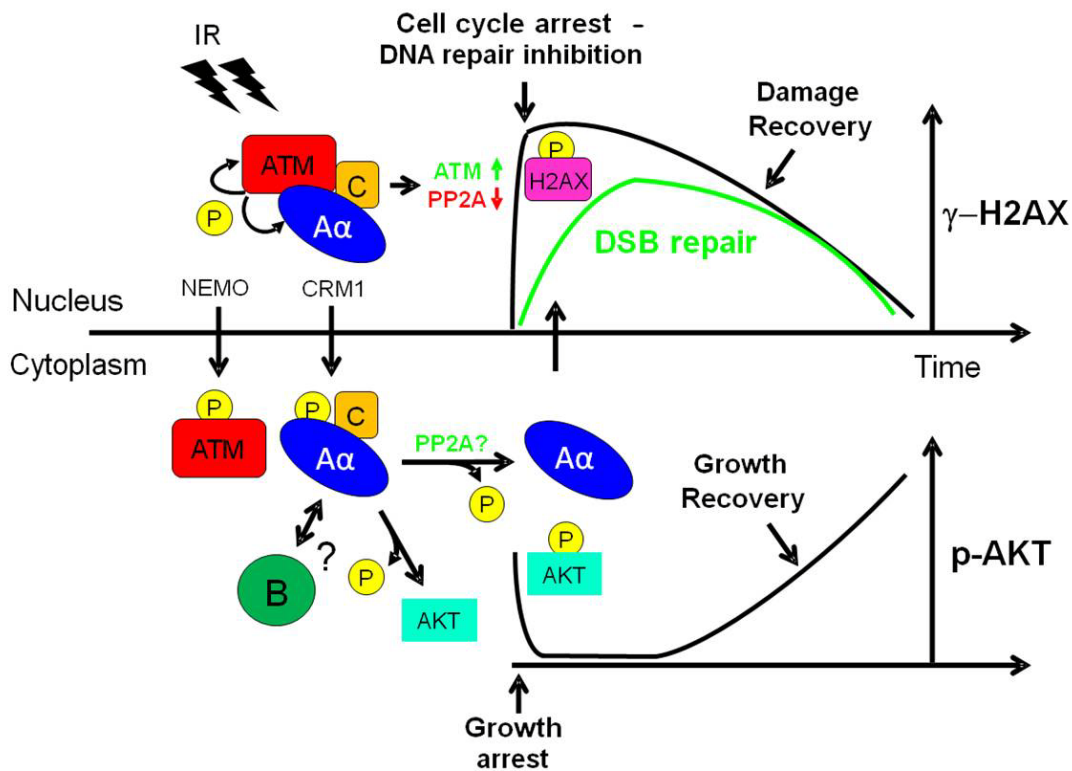
Figure 6: Model depicting how ATM mediated phosphorylation on PP2A-Aα regulates DDR and prosurvival signaling

ATM phosphorylates myriad of proteins in response to IR including H2AX at S139 and PP2A-Aα at S401 thereby initiating the DDR and triggering cell cycle checkpoints and growth arrest, and DSB repair inhibition. Soon after DDR is intiated, PP2A-Aα translocates to the cytoplasm which reduces PP2A activity in the nucleus allowing the DDR with its highly phosphorylated protein landscape to run its course. At the same time, AKT and other growth-promoting factors and signaling pathways are inhibited by the increased PP2A activity in the cytoplasm while the damage is assessed and repaired. Soon after the DDR is beginning to subside, PP2A-Aα shuttles back to the nucleus where it increases PP2A-Aα activity and begin to engage DSB repair while γ-H2AX and other DDR targets are dephosphorylated. When repair is completed and cells resume cycling, growth recovers.

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Future directions

We have unraveled a very specific mechanism by which DDR in part is orchestrated. However, there are many questions that still remain unanswered. As of now there are many ongoing experiments for this project that are not yet complete. The results from these studies will back up many of our studies as well as clarify some of the mechanisms that appear open-ended at this point.

We observed that both the S401A and S401D mutants were inefficient in HRR, whereas the S401D-expressing cell were also deficient in NHEJ. In spite of two major DSB repair pathways being impaired, the S401D cells continue to survive, concurrently accumulating aberrant chromosomes. We therefore think that a backup DSB repair pathway such as alt-EJ, which is highly error-prone, is mediating repair in these cells. We are currently in the process of verifying this by using a reporter alt-EJ cassette (Gunn and Stark, 2012).

Our studies show that the mitosis is severely dysregulated in the S401A and the S401D mutant cells. Studies have shown that inhibition of PP2A can affect HRR by aberrant CDK1 activation and as well as accumulation of active Plk-1. We therefore plan to look at several checkpoint proteins in the MEFs that express the WT, S401A and S401D forms of PP2A-Aα. The B56 subunits are crucial for regulating mitotic entry and exit. Co-immunoprecipitation for the B56 subunit and others with the WT, S401A and S401D forms of PP2A-Aα will help us understand the regulatory aspect.

Our data show that PP2A-A and CRM1 exist together in an immune-complex. We are presently in the process of looking at CRM1 and PP2A-Aα (WT/S401A/S401D) interaction using Proximity Ligation assay (PLA). PLA will give us a better insight on where the interaction is occurring and how is this interaction affected on DNA damage or CRM1 inhibition. In order to look closely at CRM1 mediated PP2A-Aα shuttling, we have generated site-specific mutations in the putative NES sequence (L373A) by itself as well as in combination with the S401A and S401D mutation. These mutants are expressed in the photoactivatable PAmCherry background. Photoactivation of these various mutants in presence and absence of DNA damage will allow us to look at nuclear export in real time.

Finally, our mouse model, in which we aim to knock out PP2A-Aα in the brain, will pave a way to many avenues. In the Nestin-Cre /PP2A- Aα (CKO/CKO) model, if the CKO/CKO mouse survives, we will be able to assess phenotypic changes when the Aα alleles are lost during development. Once we have a generated a PP2A-Aα (CKO-CKO) expressing the Nestin-CreER^{T2}, tamoxifen treatment in adult mice will facilitate knock out of PP2A-Aα. We will be primarily looking for any drastic phenotypic changes in the animals. If the animals don't survive, their brains will be analyzed for morphological changes or neurodegeneration markers. For both the models, we also plan to isolate neural progenitors from PP2A-Aα (CKO-CKO) /Nestin-CreER^{T2} pups. This will aid us in looking at neural signaling affected with PP2A-Aα in the cells.

All these studies which are currently being undertaken along with the studies performed for this project will give us an important insight on functioning of the ATM/PP2A-Aα signaling. Both, ATM and PP2A are potential therapeutic targets for treating a number of cancers and neurological disorders owing to their complex functions. Our study will help to broaden our understanding of the spatiometric regulation of ATM and PP2A in DDR.

Appendix A

	Primer name	Primer Sequence (5' to 3')
1	PPP2R1A (11609-11631) #1	GAGGACAAGTCTTGGCGTGTTCG
2	PPP2R1A (11871-11848) #2	CATGGAGTTGAAACTTGGCTTCAG
3	PPP2R1A (10709-10732) #3	TCACTGGACACTGTACTTCAGCAG
4	Cre Recombinase 5' (1083F)	GCGGCATGGTGCAAGTTGAATAA
5	Cre Recombinase 3' (1085R)	GTGAAACAGCATTGCTGTCACTT
6	Actin 5'	TCACCCACACTGTGCCCATCTACGA
7	Actin 3'	CAGCGGAACCGCTCATTGCCAATGG
8	Repair assay 5'	CGGAGCAAGCTTGATTTAGGTG
9	Repair assay 3'	CGCATGAACTCTTTGATGACCTC
10	Ampicillin 5'	TGTGCAAAAAAGCGGTTAGCT
11	Ampicillin 3'	GCGGCCAACTTACTTCTGACA

Table 1: List of Primers used

References:

Abraham, R.T. (2001). Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes dev. *15*, 2177–2196.

