Activated Akt pathway promotes genome instability through suppression of Mre11

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Summary

Activating mutations in RAS are often found in different human cancers. The expression of an oncogene is called a driver mutation because it provides the bases for tumour initiation, but it still requires additional mutations to achieve tumour progression. 90% of invasive pancreatic ductal adenocarcinoma (PDAC) present activating KRAS mutation, in conjunction with inactivation of various tumour suppressor genes such as BRCA1, TP53, SMAD4 and CDKN2A. The most important effectors of RAS are PI3K and its downstream kinases. These function as mediators of RAS-induced cell survival and proliferation. Interestingly, concurrent mutations of RAS and PI3K/PTEN/Akt pathway have been described in the same human tumour types. Endometrial cancer, thyroid cancer and acute lymphoblastic leukemia have all been shown to harbor the simultaneous mutation of RAS gene and those encoding various members of the PI3K signalling pathway. Published data suggest that 25% of human colon cancers contain mutations in both K-RAS and PI3K-associated genes. Moreover, 60% of human PDAC show PTEN loss, due to deletions, mutations or epigenetic silencing. Despite this prevalence, the molecular mechanism for the cooperation between RAS and PI3K pathway in tumourigenesis is poorly understood.

A fundamental barrier for tumourigenesis is senescence. The activation of an oncogene such as RAS in a primary cell line drives cells into unscheduled DNA synthesis, resulting in a high frequency of stalled replication forks and DNA double strand breaks (DSBs). A DSB is one of the most deleterious lesions if unrepaired, and it is the primary trigger of oncogene-induced senescence (OIS). DSBs activate the ATM/ATR signalling pathway and senescence-associated cell cycle arrest. However, various oncogenes differ in their ability to induce senescence, for example activated Akt is a weak inducer of senescence compared to RAS. Previous work from our lab and others has suggested that the co-activation of these two oncogenes may serve to bypass certain aspects of the senescence program, but the precise mechanism by which this is achieved remains unclear.

Surprisingly, detailed cell cycle analyses in this study demonstrate that the simultaneous activation of Akt in primary fibroblasts expressing oncogenic RAS reinforces RAS-induced senescence. This correlates with an increased accumulation of unrepaired damage, which is known to directly contribute to establishment of senescence. Interestingly, the expression of activated Akt in these cells correlates with reduced expression of MRN

complex components, which in presence of RAS-induced damage impairs the activation of the checkpoint kinases. The inhibition of Mre11, the nuclease component of the MRN complex, in RAS expressing cells recapitulates the phenotype of RAS/Akt cells. Thus, Akt downregulates Mre11 to exacerbate RAS-induced DNA damage and induce a qualitatively stronger senescence.

Multiple studies have previously reported the negative regulation of DDR by Akt, however a mechanism for this has not been described. Experiments on two colon cancer lines, HCT116 and DLD1, have revealed that inactivation of PTEN/activation of Akt suppresses DDR via a reduction in MRN complex expression and activity. In these cells, the components of the MRN complex display low protein stability and are rapidly degraded by an unknown mechanism. MRN complex is central in the DNA damage response to DSBs. Its suppression impairs the activation of the two checkpoint kinases Chk1 and Chk2, which mediates the G2/M arrest, and also impairs HR repair. The inhibition of these two events severely affects cell survival in presence of DSBs, and the surviving fraction present high levels of genome instability. The use of specific inhibitors targeting S6K1 activity rescues the levels of MRN complex in these cells, suggesting a role of this kinase in DDR suppression.

Thus, the enhanced RAS-induced senescence in cells caused by Akt can be ascribed to the high levels of unrepaired damage due to the suppression of MRN complex. Despite compounding senescence, the simultaneous mutation of RAS and Akt allow the cells to acquire genome instability, which *in vivo* significantly contributes to bypassing senescence and promotes tumour progression.

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Abbreviations

4OHT	4 hydroxytamoxifen
53BP1	p53-binding protein 1
A-T	Ataxia-telangiectasia
AT-LD	Ataxia-telangiectasia-like disorder
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CCl_4	Carbon tetrachloride
CDK	Cyclin-dependent kinase
CHX	Cycloheximide
CK1	Cip/Kip inhibitor
DAPI	4',6-diamidino-2-phenylindole
DDR	DNA damage response
DMSO	Dimethyl sulphoxide
DNA-PK	DNA dependent protein kinase
DSBs	DNA double-strand breaks
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
ER-α	Estrogen receptor α
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FOXO	Forkhead box O
G1 phase	Gap 1 phase
G2 phase	Gap 2 phase
GAR	Glycine-arginine rich
GSK3	Glycogen synthase kinase 3
HAT	Histone acetyl transferase
HBD	Hormone binding domain
HDFs	Human diploid fibroblasts
HLH	Helix-loop-helix
HP1	Heterochromatin protein 1
HR	Homologous recombination
HSC	Hepatic stellate cell
ILK	Integrin like kinase
IR	Ionising irradiation
M phase	Mitosis phase

MAPKAPK2	Mitogen-activated protein kinase activated protein kinase-2
MDC1	Mediator of DNA-damage checkpoint protein 1
MIPs	Macrophage inflammatory proteins
MMEJ	Microhomology mediated end-joining
MRN	Mre11-Rad50-Nbs1
mTORC1	Rapamycin complex 1
mTORC2	Rapamycin complex 2
Myr-Akt	Myristoylated-Akt
NHEJ	Non-homologous end-joining
NK	Natural killer
PanIN	Pancreatic intraepithelial neoplasia
PDAC	Pancreatic ductal adenocarcinoma
PDGF-AA	Platelet-derived growth factor AA
PDK1	3-phosphoinositide dependent protein kinase-1
PH	Pleckstrin homology
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PICS	PTEN-induced cellular senescence
PIKK	Phosphoinositide-3-kinase-related protein kinase
PIP_2	Phosphotidylinositol 4,5-bisphosphate
PIP_3	Phosphotidylinositol 3,4,5-trisphosphate
PKC-α	Protein kinase C-α
pRb	Retinoblastoma protein
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
RPA	Replication protein A
RTK	Receptor tyrosine kinase
S phase	Synthesis phase
SA-βgal	Senescence-associated β-galactosidase
SAHF	Senescence-associated heterochromatin foci
SASP	Senescence-associated secretory phenotype
SDF	Associate DNA damage foci
SGK1	Glucocorticoid-induced protein kinase 1
ssDNA	Single-stranded DNA
TMZ	Temozolomide
TSC1	Tuberous sclerosis complex 1
TSC2	Tuberous sclerosis complex 2

Declaration

I hereby declare that the thesis which follows is my own composition, that it is a record of the work done myself, and that is has not been presented in any previous application for a higher degree.

Desirée Piscitello

Acknowledgments

"I would maintain that thanks are the highest form of thought; and that gratitude is happiness doubled by wonder" (G.K. Chesterton).

First I would like to thank Professor Dave Gillespie for providing me with this great opportunity and also for his generous support throughout my PhD. I would like to express my special gratitude to my supervisor Professor Peter Adams, for welcoming me to his lab. I am extremely thankful for the years of guidance, patience and understanding of my colourful personality. I would like to acknowledge the generosity of Cancer Research UK for providing me with the financial resources necessary for enabling this work. I would also like to thank all the members of Gillespie lab, for supporting me during the difficult times at the beginning of my PhD. I would also like to thank all the current and past members of Adams lab for creating such an excellent atmosphere for doing research.

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During these four years, I believed in the green light, the orgastic research that year by year recedes before us. It eluded me then, but that's no matter - tomorrow I will work harder, stretch out my arms farther…And then one fine morning - So I beat on, boat against the current, the mission is accomplished. I can touch the green light now (Adapted from "The Great Gatsby", F. Scott Fitzgerald).