Akopiants, K., Zhou, R.-Z., Mohapatra, s., Valerie, k., Lees-Miller, S.P., Lee, K.-J., Chen, D.J., Revy, P., Villartay, J.-P. De, and Povirk, L.F. (2009). Requirement for XLF/cernunnos in alignment-based gap filling by DNA polymerases λ and μ for nonhomologous end joining in human whole-cell extracts. Nucleic acids res. *37*, 4055–4062.

Anderson, L., Henderson, C., and Adachi, Y. (2001). Phosphorylation and rapid relocalization of 53BP1 to nuclear foci upon DNA damage. Mol. Cell. Biol. *21*, 1719–1729.

Andrabi, S., Gjoerup, O.V., Kean, J.A., roberts, T.M., and Schaffhausen, b. (2007). Protein Phosphatase 2A regulates life and death decisions via AKT in a context-dependent manner. Proc. Natl. Acad. Sci. *104*, 19011–19016.

De Baere, I., Derua, R., Janssens, V., Van hoof, C., Waelkens, E., Merlevede, W., and Goris, J. (1999). Purification of porcine brain Protein Phosphatase 2A leucine carboxyl methyltransferase and cloning of the human homologue. Biochemistry (mosc.) *38*, 16539–16547.

Bakkenist, C.J., and Kastan, M.B. (2003). DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature *421*, 499–506.

Barr, F.A., Elliott, P.R., and Gruneberg, U. (2011). Protein phosphatases and the regulation of mitosis. J cell sci *124*, 2323–2334.

Bartek, J., and Lukas, J. (2001). Pathways governing G1/S transition and their response to DNA damage. Febs lett. *490*, 117–122.

Bartek, J., and Lukas, J. (2003). Chk1 and Chk2 kinases in checkpoint control and cancer. Cancer cell *3*, 421–429.

Barzel, A., and Kupiec, M. (2008). Finding a match: how do homologous sequences get together for recombination? Nat. Rev. Genet. *9*, 27–37.

Beckta, J.M., Dever, S.M., Gnawali, N., Khalil, A., sule, A., golding, S.E., Rosenberg, E., Narayanan, A., Kehn-Hall, K., Xu, B., et al. (2015). Mutation of the BRCA1 SQ-cluster results in aberrant mitosis, reduced Homologous recombination, and a compensatory increase in non-homologous end joining. Oncotarget *6*, 27674–27687.

Bennetzen, M.V., Larsen, D.H., Bunkenborg, J., Bartek, J., Lukas, J., and Andersen, J.S. (2010). Site-specific phosphorylation dynamics of the nuclear proteome during the DNA damage response. Mol. Cell. Proteomics *9*, 1314–1323.

Boehrs, J.K., He, J., Halaby, M.J., and Yang, D.Q. (2007). Constitutive expression and cytoplasmic compartmentalization of atm protein in differentiated human neuron-like sh-SY5Y cells. J. Neurochem. *100*, 337–345.

Brazil, D.P., and Hemmings, B.A. (2001). Ten years of protein kinase B signalling: a hard AKT to follow. Trends biochem. Sci. *26*, 657–664.

Bryant, H.E., Schultz, N., Thomas, H.D., Parker, K.M., Flower, D., Lopez, E., Kyle, S., Meuth, M., Curtin, N.J., and Helleday, T. (2005). Specific killing of BRCA2-deficient tumours with inhibitors of Poly(ADP-ribose) polymerase. Nature *434*, 913–917.

Bunting, S.F., Callén, E., Wong, N., Chen, H.-T., Polato, F., Gunn, A., Bothmer, A., Feldhahn, N., Fernandez-Capetillo, O., Cao, L., et al. (2010). 53BP1 inhibits homologous recombination in BRCA1-deficient cells by blocking resection of DNA breaks. Cell *141*, 243–254.

Burgess, A., Vigneron, S., Brioudes, E., Labbé, J.-C., Lorca, T., and Castro, A. (2010). Loss of human Greatwall results in G2 arrest and multiple mitotic defects due to deregulation of the cyclin B-Cdc2/PP2A balance. Proc. Natl. Acad. Sci. *107*, 12564–12569.

Campbell, K.S., Auger, K.R., Hemmings, B.A., Roberts, T.M., and Pallas, D.C. (1995). Identification of regions in polyomavirus middle t and small t antigens important for association with protein phosphatase 2A. J. Virol. *69*, 3721–3728.

Canman, C.E., Lim, D.-S., Cimprich, K.A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M.B., and Siliciano, J.D. (1998). Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science *281*, 1677–1679.

Carlson, C.J., White, M.F., and Rondinone, C.M. (2004). Mammalian target of rapamycin regulates IRS-1 Serine 307 phosphorylation. Biochem. Biophys. Res. Commun. *316*, 533–539.

Castedo, M., Perfettini, J.-L., Roumier, T., Andreau, K., Medema, R., and Kroemer, G. (2004). Cell death by mitotic catastrophe: A molecular definition. Oncogene *23*, 2825–2837.

Castilho, P.V., williams, B.C., mochida, S., Zhao, Y., and Goldberg, M.L. (2009). The m phase kinase greatwall (GWL) promotes inactivation of PP2A/b55δ, a phosphatase directed against CDK phosphosites. Mol. Biol. Cell *20*, 4777–4789.

Cattaneo, E., and Mckay, R. (1990). Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor. Nature *347*, 762–765.

Ceccaldi, R., Rondinelli, B., and d'andrea, A.D. (2016). Repair pathway choices and consequences at the double-strand break. Trends cell biol. *26*, 52–64.

Chen, J., Martin, B.L., and Brautigan, D.L. (1992). Regulation of protein serine-threonine phosphatase type-2a by tyrosine phosphorylation. Science *257*, 1261–1264.

Chen, W., Possemato, R., Campbell, K.T., Plattner, C.A., Pallas, D.C., and Hahn, W.C. (2004). Identification of specific PP2A complexes involved in human cell transformation. Cancer cell *5*, 127–136.

Chen, W., Arroyo, J.D., Timmons, J.C., Possemato, R., and Hahn, w.c. (2005). Cancer-associated PP2A Aα subunits induce functional haploinsufficiency and tumorigenicity. Cancer res. *65*, 8183–8192.

Chowdhury, D., Keogh, M.C., Ishii, H., Peterson, C.L., Buratowski, S., and Lieberman, J. (2005). Gamma-H2AX dephosphorylation by protein phosphatase 2A facilitates DNA double-strand break repair. Mol. Cell *20*, 801–809.

Ciccia, A., and Elledge, S.J. (2010). The DNA damage response: making it safe to play with knives. Mol. Cell *40*, 179–204.

Cohen, P., Klumpp, S., and Schelling, D.L. (1989). An improved procedure for identifying and quantitating protein phosphatases in mammalian tissues. Febs lett. *250*, 596–600.

Cour, t. La, Kiemer, L., Mølgaard, A., Gupta, R., Skriver, K., and Brunak, S. (2004). Analysis and prediction of leucine-rich nuclear export signals. Protein eng. Des. Sel. *17*, 527–536.

Critchlow, S.E., Bowater, R.P., and Jackson, S.P. (1997). Mammalian dna double-strand break repair protein XRCC4 interacts with DNA Ligase IV. Curr. Biol. *7*, 588–598.

Dasika, G.K., Lin, S.C., Zhao, S., Sung, P., Tomkinson, A., and Lee, E.Y. (1999). DNA damage-induced cell cycle checkpoints and DNA strand break repair in development and tumorigenesis. Oncogene *18*, 7883–7899.

Dhillon, A.S., Meikle, S., Yazici, Z., Eulitz, M., and Kolch, W. (2002). Regulation of RAF-1 activation and signalling by dephosphorylation. Embo j. *21*, 64–71.

Difilippantonio, S., and Nussenzweig, a. (2007). The Nbs1-ATM connection revisited. Cell cycle *6*, 2366–2370.

Douglas, P., Moorhead, G.B.G., Ye, R., and Lees-Miller, S.P. (2001). Protein phosphatases regulate DNA-dependent protein kinase activity. J. Biol. Chem. *276*, 18992–18998.

Dozier, C., Bonyadi, M., Baricault, L., Tonasso, L., and Darbon, J.-M. (2004). Regulation of Chk2 phosphorylation by interaction with protein phosphatase 2A via its B' regulatory subunit. Biol. Cell *96*, 509–517.

Dranovsky, A., Picchini, A.M., Moadel, T., Sisti, A.C., Yamada, A., Kimura, S., Leonardo, E.D., and Hen, R. (2011). Experience dictates stem cell fate in the adult hippocampus. Neuron *70*, 908–923.

Eichhorn, P.J.A., Creyghton, M.P., and Bernards, R. (2009). Protein phosphatase 2A regulatory subunits and cancer. Biochim. Biophys. Acta bba - rev. Cancer *1795*, 1–15.

Engelsma, D., Bernad, R., Calafat, J., and Fornerod, M. (2004). Supraphysiological nuclear export signals bind crm1 independently of rangtp and arrest at nup358. Embo j. *23*, 3643–3652.

Englmeier, L., Olivo, J.-C., and Mattaj, I.W. (1999). Receptor-mediated substrate translocation through the nuclear pore complex without nucleotide triphosphate hydrolysis. Curr. Biol. *9*, 30–41.

Farmer, H., Mccabe, N., Lord, C.J., Tutt, A.N.J., Johnson, D.A., Richardson, T.B., Santarosa, M., Dillon, K.J., Hickson, I., Knights, C., et al. (2005). Targeting the dna repair defect in brca mutant cells as a therapeutic strategy. Nature *434*, 917–921.

Favre, B., and Turowski, P. (1997). Differential inhibition and posttranslational modification of protein phosphatase 1 and 2a in MCF7 cells treated with Calyculin-A, Okadaic acid, and Tautomycin. J. Biol. Chem. *272*, 13856–13863.

Feil, R., Wagner, J., Metzger, D., and Chambon, P. (1997). Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. Biochem. Biophys. Res. Commun. *237*, 752–757.

Fernandez-Capetillo, O., Chen, H.-T., Celeste, A., Ward, I., Romanienko, P.J., Morales, J.C., Naka, K., Xia, Z., Camerini-Otero, R.D., Motoyama, N., et al. (2002). DNA damage-induced G2–M checkpoint activation by Histone H2AX and 53BP1. Nat. Cell biol. *4*, 993–997.

Fey, D., Croucher, D.R., Kolch, W., and Kholodenko, B.N. (2012). Crosstalk and signaling switches in mitogen-activated protein kinase cascades. Syst. Biol. *3*, 355.

Flegg, C.P., Sharma, M., Medina-Palazon, C., Jamieson, C., Galea, M., Brocardo, M.G., Mills, K., and Henderson, B.R. (2010). Nuclear export and centrosome targeting of the

Protein phosphatase 2A subunit B56α role of B56α in nuclear export of the catalytic subunit. J. Biol. Chem. *285*, 18144–18154.

Foley, E.A., Maldonado, M., and Kapoor, T.M. (2011). Formation of stable attachments between kinetochores and microtubules depends on the B56-PP2A phosphatase. Nat. Cell biol. *13*, 1265–1271.

Forester, C.M., Maddox, J., Louis, J.V., Goris, J., and Virshup, D.M. (2007). Control of mitotic exit by PP2A regulation of Cdc25c and Cdk1. Proc. Natl. Acad. Sci. *104*, 19867–19872.

Fornerod, m., ohno, m., yoshida, m., and mattaj, i.w. (1997). Crm1 is an export receptor for leucine-rich nuclear export signals. Cell *90*, 1051–1060.

Freeman, A.K., Dapic, V., and Monteiro, A.N.A. (2010). Negative regulation of Chk2 activity by protein phosphatase 2a is modulated by dna damage. Cell cycle *9*, 736–747.

Fu, X.Y., and Manley, J.L. (1987). Factors influencing alternative splice site utilization in vivo. Mol. Cell. Biol. *7*, 738–748.

Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M., and nishida, E. (1997). CRM1 is responsible for intracellular transport mediated by the nuclear export signal. Nature *390*, 308–311.

Gharbi-Ayachi, A., Labbé, J.-C., Burgess, A., Vigneron, S., Strub, J.-M., Brioudes, E., Van-Dorsselaer, A., Castro, A., and Lorca, T. (2010). The substrate of Greatwall kinase, Arpp19, controls mitosis by inhibiting protein phosphatase 2a. Science *330*, 1673–1677.

Goldberg, M., Stucki, M., Falck, J., d'amours, D., Rahman, D., Pappin, D., Bartek, J., and Jackson, S.P. (2003). MDC1 is required for the intra-S-phase DNA damage checkpoint. Nature *421*, 952–956.

Golding, S.E., Rosenberg, E., Khalil, A., mcewen, A., Holmes, M., Neill, S., Povirk, L.F., and Valerie, K. (2004). Double strand break repair by homologous recombination is regulated by cell cycle-independent signaling via ATM in human glioma cells. J. Biol. Chem. *279*, 15402–15410.

Golding, S.E., Rosenberg, E., Valerie, N., Hussaini, i., frigerio, m., cockcroft, x.f., chong, W.Y., Hummersone, M., Rigoreau, L., Menear, K.A., et al. (2009). Improved ATM kinase inhibitor ku-60019 radiosensitizes glioma cells, compromises insulin, AKT and ERK prosurvival signaling, and inhibits migration and invasion. Mol. Cancer ther. *8*, 2894–2902.

Goodarzi, A.A., Jonnalagadda, J.C., Douglas, P., Young, D., Ye, R., Moorhead, G.B., Lees-Miller, S.P., and Khanna, K.k. (2004). Autophosphorylation of Ataxia-Telangiectasia mutated is regulated by protein phosphatase 2A. Embo j. *23*, 4451–4461.

Görlich, D., and Kutay, and U. (1999). Transport between the cell nucleus and the cytoplasm. Annu. Rev. Cell dev. Biol. *15*, 607–660.

Görlich, D., Panté, N., Kutay, U., Aebi, U., and Bischoff, F.R. (1996). Identification of different roles for rangdp and rangtp in nuclear protein import. Embo j. *15*, 5584–5594.

Görlich, D., Dabrowski, M., Bischoff, F.R., Kutay, U., Bork, P., Hartmann, E., Prehn, S., and Izaurralde, E. (1997). A novel class of rangtp binding proteins. J. Cell biol. *138*, 65–80.

Grallert, A., Boke, E., Hagting, A., Hodgson, B., Connolly, Y., Griffiths, J.R., Smith, D.L., Pines, J., and Hagan, i.M. (2015). A PP1-PP2A phosphatase relay controls mitotic progression. Nature *517*, 94–98.

Guan, K.-L., Figueroa, C., Brtva, T.R., Zhu, T., Taylor, J., Barber, T.D., and Vojtek, A.B. (2000). Negative regulation of the Serine/Threonine kinase b-RAF by AKT. J. Biol. Chem.

Gunn, A., and Stark, J.M. (2012). I-scei-based assays to examine distinct repair outcomes of mammalian chromosomal double strand breaks. Methods mol. Biol. Clifton nj *920*, 379–391.

Guo, F., Stanevich, V., Wlodarchak, N., Sengupta, R., Jiang, l., Satyshur, K.A., and Xing, Y. (2014). Structural basis of PP2A activation by PTPA, an ATP-dependent activation chaperone. Cell res. *24*, 190–203.

Güttler, T., and Görlich, D. (2011). Ran-dependent nuclear export mediators: a structural perspective. Embo j. *30*, 3457–3474.

Güttler, T., Madl, T., Neumann, P., Deichsel, D., Corsini, L., Monecke, T., Ficner, R., Sattler, M., and Görlich, D. (2010). NES consensus redefined by structures of Pki-type and Rev-type nuclear export signals bound to CRM1. Nat. Struct. Mol. Biol. *17*, 1367–1376.

Haasen, D., Köhler, C., Neuhaus, G., and MERKle, T. (1999). Nuclear export of proteins in plants: atxpo1 is the export receptor for leucine-rich nuclear export signals in Arabidopsis thaliana. Plant j. *20*, 695–705.