Chapter 1: Introduction

1.1 Cell cycle and checkpoints

1.1.1 The cell cycle

The cell cycle is an orderly sequence of events by which a cell grows, replicates its DNA and then segregates it equally into two daughter cells (Tessema et al. (2004). This process is divided into four sequential phases: the synthesis phase (S phase) when DNA replication occurs, and the mitosis phase (M phase), during which the replicated DNA is divided between two genetically identical daughter cells (Figure 1.1). Interspersed between these two phases lie the two gap phases: gap 1 (G1), during which the cell is sensitive to the growth signals and prepares for DNA synthesis, and gap 2 (G2), when the cell prepares for entry into mitosis (Garrett et al., 2001; Norbury and Nurse, 1992). However, the majority of cells of the human body are not actively proliferating, but exist in a quiescent state called G0 or resting phase. This happens when the cells have not reached their homeostatic size or are in absence of mitogenic signals (Tessema et al., 2004).

Different classes of cellular proteins coordinate cell progression through the four phases of the cell cycle. Key components of this regulatory network are the cyclin-dependent kinase (CDK) family of Serine/Threonine kinases, which are activated at specific points of the cell cycle, in part, by the cyclins (Tessema et al., 2004; Vermeulen et al., 2003). Cyclin levels are regulated by their synthesis and degradation in a “cyclic” pattern during the cell cycle, thus periodically activating CDKs. Different cyclin-CDK combinations form catalytically active hetero-dimeric complexes that phosphorylate downstream targets and regulate their activity (Johnson and Walker, 1999; Sherr, 1996). In addition to this, a network of proteins negatively regulates cyclin-CDKs. These complexes can be bound by p16 and CIP/KIP (CKI proteins) forming inactive trimers (CKI/cyclin/CDK), or can be inhibited due to phosphorylation by Wee1-type kinases (Lee et al., 2001; Tyson et al., 1995).

Cyclin D (1-3)-CDK4/6 and cyclin E-CDK2 complexes drive cells into G1 phase through the restriction point to commit cells to cell cycle. Once cyclin D synthesis is stimulated by growth factors, it associates with CDK4/6 mediating the phosphorylation of the retinoblastoma (Rb) protein (Norbury and Nurse, 1992). Hyper-phosphorylation of pRb releases Rb-bound E2F factors, thus enabling the transcription of E2F target genes that are required for S phase, such as cyclins (A and E) and DNA polymerase (Winston and Pledger, 1993). These cells are now pushed by cyclin E-CDK2 complexes to transit from G1 to S phase (Blomen and Boonstra, 2007). Cyclin A-CDK2 phosphorylates different protein complexes responsible for the tight regulation of replication origins during S phase. This event ensures that the genome is correctly replicated only once (Blow and Gillespie, 2008). The progression to mitosis and its activation is then driven by the formation of cyclin B-CDK1.

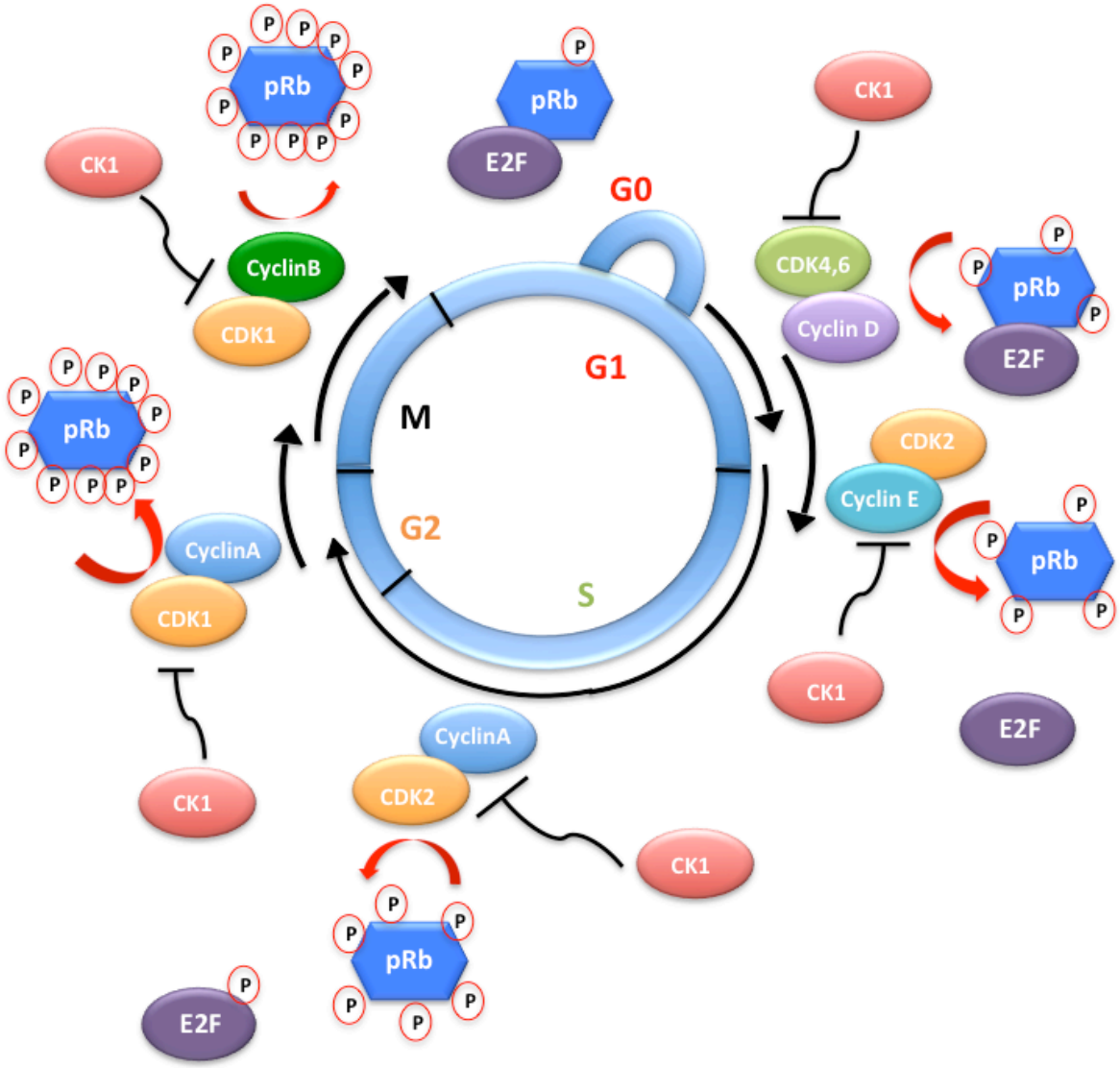


Figure 1.1: The cell cycle. A model depicting the progression of a cell through the cell cycle and the proteins that regulate the transition through each stage [Figure adapted from Zafonte et al., 2000].

1.1.2 Cell cycle checkpoints, senescence and apoptosis

Cells are continuously exposed to a variety of endogenous and exogenous insults such as replication stress, reactive oxygen species, radiation and activated oncogenes. These insults can directly damage DNA and/or cause faults in DNA replication during the cell cycle (Nurse et al., 1998; Nyberg et al., 2002). To preserve the integrity and stability of the genome, the progression from one phase to the next one of the cell cycle is tightly monitored by sensor mechanisms called checkpoints (Tyson et al., 1995). These regulatory points have the ability to transiently arrest the cell cycle and activate DNA damage response (DDR) pathways to ensure the fidelity of DNA replication and minimize the possibility of genetic damage (Yu, 2007). Key players in this process are the ATM/Chk2 and ATR/Chk1 pathways (Reinhardt and Yaffe, 2009). Their activation is crucial for the proper coordination of checkpoint activation and the DNA repair mechanisms (Smith et al., 2010). Another important factor activated in response to ATM/Chk2 and ATR/Chk1 is p53, which is pivotal in shaping the appropriate cellular outcome in response to damage (Figure 1.2). There are several cellular outcomes, most of which are severely influenced by cell type, cellular environment and the severity of the DNA damage. If the damage is not severe, the outcome will be transient cell cycle arrest due to activation of the checkpoint and appropriate repair. When the damage is unrepairable, the cell may undergo senescence, a naturally irreversible cell cycle arrest, or even programmed cell death (apoptosis). In the worst-case scenario the repair attempt fails, however the cell still survives and does not arrest permanently, which may results in accumulation of mutations or/and chromosomal aberrations, promoting the development of cancer. Thus p53 is called the "guardian of the genome", because it prevents the passage of genetic alterations to the next generation suppressing tumourigenesis (Sancar et al., 2004; Yu, 2007).