Hagstrom, K.A., and Meyer, B.J. (2003). Condensin and cohesin: more than chromosome compactor and glue. Nat. Rev. Genet. *4*, 520–534.

Hahn, W.C., Counter, C.M., Lundberg, A.S., Beijersbergen, R.L., Brooks, M.W., and Weinberg, r.a. (1999). Creation of human tumour cells with defined genetic elements. Nature *400*, 464–468.

Hamamoto, T., Seto, H., and Beppu, T. (1983a). Leptomycins A and B, new antifungal antibiotics. Ii. Structure elucidation. J. Antibiot. (tokyo) *36*, 646–650.

Hamamoto, T., Gunji, S., Tsuji, H., and Beppu, T. (1983b). Leptomycins A and B, new antifungal antibiotics. I. Taxonomy of the producing strain and their fermentation, purification and characterization. J. Antibiot. (tokyo) *36*, 639–645.

Hamamoto, T., Uozumi, T., and Beppu, T. (1985). Leptomycins A and B, new antifungal antibiotics. Iii. Mode of action of leptomycin B on Schizosaccharomyces pombe. J. Antibiot. (tokyo) *38*, 1573–1580.

Hauf, S., Roitinger, E., Koch, B., Dittrich, C.M., Mechtler, K., and Peters, J.-M. (2005). Dissociation of cohesin from chromosome arms and loss of arm cohesion during early mitosis depends on phosphorylation of Sa2. Plos biol *3*, e69.

Hemmings, B.A., Adams-Pearson, C., Maurer, F., Müller, P., Goris, J., Merlevede, w., Hofsteenge, J., and Stone, S.R. (1990). A- and beta-forms of the 65-Kda subunit of protein phosphatase 2A have a similar 39 amino acid repeating structure. Biochemistry (mosc.) *29*, 3166–3173.

Hendrix, P., Turowski, P., Mayer-Jaekel, R.E., Goris, J., Hofsteenge, J., Merlevede, W., and hemmings, B.A. (1993). Analysis of subunit isoforms in protein phosphatase 2a holoenzymes from rabbit and xenopus. J. Biol. Chem. *268*, 7330–7337.

Hiraga, A., and Tamura, S. (2000). Protein phosphatase 2A is associated in an inactive state with microtubules through 2a1-specific interaction with tubulin. Biochem. J. *346*, 433–439.

Houge, G., Haesen, D., Vissers, L.E.L.M., Mehta, S., Parker, M.J., Wright, M., Vogt, J., Mckee, S., Tolmie, J.L., Cordeiro, N., et al. (2015). B56δ-related protein phosphatase 2A dysfunction identified in patients with intellectual disability. J. Clin. Invest. *125*, 3051–3062.

Hu, Y., Petit, S.A., Ficarro, S.B., Toomire, K.J., Xie, A., Lim, E., Cao, S.A., Park, E., Eck, M.J., Scully, R., et al. (2014). PARP1-driven poly-adp-ribosylation regulates BRCA1 function in homologous recombination–mediated DNA repair. Cancer discov. *4*, 1430–1447.

Hunter, T. (1995). Protein kinases and phosphatases: the Yin and Yang of protein phosphorylation and signaling. Cell *80*, 225–236.

Izaurralde, E., Kutay, U., Von kobbe, C., Mattaj, I.W., and görlich, D. (1997). The asymmetric distribution of the constituents of the Ran system is essential for transport into and out of the nucleus. Embo j. *16*, 6535–6547.

Jackson, S.P., and Bartek, J. (2009). The DNA-Damage response in human biology and disease. Nature *461*, 1071–1078.

Janssens, V., and Goris, j. (2001). Protein phosphatase 2A: a highly regulated family of Serine/Threonine phosphatases implicated in cell growth and signalling. Biochem j *353*, 417–439.

Janssens, V., Longin, S., and Goris, J. (2008). PP2A holoenzyme assembly: in Cauda venenum (the sting is in the tail). Trends biochem sci *33*, 113–121.

Jin, Z., Shi, J., SaRAF, A., Mei, W., Zhu, G.-Z., Strack, S., and Yang, J. (2009). The 48-kda alternative translation isoform of PP2A:B56ϵ is required for Wnt signaling during midbrain-hindbrain boundary formation. J. Biol. Chem. *284*, 7190–7200.

Johansson, C.B., Momma, S., Clarke, D.L., Risling, M., Lendahl, U., and Frisén, j. (1999). Identification of a neural stem cell in the adult mammalian central nervous system. Cell *96*, 25–34.

Kalev, P., Simicek, M., Vazquez, I., Munck, S., Chen, L., Soin, T., Danda, N., Chen, W., and Sablina, A. (2012). Loss of PPP2R2A inhibits homologous recombination DNA repair and predicts tumor sensitivity to PARP inhibition. Cancer res. *72*, 6414–6424.

Kastan, M.B., Lim, D.S., Kim, S.T., and Yang, D. (2001). ATM--a key determinant of multiple cellular responses to irradiation. Acta oncol. Stockh. Swed. *40*, 686–688.

Kehlenbach, R.H., Dickmanns, A., Kehlenbach, A., Guan, T., and Gerace, L. (1999). A role for Ranbp1 in the release of CRM1 from the nuclear pore complex in a terminal step of nuclear export. J. Cell biol. *145*, 645–657.

Kellogg, G.E., Semus, S.F., and Abraham, D.J. (1991). HINT: a new method of empirical hydrophobic field calculation for comfa. J. Comput. Aided mol. Des. *5*, 545–552.

Keogh, M.-C., Kim, J.-A., Downey, M., Fillingham, J., Chowdhury, D., Harrison, J.C., Onishi, M., Datta, N., Galicia, S., Emili, A., et al. (2006). A phosphatase complex that dephosphorylates gamma H2AX regulates DNA damage checkpoint recovery. Nature *439*, 497–501.

Khalil, A., Morgan, R.N., Adams, B.R., golding, S.E., Dever, S.M., Rosenberg, E., Povirk, L.F., and Valerie, K. (2011). ATM-dependent ERK signaling via AKT in response to DNA double-strand breaks. Cell cycle *10*, 481–491.

Khew-Goodall, Y., and Hemmings, B.A. (1988). Tissue-specific expression of mrnas encoding α- and beta-catalytic subunits of protein phosphatase 2A. Febs lett. *238*, 265–268.

Kitajima, T.S., Sakuno, T., Ishiguro, K., Iemura, S., Natsume, T., kawashima, S.A., and watanabe, Y. (2006). Shugoshin collaborates with protein phosphatase 2A to protect cohesin. Nature *441*, 46–52.

Klebe, C., Bischoff, F.R., Ponstingl, H., and Wittinghofer, A. (1995). Interaction of the nuclear GTP-binding protein Ran with its regulatory proteins RCC1 and Rangap1. Biochemistry (mosc.) *34*, 639–647.

Kolupaeva, V., Daempfling, L., and Basilico, C. (2013). The B55α regulatory subunit of protein phosphatase 2A mediates fibroblast growth factor-induced p107 dephosphorylation and growth arrest in chondrocytes. Mol. Cell. Biol. *33*, 2865–2878.

Kong, X., Mohanty, S.K., Stephens, J., Heale, J.T., Gomez-Godinez, V., Shi, L.Z., Kim, J.-S., Yokomori, K., and Berns, M.W. (2009). Comparative analysis of different laser systems to study cellular responses to dna damage in mammalian cells. Nucleic acids res. *37*, e68–e68.

Kosugi, S., Hasebe, M., Tomita, M., and Yanagawa, H. (2008). Nuclear export signal consensus sequences defined using a localization-based yeast selection system. TRAFfic *9*, 2053–2062.

Kotlo, K., Xing, Y., Lather, S., Grillon, J.M., Johnson, K., Skidgel, R.A., Solaro, R.J., and danziger, R.S. (2014). PR65A phosphorylation regulates PP2A complex signaling. Plos one *9*, e85000.