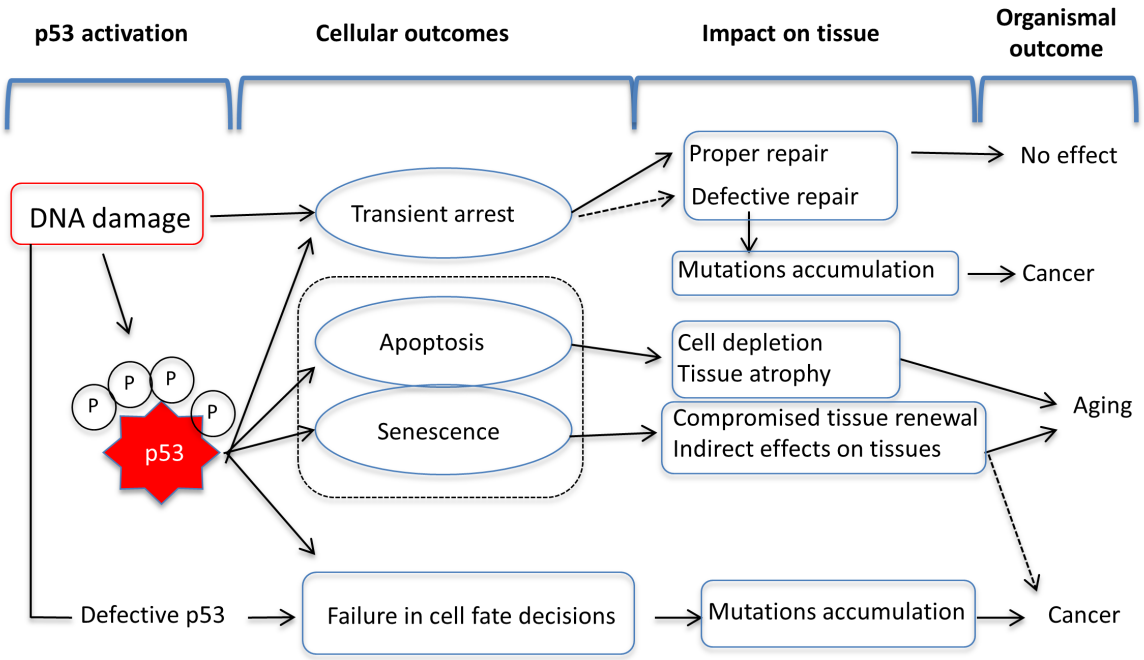


Figure 1.2: Cell cycle arrest, senescence or apoptosis? A model depicting the decision flow determining cell fate following DNA damage [Figure adapted from Rodier et al., 2007].

1.1.2.1 DNA damage and checkpoints: transient cell cycle arrest

The progression of cells from G1 to S phase is controlled by the G1/S checkpoint (Figure 1.3). Its function is to prevent cells with damaged DNA from initiating DNA replication. DNA damage activates ATM/Chk2 (in presence of DNA Double-Strand Breaks (DSBs)) and ATR/Chk1 (in presence of Single-Stranded DNA (ssDNA)), triggering a cascade of inhibitory events to block the activation of cyclin E-CDK2, thus inducing a rapid and sustained activation of G1/S checkpoint. Activated Chk1/Chk2 phosphorylates and inhibits the phosphatase CDC25A, marking it for ubiquitination and rapid degradation by proteasome (Falck et al., 2002). The lack of CDC25A locks cyclin E-CDK2 complex in a hyper-phosphorylated and inactive form, which is unable to promote the loading of CDC45 into chromatin and formation of the pre-replication complex, thereby inhibiting the initiation of DNA replication (Mimura and Takisawa, 1998). This is considered the early response, which has the role to quickly block the cell cycle for the repair of the damage. As previously mentioned, ATM/Chk2 and ATR/Chk1 proteins are also pivotal for the phosphorylation and stabilization of p53, preventing its binding with MDM2, a p53 specific ubiquitin-ligase that triggers p53 degradation (Shieh et al., 2000). p53 has a number of transcriptional targets that contribute to checkpoint activation, including the CK1A (p21). In turn p21 binds and inhibits cyclin E-CDK2 securing the maintenance of the G1/S checkpoint arrest (Mirza et al., 2003). This is considered the later response, which has the role to maintain the arrest if the damage is still not repaired.

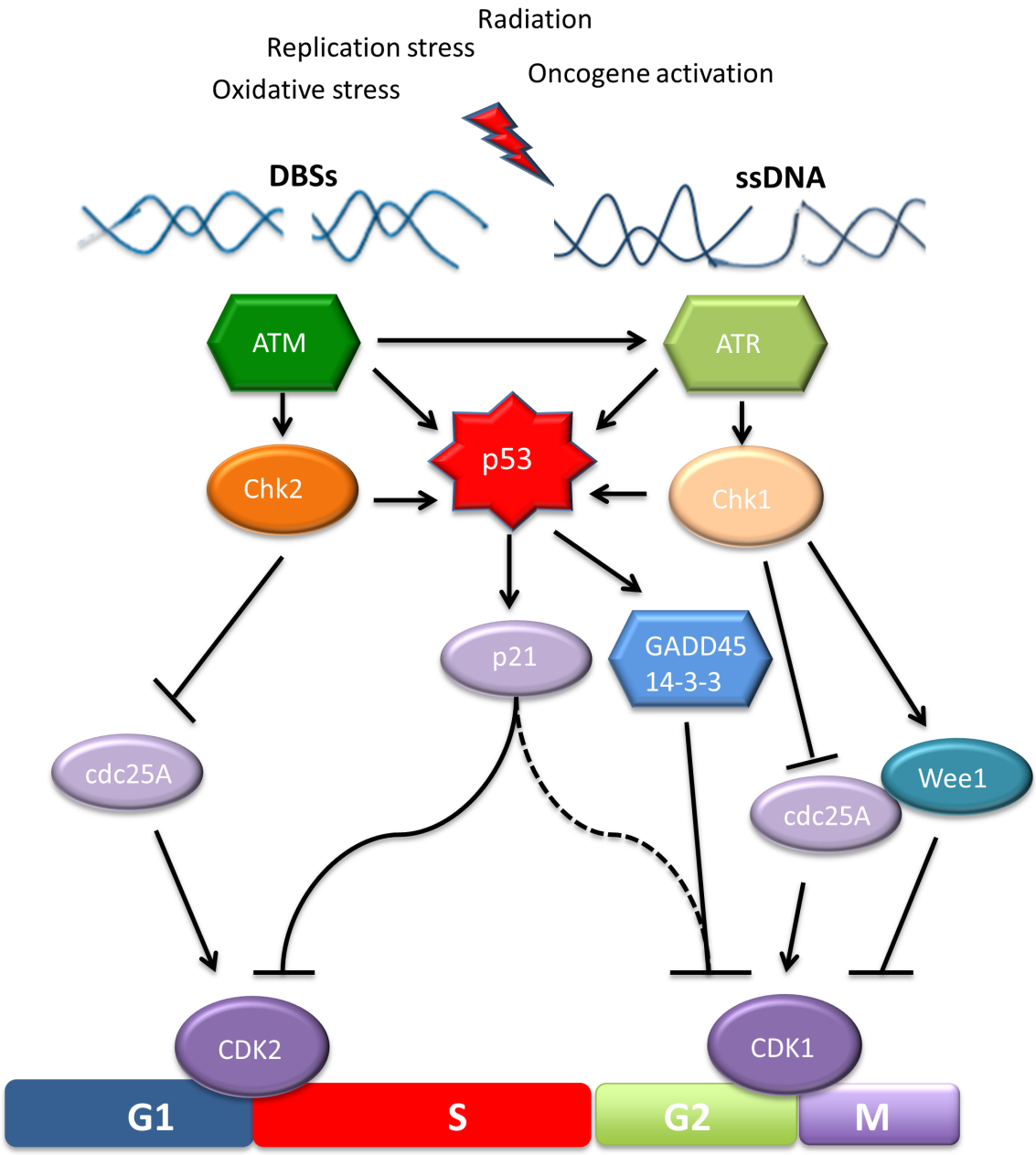


Figure 1.3: DNA damage and checkpoints. A schematic showing the checkpoints that control the progression of a cell through the cell cycle and the proteins involved [Figure adapted from Lopez-Contreras, 2012].

Stalled replication forks, latent replication origins or UV-light can cause the formation of long tracks of ssDNA during S phase. This, in turn can activate the ATR/Chk1 pathway, the major regulator of the intra-S checkpoint (Choi et al., 2010). This checkpoint has the ability to delay the onset of mitosis until DNA replication is correctly completed (Bartek and Lukas, 2003). In this case, Chk1 inhibits the S/M transition through inhibition of CDC25A, which otherwise activates cyclin B-CDK1 and entry into mitosis. However, Chk1 can also activate this checkpoint through a CDC25A/CDK1 independent pathway. The main target of this pathway is CDC45, a key protein in the regulation of DNA replication. Chk1 inhibits both CDC45 loading on the replication origins and its interaction with MCM7, affecting DNA replication initiation and elongation (Broderick and Nasheuer, 2009).

Cyclin B-CDK1 is crucial for the G2/M transition of the cell cycle. It is specifically required for mitosis as it is responsible for the disruption of the nuclear envelope and initiation of prophase. During the G2 phase, this complex is maintained in a hyper-phosphorylated inactive form by Wee1 and Myt1 kinases. Approaching M phase CDC25A/C are activated, which leads to the de-phosphorylation and activation of cyclin B-CDK1, promoting the transition to mitosis. If DNA damage occurs during G2, ATM/ATR kinases and Chk1/Chk2 kinases activate the G2/M checkpoint to prevent damaged cells from propagating. Chk1/Chk2, once activated, target the phosphorylation of CDC25A and CDC25C phosphatases. CDC25A phosphorylation on serine 123 mediates its ubiquitination and degradation (Falck et al., 2001). Instead CDC25C phosphorylation on serine 216 promotes its binding with 14-3-3 protein, causing its export from the nucleus (Peng et al., 1997). Hence, Chk1 and Chk2 cause cell cycle arrest in both G1 and G2 phases by shifting the balance of the cyclin-CDK complexes towards phosphorylated and inactive forms (Tyson et al., 1995). At the same time p53 stabilization, driven by ATM/ATR activation, stimulates the transcription of genes that contribute directly to the maintenance of the G2/M arrest. These genes include: 14-3-3 itself, GADD45 that binds and dissociates cyclin B-CDK1, and p21 inhibitor of CDKs (Falck et al., 2001). Collectively these mechanisms prevent the activation of cyclin B-CDK1 causing the arrest of the cell cycle in G2-phase.

1.1.2.2 Senescence: permanent cell cycle arrest

A checkpoint is a transient and reversible cell cycle arrest, from which cells can exit if the damage is repaired. On the contrary, senescence is described as permanent and irreversible cell cycle arrest due to unrepaired damaged DNA.

Senescence was first observed by L. Hayflick in a culture of human primary cell lines. It was described as growth arrest due to exhausted capacity of the cells to divide *in vitro* and called replicative senescence (Hayflick, 1965; Hayflick and Moorhead, 1961). However, the DSBs caused by the erosion of the telomeres, which progressively shorten every time the cell divides, was later identified as the determinant for this irreversible block of these cells in G1 phase of the cell cycle (d'Adda di Fagagna et al., 2003; Harley et al., 1990; Herbig et al., 2004). The activation of an oncogene leads to the production of high level of reactive species, more active replicons, anomalies in DNA replication forks progression and then the formation of single and double strand breaks that initiates DDR response (Di Micco et al., 2007; Moiseeva et al., 2006; Passos et al., 2010). This can lead to non-replicative, i.e. premature senescence (Di Micco et al., 2006; Mallette et al., 2007; Michaloglou et al., 2005; Serrano et al., 1997; Zhu et al., 1998). Collectively, regardless of the stimulus, the response is the activation of ATM/Chk2 and ATR/Chk1 pathways that mediate the activation of the cell cycle checkpoints associated with cellular senescence. This occurs via p53 and with the participation of p21, p16 and pRb (Schmitt, 2007). Thus, senescence is a physiological barrier against tumourigenesis that could provide a potential avenue for targeting DDR in new cancer therapies (Halazonetis et al., 2008).

1.1.2.3 Apoptosis: cell death

Cells with intolerable amount of DNA damage are eliminated by apoptosis, a form of programmed cell death. It is one of the most dramatic p53-mediated responses that has a pivotal role in various physiological processes. Not only is it important for normal development, but also for removing damaged cells in aging and is often the mechanism of action for genotoxic cancer therapies.

The induction of apoptosis by p53 includes the transcriptional upregulation of pro-apoptotic factors of BH3-only family of proteins (PUMA and NOXA), and downregulation of anti-apoptotic factors such as BCL-2 and SURVIVIN (Oren, 2003). BH3-only proteins translocate to the mitochondrial membrane where they stimulate an apoptotic-cascade (Oda et al., 2000). This cascade is further amplified by the translocation of p53 to the

mitochondria and inhibition of BCL-2 family members (Murphy et al., 2004). These events lead to the permeabilization of the mitochondrial membrane, release of cytochrome C into the cytosol, formation of the apoptosome, and ultimately causing the activation of effector caspases (Ferri and Kroemer, 2001).

1.2 DNA damage response pathways

Different type of DNA lesions can activate DDR pathway, which involves the activation of various kinases that constitute a complex network of signalling cascade (Figure 1.4). The aim is to coordinate checkpoint activation, proper repair mechanism, senescence and apoptosis, thereby preventing the transmission of any genetic lesions to daughter cells (Ciccia and Elledge, 2010). Defects in any of the DDR pathway components contributes to aging, various disorders such as development defects, and therefore, cancer (Jackson and Bartek, 2009). The two main signalling pathways activated by DDR are ATM/Chk2 and ATR/Chk1 protein kinase pathways, which respond to different DNA lesions.

1.2.1 ATM/Chk2 pathway

The ATM/Chk2 pathway is primarily activated by DSBs caused by radiation and genotoxins. ATM is a Ser/Thr protein kinase that belongs to phosphoinositide-3-kinase-related protein kinase (PIKK) family. Its importance is documented by the fact that ATM deficiency causes the genetic disorder ataxia-telangiectasia (A-T), characterized by immunodeficiency, cerebellar degeneration, radiation sensitivity, chromosomal instability and cancer predisposition (Uziel et al., 2003).

In undamaged cells, ATM exists as inactive homodimers. Following DSBs, these homodimers auto-phosphorylates in trans, resulting in partially active monomers. Three auto-phosphorylation sites have been documented for ATM. Serine 1981 was the first to be discovered and its phosphorylation is tightly associated with ATM activation in most of the circumstances (Bakkenist and Kastan, 2003). Serine 367 and 1893 are considered additional phosphorylations that may contribute to its activation (Kozlov et al., 2006). However, the precise mechanism for ATM auto-phosphorylation and activation is still unclear. Alterations in the chromatin structure surrounding a DSB have been proposed to drive the association of ATM with DNA and initiate its activation (Bakkenist and Kastan, 2003). Following ATM activation, the first event is ATM-dependent phosphorylation of the histone variant H2AX, forming the DNA damage histone mark γ-H2AX (Fernandez-Capetillo et al., 2004). This is a crucial event because it functions as signal for the

subsequent accumulation of DNA damage-response proteins (Bassing et al., 2003). The second component of the pathway is the mediator of DNA-damage checkpoint protein 1 (MDC1) that binds H2AX and acts as platform for the retention of other DNA-damage recognition/repair proteins on the chromatin (Kastan and Lim, 2000). MDC1 also contributes to the long-range phosphorylation of H2AX and the maintenance of the response (Huen and Chen, 2008). The RING-finger ubiquitin ligase RFN8 then associates to phosphorylated MDC1, and ubiquitinates H2AX inducing the accumulation of p53-binding protein (53BP1) and BRCA1 at the sites of the damage.