Kozlov, S.V., Graham, M.E., Peng, C., Chen, P., Robinson, P.J., and Lavin, M.F. (2006). Involvement of novel autophosphorylation sites in atm activation. Embo j. *25*, 3504–3514.

Kozlov, S.V., Graham, M.E., Jakob, B., Tobias, F., Kijas, A.W., Tanuji, M., Chen, P., Robinson, P.J., Taucher-scholz, G., Suzuki, K., et al. (2011). Autophosphorylation and ATM activation additional sites add to the complexity. J. Biol. Chem. *286*, 9107–9119.

Kudo, N., Wolff, B., Sekimoto, T., Schreiner, E.P., Yoneda, Y., Yanagida, M., Horinouchi, S., and Yoshida, M. (1998). Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1. Exp. Cell res. *242*, 540–547.

Kudo, N., Matsumori, N., Taoka, H., Fujiwara, D., Schreiner, E.P., Wolff, B., Yoshida, M., and Horinouchi, S. (1999). Leptomycin B Inactivates Crm1/Exportin 1 by covalent modification at a cysteine residue in the central conserved region. Proc. Natl. Acad. Sci. *96*, 9112–9117.

Kuo, Y.C., Huang, K.Y., Yang, C.H., Yang, Y.S., lee, W.Y., and Chiang, C.W. (2008). Regulation of phosphorylation of Thr-308 of AKT, cell proliferation, and survival by the b55α regulatory subunit targeting of the protein phosphatase 2A holoenzyme to AKT. J. Biol. Chem. *283*, 1882–1892.

Kutay, U., and Güttinger, s. (2005). Leucine-rich nuclear-export signals: born to be weak. Trends cell biol. *15*, 121–124.

Lao, d.-h., yusoff, p., chandramouli, s., philp, R.J., Fong, C.W., Jackson, R.A., Saw, T.Y., Yu, C.Y., and Guy, G.r. (2007). Direct binding of PP2A to Sprouty2 and phosphorylation changes are a prerequisite for ERK inhibition downstream of fibroblast growth factor receptor stimulation. J. Biol. Chem. *282*, 9117–9126.

Lavin, M.F. (2008). Ataxia-telangiectasia: from a rare disorder to a paradigm for cell signalling and cancer. Nat. Rev. Mol. Cell biol. *9*, 759–769.

Lee, D.H., and Chowdhury, D. (2011). What goes on must come off: phosphatases gate-crash the dna damage response. Trends biochem. Sci. *36*, 569–577.

Lee, J.-H., and Paull, T.T. (2005). ATM activation by dna double-strand breaks through the MRE11-RAD50-NBS1 complex. Scence *308*, 551–554.

Lee, J., Chen, Y., Tolstykh, T., and Stock, J. (1996). A specific protein carboxyl methylesterase that demethylates phosphoprotein phosphatase 2A in bovine brain. Proc. Natl. Acad. Sci. U. S. A. *93*, 6043–6047.

Lehr, s., Kotzka, J., Avci, H., Sickmann, A., Meyer, H.E., HERKner, A., and Muller-Wieland, D. (2004). Identification of major ERK-related phosphorylation sites in Gab1. Biochemistry (mosc.) *43*, 12133–12140.

Lei, Y. (2013). Generation and culture of mouse embryonic fibroblasts. Methods mol. Biol. Clifton nj *1031*, 59–64.

Lendahl, U., Zimmerman, L.B., and Mckay, R.D.G. (1990). CNS stem cells express a new class of intermediate filament protein. Cell *60*, 585–595.

Leung-Pineda, V., Ryan, C.E., and Piwnica-Worms, H. (2006). Phosphorylation of Chk1 by ATR is antagonized by a Chk1-regulated protein phosphatase 2A circuit. Mol. Cell. Biol. *26*, 7529–7538.

Li, J., and Jiang, D. (2015). The role of epigenomics in the neurodegeneration of Ataxia-Telangiectasia. Epigenomics *7*, 137–141.

Li, G., Elder, R.T., Qin, K., Park, H.U., Liang, D., and Zhao, R.Y. (2007). Phosphatase type 2A-dependent and -independent pathways for atr phosphorylation of Chk1. J. Biol. Chem. *282*, 7287–7298.

Li, j., Chen, J., Ricupero, C.L., Hart, R.P., Schwartz, M.S., Kusnecov, A., and Herrup, K. (2012). Nuclear accumulation of HDAC4 in atm deficiency promotes neurodegeneration in ataxia telangiectasia. Nat. Med. *18*, 783–790.

Li, X., Nan, A., Xiao, Y., Chen, Y., and Lai, Y. (2015). PP2A–B56ϵ complex is involved in dephosphorylation of γ-H2AX in the repair process of Cpt-induced DNA double-strand breaks. Toxicology *331*, 57–65.

Liu, R., Zhou, X.-W., Tanila, H., Bjorkdahl, C., Wang, J.-Z., Guan, Z.-Z., Cao, Y., Gustafsson, J.-Å., Winblad, B., and Pei, J.-J. (2008). Phosphorylated PP2A (Tyrosine 307) is associated with alzheimer neurofibrillary pathology. J. Cell. Mol. Med. *12*, 241–257.

Lliakis, G. (1991). The role of DNA double strand breaks in Ionizing radiation-induced killing of eukaryotic cells. Bioessays *13*, 641–648.

Lorca, T., Bernis, C., Vigneron, S., Burgess, A., Brioudes, E., Labbé, J.-C., and Castro, A. (2010). Constant regulation of both the Mpf amplification loop and the Greatwall-PP2A pathway is required for metaphase II arrest and correct entry into the first embryonic cell cycle. J cell sci *123*, 2281–2291.

Lowe, M., Gonatas, N.K., and Warren, G. (2000). The mitotic phosphorylation cycle of the Cis-golgi matrix protein Gm130. J. Cell biol. *149*, 341–356.

Ma, Y., LU, H., Tippin, B., Goodman, M.F., Shimazaki, N., Koiwai, O., Hsieh, C.-l., Schwarz, K., and Lieber, M.R. (2004). A biochemically defined system for mammalian nonhomologous DNA end joining. Mol. Cell *16*, 701–713.

Mailand, N., Falck, J., Lukas, C., Syljuåsen, R.G., Welcker, M., Bartek, J., and Lukas, J. (2000). Rapid destruction of human Cdc25a in response to dna damage. Science *288*, 1425–1429.

Maiuri, T., Woloshansky, T., Xia, J., and Truant, R. (2013). The huntingtin N17 domain is a multifunctional Crm1 and Ran-dependent nuclear and cilial export signal. Hum. Mol. Genet. *22*, 1383–1394.

Margolis, S.S., Perry, J.A., Forester, C.M., Nutt, L.K., Guo, Y., Jardim, M.J., Thomenius, M.J., Freel, C.D., Darbandi, R., Ahn, J.-H., et al. (2006a). Role for the PP2A/B56δ phosphatase in regulating 14-3-3 release from Cdc25 to control mitosis. Cell *127*, 759–773.

Margolis, S.S., Perry, J.A., Weitzel, D.H., Freel, C.D., Yoshida, M., Haystead, T.A., and Kornbluth, S. (2006b). A role for PP1 in the Cdc2/Cyclin B–mediated positive feedback activation of Cdc25. Mol. Biol. Cell *17*, 1779–1789.

Marková, E., Schultz, N., and Belyaev, D.I.Y. (2007). Kinetics and dose-response of residual 53BP1/γ-H2AX foci: co-localization, relationship with dsb repair and clonogenic survival. Int. J. Radiat. Biol. *83*, 319–329.

Matsuoka, S., Ballif, B.A., Smogorzewska, A., Mcdonald, E.R., 3rd, Hurov, K.E., Luo, j., Bakalarski, C.E., Zhao, Z., Solimini, N., Lerenthal, y., et al. (2007). ATM and ATR substrate analysis reveals extensive protein networks responsive to dna damage. Science *316*, 1160–1166.