However, the full activation of ATM is achieved by the association of the Mre11-Rad50-Nbs1 (MRN) sensor complex, through its interaction with MDC1-Nbs1 (Berkovich et al., 2007). ATM phosphorylates a series of downstream targets, which include a multitude of proteins involved in DNA repair, cell cycle checkpoint activation and transcription (Matsuoka et al., 2007). The phosphorylation of Nbs1, BRCA1 and CTIP are crucial for the initiation of the homologous recombination (HR) repair. ATM also targets 53BP1 (Ward et al., 2003), the cohesin SMC1 (Kitagawa et al., 2004) and its kinase effector Chk2 (Bartek and Lukas, 2003). In addition to this, ATM also targets proteins localized in the nucleoplasm. ATM is important for the activation p53-mediated response to DNA damage. ATM stabilizes p53 through its phosphorylation and the stability of its regulators, MDMX and MDM2 (Chen et al., 2005a; Lavin, 2008).

Chk2 is the main kinase effector of ATM in the DDR pathway. ATM activates Chk2 via phosphorylation at Threonine 68, which is located in N-terminal serine/threonine-glutamine (SQ/TQ)-rich motif (Ahn et al., 2000). Phosphorylated Chk2 proteins can associate to each other through their fork-head associated domains, forming transient homodimers, which leads to intermolecular activation loop auto-phosphorylation and then its full activation (Ahn et al., 2002; Cai et al., 2009). Once activated, Chk2 dissociates from the DSBs and rapidly disperses as a monomer into the nucleoplasm, where it regulates the activation of substrates involved in various cellular processes (Bartek and Lukas, 2003). As previously mentioned, Chk2 not only regulates the stability of p53 and its regulator MDMX, but also inhibits CDC25 phosphatase family, thus controlling the cell cycle progression and apoptosis (Chehab et al., 2000; Chen et al., 2005a). It also regulates FOXM1 and E2F1, thereby regulating gene transcription (Stevens et al., 2003; Tan et al., 2007), and BRCA1, thus contributing to HR regulation (Zhang et al., 2004).

1.2.2 ATR/Chk1 pathway

ATR, as ATM, is a Ser/Thr kinase that belongs to PIKK family. It is activated in response to a broad spectrum of DNA damage that induces the formation of ssDNA (Ciccia and Elledge, 2010; Flynn and Zou, 2011). The formation of these ssDNA regions can occur by multiple mechanisms. For example, chemical agents can directly or indirectly affect active replication forks causing them to stall or collapse forming ssDNA. Moreover, single-stranded DNA can also be formed as DNA repair intermediate of DSBs activating ATR (Hurley and Bunz, 2007). Its unusual versatility makes ATR essential for cell survival, even in absence of genomic insults. Indeed, ATR knockout in mice results in embryonic lethality at day 7.5 (Brown and Baltimore, 2000).

Following ssDNA formation, ATR is rapidly recruited in association with its binding partner ATRIP. Tracts of ssDNA are rapidly covered with the trimeric ssDNA binding protein complex, which recruits ATRIP through its 70KD RPA1 subunit (Zou and Elledge, 2003). Like ATM, ATR activation requires an auto-phosphorylation event on Thr 1989, which occurs amongst ATR-ATRIP complexes in trans on RPA-ssDNA (Liu et al., 2011). However, its full activation is achieved by ATR interaction with two mediator proteins: TOBP1 and Claspin. TOBP1 is recruited on ssDNA via PCNA-like Rad9:Rad1:Hus1 "checkpoint clamp" (Delacroix et al., 2007). It recognizes and binds ATR at its phosphorylated Thr 1989, stimulating its kinase activity. Claspin, instead, is normally associated with the replication forks and is phosphorylated in an ATR-dependent manner within a short repeated motif (Lee et al., 2003). Its phosphorylation induces a structural modification that stimulates its binding to Chk1 (Jeong et al., 2003). Both these mediators work as a platform for ATR substrates activation.

Once Chk1 is recruited at ssDNA by Claspin and brought in proximity of ATR, it is phosphorylated at multiple S/T-Q sites within its C-terminal regulatory domain by ATR (Jeong et al., 2003). The first phosphorylation event is on Ser 317, and is specifically DNA damage induced (Niida et al., 2007). This modification is required for the subsequent phosphorylation on other sites including Ser 345, achieving its full activation. S345 is normally found phosphorylated even during an unperturbed cell cycle. A pool of activated Chk1 is normally associated to the chromatin, stimulating the transcription of growth promoting genes (Niida et al., 2007; Wilsker et al., 2008). When Chk1 is phosphorylated at both S317 and S345, it dissociates from chromatin and diffuses into the nucleus and the cytosol targeting proteins for phosphorylation (Smits, 2006).

Active Chk1 targets include multiple proteins involved in cell cycle progression, repair and gene transcription. As already mentioned, activated Chk1 regulates CDC25 family phosphatase (CDC25A and C) and Wee1 activity, controlling cell cycle transition. Chk1 mediates the inhibition of both CDC25 phosphatases, whereas Chk1-directed phosphorylation of Wee1 stimulates its activity (Lee et al., 2001). Chk1 also targets BRCA2 and RAD51 for phosphorylation to govern HR repair (Sorensen et al., 2005), and mediates H3 phosphorylation to repress gene transcription of factors that promote cell cycle progression (Shimada et al., 2008).

In conclusion, ATM/Chk2 and ATR/Chk1 pathways are activated in response to different stimuli. The ATM/Chk2 pathway is specifically activated in presence of DNA damage. On the contrary, ATR/Chk1 pathway is also found activated at low levels also during S phase of the cell cycle, ensuring the correct DNA replication (Syljuasen et al., 2005). These pathways together coordinate the cell cycle checkpoints and the DNA repair process.