Mattaj, I.W., and Englmeier, and l. (1998). Nucleocytoplasmic transport: the soluble PHASE. ANNU. REV. BIOCHEM. *67*, 265–306.

Mayer-jaekel, r.e., and hemmings, b.a. (1994). Protein phosphatase 2a--a "menage a trois." Trends cell biol *4*, 287–291.

Mccright, B., Rivers, A.M., Audlin, S., and Virshup, D.M. (1996). The B56 family of protein phosphatase 2A (pp2a) regulatory subunits encodes differentiation-induced phosphoproteins that target PP2A to both nucleus and cytoplasm. J. Biol. Chem. *271*, 22081–22089.

Melander, F., Bekker-Jensen, S., Falck, J., Bartek, J., Mailand, N., and Lukas, J. (2008). Phosphorylation of SDT repeats in the MDC1 N terminus triggers retention of NBS1 at the DNA damage–modified chromatin. J. Cell biol. *181*, 213–226.

Mitra, a., menezes, m.e., pannell, l.k., mulekar, m.s., honkanen, r.e., shevde, l.a., and samant, r.s. (2012). Dnajb6 chaperones pp2a mediated dephosphorylation of gsk3β to downregulate β-catenin transcription target, osteopontin. Oncogene *31*, 4472–4483.

Mochida, S., Ikeo, S., Gannon, J., and Hunt, T. (2009). Regulated activity of PP2A–B55δ is crucial for controlling entry into and exit from mitosis in xenopus egg extracts. Embo j. *28*, 2777–2785.

Mochida, S., Maslen, S.L., Skehel, M., and Hunt, T. (2010). Greatwall phosphorylates an inhibitor of protein phosphatase 2α that is essential for mitosis. Science *330*, 1670–1673.

Mumby, M. (1995). Regulation by tumour antigens defines a role for PP2A in signal transduction. Semin. Cancer biol. *6*, 229–237.

Neville, M., and ROsbash, M. (1999). The NES–Crm1p export pathway is not a major mRNA export route in Saccharomyces cerevisiae. Embo j. *18*, 3746–3756.

Nishi, K., Yoshida, M., Fujiwara, D., Nishikawa, M., Horinouchi, S., and Beppu, T. (1994). Leptomycin B targets a regulatory cascade of CRM1, a fission yeast nuclear protein, involved in control of higher order chromosome structure and gene expression. J. Biol. Chem. *269*, 6320–6324.

O'connor, M.J. (2015). Targeting the dna damage response in cancer. Mol. Cell *60*, 547–560.

Ory, S., Zhou, M., Conrads, T.P., Veenstra, T.D., and Morrison, D.K. (2003). Protein phosphatase 2A positively regulates RAS signaling by dephosphorylating KSR1 and RAF-1 on critical 14-3-3 binding sites. Curr. Biol. *13*, 1356–1364.

Ossareh-Nazari, B., Gwizdek, C., and Dargemont, C. (2001). Protein export from the nucleus. TRAFfic *2*, 684–689.

Pallas, D.C., Shahrik, L.K., Martin, B.L., Jaspers, S., Miller, T.B., Brautigan, D.L., and Roberts, T.M. (1990). Polyoma small and middle T antigens and sv40 small t antigen form stable complexes with protein phosphatase 2A. Cell *60*, 167–176.

Paull, T.T., Rogakou, E.P., Yamazaki, V., Kirchgessner, C.U., Gellert, M., and Bonner, W.M. (2000). A critical role for histone H2Ax in recruitment of repair factors to nuclear foci after DNA damage. Curr. Biol. *10*, 886–895.

Pawson, T., and Scott, J.D. (1997). Signaling through scaffold, anchoring, and adaptor proteins. Science *278*, 2075–2080.

Pear, W.S., Nolan, G.P., Scott, M.L., and Baltimore, D. (1993). Production of high-titer helper-free retroviruses by transient transfection. Proc. Natl. Acad. Sci. U. S. A. *90*, 8392–8396.

Peng, A., and Maller, J.L. (2010). Serine/Threonine phosphatases in the DNA damage response and cancer. Oncogene *29*, 5977–5988.

Perrotti, D., and NEviani, P. (2013). Protein phosphatase 2A: a target for anticancer therapy. Lancet oncol. *14*, e229–e238.

Petersen, P., Chou, D.M., You, Z., Hunter, T., Walter, J.C., and Walter, G. (2006). Protein phosphatase 2A antagonizes ATM and ATR in a Cdk2- and Cdc7-independent dna damage checkpoint. Mol. Cell. Biol. *26*, 1997–2011.

Pierce, A.J., Johnson, R.D., Thompson, L.h., and Jasin, M. (1999). XRCC3 promotes homology-directed repair of DNA damage in mammalian cells. Genes dev. *13*, 2633–2638.

Povirk, L.F., Zhou, T., Zhou, R., Cowan, M.J., and Yannone, S.M. (2007). Processing of 3′-phosphoglycolate-terminated DNA double strand breaks by Artemis nuclease. J. Biol. Chem. *282*, 3547–3558.

Purev, E., Soprano, D.R., and Soprano, K.J. (2011). PP2A interaction with Rb2/p130 mediates translocation of Rb2/p130 into the nucleus in all-trans retinoic acid-treated ovarian carcinoma cells. J. Cell. Physiol. *226*, 1027–1034.

Rappold, I., Iwabuchi, K., Date, T., and Chen, J. (2001). Tumor suppressor p53 binding protein 1 (53BP1) is involved in DNA damage–signaling pathways. J. Cell biol. *153*, 613–620.

Rexach, M., and Blobel, G. (1995). Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. Cell *83*, 683–692.

Ribbeck, K., Lipowsky, G., Kent, H.M., stewart, M., and Görlich, D. (1998). Ntf2 mediates nuclear import of ran. Embo j. *17*, 6587–6598.

Riedel, C.G., Katis, V.L., Katou, Y., Mori, S., Itoh, T., Helmhart, W., Gálová, M., Petronczki, M., Gregan, J., Cetin, B., et al. (2006). Protein phosphatase 2A protects centromeric sister chromatid cohesion during meiosis I. Nature *441*, 53–61.

Robins, P., and Lindahl, T. (1996). DNA ligase IV from Hela cell nuclei. J. Biol. Chem. *271*, 24257–24261.

Rodriguez-Viciana, P., Collins, C., and Fried, M. (2006). Polyoma and sv40 proteins differentially regulate PP2A to activate distinct cellular signaling pathways involved in growth control. Proc. Natl. Acad. Sci. *103*, 19290–19295.

Rogakou, E.P., Pilch, D.R., Orr, A.H., Ivanova, V.S., and Bonner, W.M. (1998). DNA double-stranded breaks induce Histone H2AX phosphorylation on serine 139. J. Biol. Chem. *273*, 5858–5868.

Rogakou, E.P., Boon, C., Redon, C., and Bonner, W.M. (1999). Megabase chromatin domains involved in DNA double-strand breaks in vivo. J. Cell biol. *146*, 905–916.

Ruediger, R., Roeckel, D., Fait, J., Bergqvist, A., Magnusson, G., and Walter, G. (1992). Identification of binding sites on the regulatory a subunit of protein phosphatase 2A for the catalytic c subunit and for tumor antigens of simian virus 40 and polyomavirus. Mol cell biol *12*, 4872–4882.

Ruediger, R., Ruiz, J., and Walter, G. (2011). Human cancer-associated mutations in the Aα subunit of protein phosphatase 2A increase lung cancer incidence in Aα knock-in and knockout mice. Mol. Cell. Biol. *31*, 3832–3844.

Sablina, A.A., Hector, M., Colpaert, N., and Hahn, W.c. (2010). Identification of PP2A complexes and pathways involved in cell transformation. Cancer res. *70*, 10474–10484.

Salic, A., Waters, J.C., and Mitchison, T.J. (2004). Vertebrate Shugoshin links sister centromere cohesion and kinetochore microtubule stability in mitosis. Cell *118*, 567–578.

Sauer, B. (1987). Functional expression of the Cre-lox site-specific recombination system in the yeast saccharomyces cerevisiae. Mol. Cell. Biol. *7*, 2087–2096.

Schmitz, M.H.A., Held, M., Janssens, V., Hutchins, J.R.A., Hudecz, O., Ivanova, E., Goris, J., Trinkle-Mulcahy, L., Lamond, A.I., POser, I., et al. (2010). Live-cell imaging RNAi screen identifies PP2A-B55α and Importin-β1 as key mitotic exit regulators in human cells. Nat. Cell biol. *12*, 886–893.

Schönthal, A.H. (2001). Role of Serine/Threonine protein phosphatase 2A in cancer. Cancer lett. *170*, 1–13.

Schultz, L.B., Chehab, N.H., Malikzay, A., and Halazonetis, T.D. (2000). P53 binding protein 1 (53BP1) is an early participant in the cellular response to dna double-strand breaks. J. Cell biol. *151*, 1381–1390.

Seshacharyulu, P., Pandey, P., Datta, K., and Batra, S.K. (2013). Phosphatase: PP2A structural importance, regulation and its aberrant expression in cancer. Cancer lett. *335*, 9–18.

Shiloh, y. (2003). ATM and related protein kinases: safeguarding genome integrity. Nat. Rev. Cancer *3*, 155–168.

Shiloh, Y., and Ziv, Y. (2013). The ATM protein kinase: regulating the cellular response to genotoxic stress, and more. Nat. Rev. Mol. Cell biol. *14*, 197–210.

Silverstein, A.M., Barrow, C.A., Davis, A.J., and Mumby, M.C. (2002). Actions of PP2A on the MAP kinase pathway and apoptosis are mediated by distinct regulatory subunits. Proc. Natl. Acad. Sci. U. S. A. *99*, 4221–4226.

Smits, V.A.J., Reaper, P.M., and Jackson, S.P. (2006). Rapid PIKK-dependent release of Chk1 from chromatin promotes the DNA-damage checkpoint response. Curr. Biol. *16*, 150–159.

Sontag, J.-M., and Sontag, E. (2014). Protein phosphatase 2A dysfunction in Alzheimer's disease. Front. Mol. Neurosci. *7*, 16.

Sontag, E., Fedorov, S., Kamibayashi, C., Robbins, D., Cobb, M., and Mumby, M. (1993a). The interaction of SV40 small tumor antigen with protein phosphatase 2A stimulates the map kinase pathway and induces cell proliferation. Cell *75*, 887–897.

Sontag, e., Fedorov, S., Kamibayashi, C., Robbins, D., Cobb, M., and Mumby, M. (1993b). The interaction of SV40 small tumor antigen with protein phosphatase 2A stimulates the MAP kinase pathway and induces cell proliferation. Cell *75*, 887–897.

Sontag, E., Nunbhakdi-Craig, V., Bloom, G.S., and Mumby, M.C. (1995). A novel pool of protein phosphatase 2A is associated with microtubules and is regulated during the cell cycle. J cell biol *128*, 1131–1144.

Sontag, E., Luangpirom, A., Hladik, C., Mudrak, I., Ogris, E., Speciale, S., and White, C.L. (2004). Altered expression levels of the protein phosphatase 2A abαc enzyme are associated with Alzheimer disease pathology. J. Neuropathol. Exp. Neurol. *63*, 287–301.

Soprano, K.J., Purev, E., Vuocolo, S., and Soprano, D.r. (2006). Rb2/p130 and protein phosphatase 2A: key mediators of ovarian carcinoma cell growth suppression by all-trans retinoic acid. Oncogene *25*, 5315–5325.

Stade, K., ford, C.S., Guthrie, C., and Weis, K. (1997). Exportin 1 (Crm1p) is an essential nuclear export factor. Cell *90*, 1041–1050.

Strack, S., Zaucha, J.A., Ebner, F.F., Colbran, R.J., and Wadzinski, B.E. (1998). Brain protein phosphatase 2A: Developmental regulation and distinct cellular and subcellular localization by b subunits. J. Comp. Neurol. *392*, 515–527.

Strack, S., Chang, D., Zaucha, J.A., Colbran, R.J., and Wadzinski, B.E. (1999). Cloning and characterization of Bδ, a novel regulatory subunit of protein phosphatase 2A. Febs lett. *460*, 462–466.

Stucki, M., Clapperton, J.A., Mohammad, D., Yaffe, M.B., Smerdon, S.J., and Jackson, S.P. (2005). MDC1 directly binds phosphorylated histone H2AX to regulate cellular responses to DNA double-strand breaks. Cell *123*, 1213–1226.

Subach, F.V., Malashkevich, V.N., Zencheck, W.D., Xiao, H., Filonov, G.S., Almo, S.C., and VERKhusha, v.v. (2009). Photoactivation mechanism of pamcherry based on crystal structures of the protein in the dark and fluorescent states. Proc. Natl. Acad. Sci. *106*, 21097–21102.

Suijkerbuijk, S.J.E., Vleugel, M., Teixeira, A., and Kops, G.J.P.L. (2012). Integration of kinase and phosphatase activities by bubr1 ensures formation of stable kinetochore-microtubule attachments. Dev. Cell *23*, 745–755.

Sun, y., Jiang, X., Chen, S., Fernandes, N., and Price, B.D. (2005). A role for the Tip60 histone acetyltransferase in the acetylation and activation of atm. Proc. Natl. Acad. Sci. U. S. A. *102*, 13182–13187.

Sun, y., Xu, Y., Roy, K., and Price, B.D. (2007). DNA damage-induced acetylation of lysine 3016 of atm activates atm kinase activity. Mol. Cell. Biol. *27*, 8502–8509.

Swingle, M., Ni, L., and Honkanen, r.e. (2007). Small-molecule inhibitors of Ser/Thr protein phosphatases: specificity, use and common forms of abuse. Methods mol. Biol. Clifton nj *365*, 23–38.

Takemoto, A., Maeshima, K., Ikehara, T., Yamaguchi, K., Murayama, A., Imamura, S., imamoto, N., Yokoyama, S., Hirano, T., Watanabe, Y., et al. (2009). The chromosomal association of condensin ii is regulated by a noncatalytic function of PP2A. Nat. Struct. Mol. Biol. *16*, 1302–1308.

Tang, Z., Sun, Y., Harley, S.E., Zou, H., and Yu, H. (2004). Human Bub1 protects centromeric sister-chromatid cohesion through Shugoshin during mitosis. Proc. Natl. Acad. Sci. *101*, 18012–18017.

Taylor, W.R., and Stark, G.R. (2001). Regulation of the G2/M transition by p53. Oncogene *20*, 1803–1815.

Taylor, A.M., Harnden, D.G., Arlett, C.F., Harcourt, S.A., Lehmann, A.R., Stevens, S., and bridges, B.A. (1975). Ataxia Telangiectasia: a human mutation with abnormal radiation sensitivity. Nature *258*, 427–429.

The deciphering developmental disorders study (2015). Large-scale discovery of novel genetic causes of developmental disorders. Nature *519*, 223–228.

Tronche, F., Kellendonk, C., Kretz, O., Gass, P., Anlag, K., Orban, P.C., Bock, R., Klein, R., and Schütz, G. (1999). Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nat. Genet. *23*, 99–103.

Tsuchiya, A., Tashiro, E., Yoshida, M., and Imoto, M. (2006). Involvement of protein phosphatase 2A nuclear accumulation and subsequent inactivation of activator protein-1 in leptomycin B-inhibited Cyclin D1 expression. Oncogene *26*, 1522–1532.

Ugi, S., Imamura, T., Ricketts, W., and Olefsky, J.M. (2002). Protein phosphatase 2A forms a molecular complex with Shc and regulates Shc tyrosine phosphorylation and downstream mitogenic signaling. Mol. Cell. Biol. *22*, 2375–2387.

Valerie, K., and Povirk, L.F. (2003). Regulation and mechanisms of mammalian double-strand break repair. Oncogene *22*, 5792–5812.

Vigneron, S., Brioudes, E., Burgess, A., Labbé, J.-C., Lorca, T., and Castro, A. (2009). Greatwall maintains mitosis through regulation of pp2a. Embo j. *28*, 2786–2793.