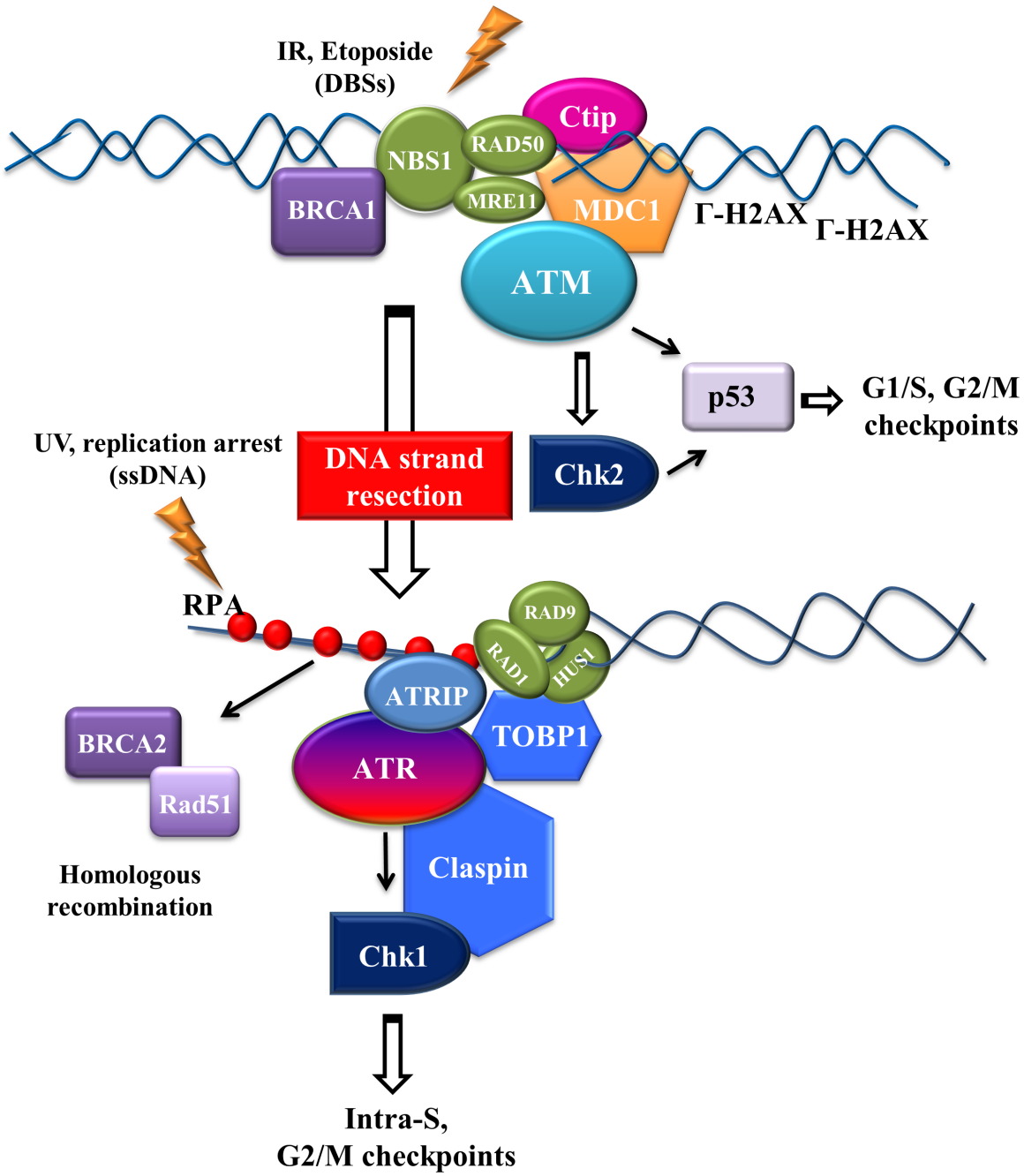


Figure 1.4: The activation of ATM/Chk2 and ATR/Chk1 pathways. A model showing the pathway involved in triggering the G1/S, intra-S or G2/M checkpoints [Figure adapted from Smith et al., 2010].

1.3 MRN complex and DNA repair mechanisms

1.3.1 MRN complex, the DSB sensor and activator

MRN complex is amongst the first proteins to be rapidly recruited on DNA-double strand breaks. It is a DSB sensor and directly binds the broken DNA independently without sequence specificity (de Jager et al., 2001b). It is a global player in DDR response, because it functions as co-activator of DSB-induced cell cycle checkpoint signalling, and an effector of both HR and non-homologous end-joining (NHEJ) repair mechanisms (D'Amours and Jackson, 2002; Zha et al., 2009). MRN complex is also responsible for the telomeres maintenance, thus contributing to genome integrity (Assenmacher and Hopfner, 2004). Moreover, MRN complex has also been found associated with the chromatin during unperturbed cell cycle, preventing the accumulation of DSBs during replication (Mirzoeva and Petrini, 2003).

The MRN complex is formed of three proteins: Mre11, Rad50 and Nbs1. Mre11 and Rad50 are well conserved in all taxonomic kingdoms, instead Nbs1 is specific to eukaryotes (Stracker et al., 2004). Mre11 is the key component of the complex, because it contains numerous biochemical activities (de Jager et al., 2001a; Usui et al., 1998). It has DNA nuclease, strand dissociation and strand annealing activities, which are all crucial for HR and NHEJ repair (Milman et al., 2009; Zhuang et al., 2009). The DNA nuclease activity is 3'➔5' endo and exonuclease that works in association with both DSBs and ssDNA (Trujillo and Sung, 2001; Trujillo et al., 1998). However, in order to repair DSBs by HR, the 5'-terminated DNA strand must be resected to produce the 3' ssDNA overhang. Mre11 lacks of the 5'➔3' nuclease activity to catalyse this, suggesting the involvement of other DSB processing factors (Farah et al., 2009). Mre11 is able to form dimers and large multimers with itself (Desai-Mehta et al., 2001), and can interact independently with both Rad50 and Nbs1 (Johzuka and Ogawa, 1995). It also has an intrinsic capacity to bind DNA that is stimulated by Rad50 on its own or in combination with Nbs1 (Paull and Gellert, 1999).

Rad50 belongs to a group of proteins known as the structural maintenance of chromosome (SMC) proteins, because they regulate chromosome condensation and sister-chromatid cohesion (Hirano, 2002). This protein contains Walker A and B nucleotide (NTP)-binding motifs (ATPase domains) at the N and C terminus respectively (Alani et al., 1989). Rad50 proteins associate to form homodimers, which in turn bind Mre11 dimers forming

heterotetramers called the “core” complex (Figure 1.5). The function of the core complex is to mediate the spatial juxtaposition of DNA molecules to achieve spatial proximity more conducive for homology-mediated repair (Hopfner et al., 2002). Mre11 dimers mediate short-range DNA end interactions, whereas Rad50 dimers, with the two large coiled arms, mediate long-range interactions. Rad50 association with Mre11 also stimulates its endo and exonuclease activity (Paull and Gellert, 1998).

The last component to associate to the core complex is Nbs1. It contributes to checkpoint activation and DSB repair functioning as an adaptor for various protein-protein interactions. Indeed, Nbs1 consists of an FHA and two BRCT domains essential for recognising phosphorylated proteins involved in DDR response, most importantly ATM (Lloyd et al., 2009). However, Nbs1 is itself phosphorylated by ATM at these breakage sites as part of the DDR response (You et al., 2005). Nbs1 retains also an Mre11-binding domain. Its association with the $Mre11_2$-$Rard50_2$ core complex not only is fundamental for MRN complex translocation from the cytosol to the nuclei, but also for stimulating its DNA binding capacity and endonuclease activity (Desai-Mehta et al., 2001; Paull and Gellert, 1999).

The role of MRN complex in DDR signalling is to activate it, however, the master regulator of cellular response to DSBs is ATM (Shiloh, 2003b). MRN leads to the activation of ATM in a two-step process. MRN binds DSBs and this leads to an increase in the local concentration of DNA ends to such a level that induces dimeric ATM to dissociate into monomers. These monomers are then recruited at the DSBs by Nbs1-mediated interaction, which at the same time stimulates ATM enzymatic activity leading to its auto-phosphorylation (Dupre et al., 2006).

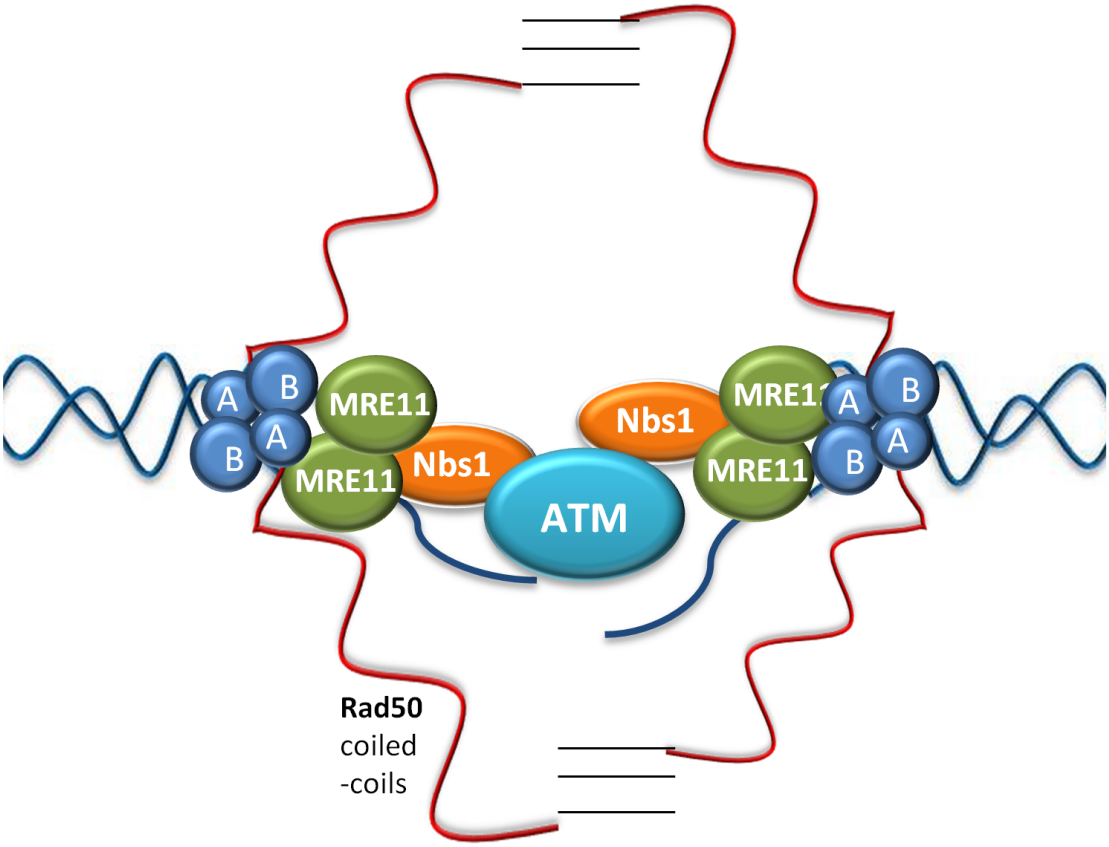


Figure 1.5: MRN complex assembly at a double strand break. A model depicting the assembly of the core subunits of the MRN complex and its associated proteins at a DSB [Figure adapted from Nakamura et al., 2010].

1.3.2 DSB repair mechanisms

DSBs are the most toxic and deleterious form of DNA damage, as both of the DNA strands are broken simultaneously. They are really dangerous lesions because they leave cells without an intact template to use for the repair. Moreover, these broken DNA ends can also subjected to further physical and chemical assault, which can cause the loss or the damage of bases or formation of abnormal DNA structure, all them concurring in loss of genetic information (Hiom, 2010). Various types of insults can cause DSBs. Among those, radiation or radiomimetic drugs cause DSBs by generating free radicals that attack the deoxyribose, whereas DSBs can also arise from the inhibition or failed DNA end ligation by topoisomerase II (Jackson and Bartek, 2009). Any errors during DNA replication or replication during the repair of single stranded DNA breaks is another source of DSBs in cells. DSBs can also originate due to free-oxygen radicals formed by normal aerobic metabolism (Chance et al., 1979).

Two major pathways are used by the cells to repair these DNA lesions, which differ in template and fidelity requirements: HR and NHEJ (Figure 1.6) (Pardo et al., 2009). The NHEJ is the most used pathway in mammalian cells for repairing DSBs, as it can operate quickly and through all cell cycle phases (Rothkamm et al., 2003). However it is an error-prone mechanism, since it mediates the ligation of broken DNA ends together without considering their homology, often generating deletions or insertions (Lieber, 2010). An alternative repair mechanism called microhomology mediated end-joining (MMEJ) is used when NHEJ cannot occur, but this is also prone to errors (McVey and Lee, 2008). Contrary to these, HR is a more accurate repair mechanism because it uses a homologous template for repairing the damage. Since a sister chromatid is usually used as template, this pathway acts on post-replicative DNA in S and G2 phase of the cell cycle (Moynahan and Jasin, 2010; San Filippo et al., 2008).

1.3.2.1 HR repair mechanism

The MRN complex has a pivotal role in the initial processing of a DSB, although its nuclease activity works in the opposite polarity of that required for HR. Thus, additional factors are required to work in conjunction with MRN complex for the 5'-end nucleolytic resection (Rupnik et al., 2010). A recent study in yeast has proposed a bidirectional model, by which MRN complex could overcome this problem. In the first step, the endonuclease Sae2 (orthologl of the mammalian Ctip) stimulates the endonuclease activity of Mre11 to mediate a cleavage of double-stranded DNA downstream of the DSB to form an intermediate. In the second step, MRN complex then proceeds back to dsDNA using its 3´ to 5´ exonuclease activity (Cannavo and Cejka, 2014). Following this, nucleases ExoI and RECQ1, in conjunction with Dna2 are recruited to carry out the long-range resection in the 5´ to 3´ direction to facilitate the generation of the 3´-single stranded DNA (Bernstein and Rothstein, 2009; Mimitou and Symington, 2009; Sartori et al., 2007). The resultant ssDNA is then rapidly coated by the ssDNA-binding protein RPA, in order to avoid the formation of secondary structures (Starczynowski et al., 2010). BRCA2 then mediates the displacement of RPA from the ssDNA, allowing the binding of RAD51 recombinase, which associates in multimers to form a nucleoprotein filament. BRCA2 is essential for this step, since RPA has higher affinity than RAD51 in binding ssDNA. RAD51 together with various HR factors mediates the search for the homologous sister chromatid, followed by DNA strand invasion and finally extension of the DNA end using intact homologous sequence as a template. Lastly, the second end of the broken DNA is captured and the junctions are solved by the activities of DNA polymerases δ/η, DNA ligase I, DNA helicase and resolvase enzymes (Mazon et al., 2010; Pardo et al., 2009).

The strand resection step is regulated in a cell cycle dependent manner. Since HR is mainly used to repair DSBs in S and G2 phases, resection is indeed tightly regulated by S/G2 phase CDKs, ensuring maximal resection activity. For example, it has been shown that Ctip becomes more abundant in S phase and its enzymatic activity is directly regulated by CDK-mediated phosphorylation. Ctip upregulates DNA-ends resection, which in-turn increases the number of DSBs repaired by HR (Huertas and Jackson, 2009; Sartori et al., 2007; Yun and Hiom, 2009). Nbs1 is also phosphorylated by cyclin B-CDK1 in S/G2/M phase, potentiating HR-mediated resection (Falck et al., 2012). However, the cell cycle regulation of the resection mechanism is insufficient to ensure the safe use of HR. In addition to this, SMC proteins help to restrain HR repair to the sister chromatids, thus avoiding its use on other sequences (Cortes-Ledesma et al., 2007; Covo et al., 2010). The

mutation of most proteins involved HR repair predispose to cancer, suggesting the critical role of HR in the maintenance of genome stability.

1.3.2.2 NHEJ repair mechanism

MRN complex is also required in the NHEJ pathway and functions within the first step of this pathway. The ring hook domain of RAD50 is thought to assist Ku70/80 and DNA-PK in tethering DNA ends and bringing them in proximity to each other (Hopfner et al., 2002). NHEJ is a relatively simpler DSB repair pathway than HR and it is mediated by a different set of proteins. Ku70 and Ku80 combine to form a heterodimer that binds with high affinity to the two-ended DSBs promoting the alignment of the two DNA ends (Cary et al., 1997; Walker et al., 2001). The complex then recruits the catalytic subunit of the DNA dependent protein kinase stimulating its kinase activity (DNA-PKcs) (Ciccia and Elledge, 2010; Yaneva et al., 1997). DNA-PK once active together with the nuclease Artemis can trim the DNA ends, or with DNA polymerases (Polμ or Polλ) can fill in to create compatible ends. Finally, the XRCC4, Xrcc4 like factor, and DNA ligase IV form the ligation complex that, through its interaction with Ku-DNA, ligates the DNA ends completing the repair process (Grawunder et al., 1997; Hentges et al., 2006).