Viniegra, J.G., Martinez, N., Modirassari, P., Hernandez Losa, J., Parada Cobo, C., Sanchez-Arevalo Lobo, V.j., Aceves Luquero, C.I., Alvarez-Vallina, L., RAmon Y Cajal, S., Rojas, J.M., et al. (2005a). Full activation of pkB/AKT in response to insulin or ionizing radiation is mediated through ATM. J. Biol. Chem. *280*, 4029–4036.

Viniegra, J.G., Martínez, N., Modirassari, P., Losa, J.H., Cobo, C.P., Lobo, V.J.S.-A., Luquero, C.I.A., Álvarez-Vallina, L., Cajal, S.R. Y, Rojas, J.M., et al. (2005b). Full activation of pkb/AKT in response to insulin or ionizing radiation is mediated through ATM. J. Biol. Chem. *280*, 4029–4036.

Walter, G., Ferre, F., Espiritu, O., and Carbone-Wiley, A. (1989). Molecular cloning and sequence of cDNA encoding polyoma medium tumor antigen-associated 61-Kda protein. Proc. Natl. Acad. Sci. U. S. A. *86*, 8669–8672.

Walter, G., Ruediger, R., Slaughter, C., and Mumby, M. (1990). Association of protein phosphatase 2A with polyoma virus medium tumor antigen. Proc. Natl. Acad. Sci. U. S. A. *87*, 2521–2525.

Wang, Q., Gao, F., Wang, T., Flagg, T., and Deng, X. (2009). A nonhomologous end-joining pathway is required for protein phosphatase 2A promotion of dna double-strand break repair. Neoplasia n. Y. N *11*, 1012–1021.

Wang, W., Seki, M., Otsuki, M., Tada, S., Takao, N., Yamamoto, K., Hayashi, M., Honma, M., and Enomoto, T. (2004). The absence of a functional relationship between ATM and Blm, the components of BASC, in DT40 cells. Biochim. Biophys. Acta bba - mol. Basis dis. *1688*, 137–144.

Wei, D., Parsels, L.A., Karnak, D., Davis, M.A., Parsels, J.D., Marsh, A.C., Zhao, L., Maybaum, J., Lawrence, T.S., Sun, Y., et al. (2013). Inhibition of protein phosphatase 2A radiosensitizes pancreatic cancers by modulating Cdc25c/Cdk1 and homologous recombination repair. Clin. Cancer res. Off. J. Am. Assoc. Cancer res. *19*, 4422–4432.

Welburn, J.P.I., Vleugel, M., Liu, D., Yates III, J.R., Lampson, M.A., Fukagawa, T., and cheeseman, I.M. (2010). Aurora B phosphorylates spatially distinct targets to differentially regulate the kinetochore-microtubule interface. Mol. Cell *38*, 383–392.

Westermarck, J., Holmström, T., Ahonen, M., Eriksson, J.E., and Kähäri, V.-M. (1998). Enhancement of fibroblast collagenase-1 (MMP-1) gene expression by tumor promoter okadaic acid is mediated by stress-activated protein kinases Jun N-terminal kinase and p38. Matrix biol. *17*, 547–557.

Williams, R.S., Dodson, G.E., Limbo, O., Yamada, Y., Williams, J.S., Guenther, G., Classen, s., glover, J.N.M., Iwasaki, H., Russell, P., et al. (2009). Nbs1 flexibly tethers ctp1 and Mre11-Rad50 to coordinate DNA double-strand break processing and repair. Cell *139*, 87–99.

Wlodarchak, N., and Xing, Y. (2016). PP2A as a master regulator of the cell cycle. Crit. Rev. Biochem. Mol. Biol. *51*, 162–184.

Wu, J.Q., Guo, J.Y., Tang, W., Yang, C.-S., Freel, C.D., Chen, C., Nairn, A.C., and Kornbluth, s. (2009). PP1-mediated dephosphorylation of phosphoproteins at mitotic exit is controlled by inhibitor-1 and pp1 phosphorylation. Nat. Cell biol. *11*, 644–651.

Xing, Y., Li, Z., Chen, Y., Stock, J.B., Jeffrey, P.D., and Shi, Y. (2008). Structural mechanism of demethylation and inactivation of protein phosphatase 2A. Cell *133*, 154–163.

Xu, y., Ayrapetov, M.K., XU, C., Gursoy-YUzugullu, O., Hu, Y., and Price, B.D. (2012). Histone H2A.Z controls a critical chromatin remodeling step required for DNA double-strand break repair. Mol. Cell *48*, 723–733.

Yan, Y., Cao, P.T., Greer, P.M., Nagengast, E.S., Kolb, R.H., Mumby, M.C., and Cowan, K.H. (2010). Protein phosphatase 2A has an essential role in the activation of gamma-irradiation-induced G2/M checkpoint response. Oncogene *29*, 4317–4329.

Yang, D.-Q., and Kastan, M.B. (2000). Participation of ATM in insulin signalling through phosphorylation of eif-4e-binding protein 1. Nat. Cell biol. *2*, 893–898.

Yang, J., and Phiel, C. (2010). Functions of B56-containing PP2As in major developmental and cancer signaling pathways. Life sci. *87*, 659–666.

Yi, R., Bogerd, H.P., Wiegand, H.L., and Cullen, B.R. (2002). Both Ran and Importins have the ability to function as nuclear mRNA export factors. Rna *8*, 180–187.

Yu, C.F., Roshan, B., Liu, Z.-X., and Cantley, L.G. (2001a). ERK regulates the hepatocyte growth factor-mediated interaction of Gab1 and the phosphatidylinositol 3-kinase. J. Biol. Chem. *276*, 32552–32558.

Yu, C.F., Liu, Z.-X., and Cantley, L.G. (2002). ERK negatively regulates the epidermal growth factor-mediated interaction of Gab1 and the phosphatidylinositol 3-kinase. J. Biol. Chem. *277*, 19382–19388.

Yu, J., Boyapati, A., and Rundell, K. (2001b). Critical role for sv40 small-t antigen in human cell transformation. Virology *290*, 192–198.

Zhang, M.J., and Dayton, A.I. (1998). Tolerance of diverse amino acid substitutions at conserved positions in the nuclear export signal (NES) of hiv-1 rev. Biochem. Biophys. Res. Commun. *243*, 113–116.

Zhang, N., Chen, P., Gatei, M., Scott, S., Khanna, K.K., and Lavin, M.F. (1998). An anti-sense construct of full-length atm cDNA imposes a radiosensitive phenotype on normal cells. Oncogene *17*, 811–818.

Zhang, W., Yang, J., Liu, Y., Chen, X., Yu, T., Jia, J., and Liu, C. (2009). PR55α, a regulatory subunit of PP2A, specifically regulates PP2A-mediated β-catenin dephosphorylation. J. Biol. Chem. *284*, 22649–22656.

Zhou, J., Pham, H.T., Ruediger, R., and Walter, G. (2003). Characterization of the Aα and Abeta subunit isoforms of protein phosphatase 2A: differences in expression, subunit interaction, and evolution. Biochem. J. *369*, 387–398.

Zimmermann, M., and Lange, T. De (2014). 53BP1: pro choice in DNA repair. Trends cell biol. *24*, 108–117.

Zimmermann, S., and Moelling, K. (1999). Phosphorylation and regulation of RAF by AKT (protein kinase B). Science *286*, 1741–1744.

Ziv, Y., Bar-Shira, A., Pecker, I., Russell, P., Jorgensen, T.J., Tsarfati, I., and shiloh, Y. (1997). Recombinant ATM protein complements the cellular A-T phenotype. Oncogene *15*, 159–167.

Zolnierowicz, S., Csortos, C., Bondor, J., Verin, A., Mumby, M.C., and Depaoli-Roach, A.a. (1994). Diversity in the regulatory B-subunits of protein phosphatase 2A: identification of a novel isoform highly expressed in brain. Biochemistry (mosc.) *33*, 11858–11867.

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