Altogether, the activation of repair pathways following DNA damage is critical for genome stability and tumour prevention. This event is influenced by different factors, such as cell cycle phase, complexity of the breaks, genetic factors or even genome complexity. In higher eukaryotes, the genome is characterized by a large number of highly repetitive sequences making HR prone to misalignment (Karanam et al., 2012). Indeed, mammals tend to use NHEJ as a preferred mechanism to repair DSBs, whereas HR is more often used in organisms where the genome has low amount of repetitive sequences. However, HR is mainly used if the damage occurs in S and G2 phases of the cell cycle (Brandsma and Gent, 2012; Karanam et al., 2012). In conclusion, the choice of the correct pathway for repairing that type of damage is crucial and can be irreversible, and wrong decisions may lead to dramatic consequences.

1.3.2.3 MMEJ repair mechanism

MMEJ is used as an alternative repair mechanism to NHEJ, when polypeptides bound to the broken DNA ends hamper Ku proteins from binding them. It is a poorly defined mechanism, which requires the nucleolytic excision of the blocked DNA followed by the resection of one of the strand until a 5-20 nucleotide long complimentary sequence is found. This event is called a microhomology search and is necessary to achieve the base-pairing required to stabilize the DNA ends. The displaced DNA ends are then removed and ligation occurs (McVey and Lee, 2008). Both MRN complex and Ctip have been reported to play a pivotal role in this repair mechanism, but their exact functions are still not clear (Yun and Hiom, 2009). However, like NHEJ, MMEJ is error-prone and implies the loss of genetic information.

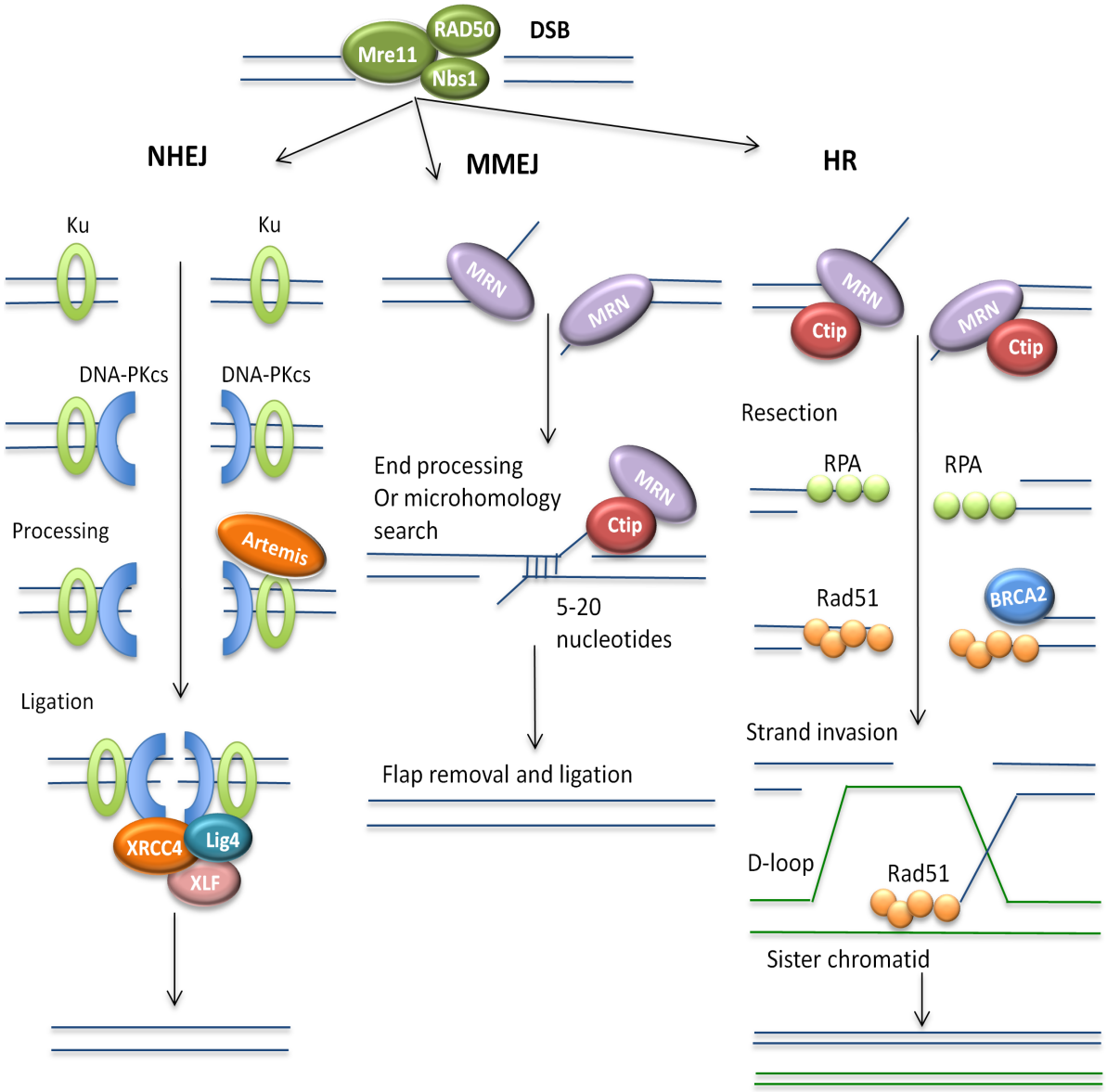


Figure 1.6: DSB repair mechanisms. A model showing the mechanism and proteins involved in HR, NHEJ and MMEJ repair pathways [Figure adapted from Brandsma and Gent, 2012].

1.4 Alterations in DDR and cancer

DDR plays a crucial role in the maintenance of genome stability. In response to DNA damage, cells normally arrest or die. However, mutations in apoptosis, DDR-response pathway or mitotic-checkpoint genes allow the survival and even the proliferation of cells with genomic abnormalities, promoting oncogenic transformation. Therefore, loss of function mutations in DDR response genes, involved in both checkpoint and repair regulation, results in human cancer predisposition syndromes and cancer (Kastan and Bartek, 2004). Thus, DDR pathway is a powerful anti-cancer barrier that blocks oncogenic progression (Halazonetis et al., 2008).

1.4.1 DDR component mutations predispose to cancer

ATM is the master regulator of all the cellular responses to DSBs. Therefore, germline mutations in ATM with loss of function cause the genetic disorder ataxia-telangiectasia (A-T). As in humans, ATM-null mice are predisposed to develop T-cell lymphoma, whereas loss of ATM in APC mutant mice increases the incidence of intestinal tumours (Barlow et al., 1996; Liao and Van Dyke, 1999; Xu et al., 1996). Interestingly even though A-T is a recessive disease, heterozygous mutations for ATM make the cells more sensitive to DSB-inducing agents. These cells are characterized by high chromosomal instability and radioresistance to DNA synthesis, suggesting an inability to fully activated intra-S checkpoint (Shiloh, 2003a; Swift et al., 1991). However, ATM is not the only protein in which mutations cause syndromes and predisposition to cancer.

It has been shown that the inactivation of components of the MRN complex not only severely affect DSB processing (HR and NHEJ repair) and checkpoint regulation, but also DNA metabolic processes, telomere maintenance and the formation of meiotic DSBs (van den Bosch et al., 2002). This suggests that MRN complex is fundamental for several processes in cells. Germline knockout of Mre11 or Rad50 or Nbs1 genes in mice is embryonically lethal (Luo et al., 1999; Xiao and Weaver, 1997; Zhu et al., 2001). Hypomorphic mutations of Nbs1 cause the Nijmegen Breakage Syndrome (NBS), whereas hypomorphic mutations of Mre11 cause a milder form of A-T disease, called Ataxia-Telangiectasia-like disorder (AT-LD). Both these syndromes show similar cellular and animal phenotypes to A-T disease, such as hypersensitivity to radiation, immunodeficiency and predisposition to cancer (Shiloh, 1997). NBS patients are also found to have mutations in Rad50. Altogether, these observations suggest how important the MRN complex is in

orchestrating the right response to DNA damage, and in particular for DSB-specific ATM activation (Shiloh, 2003a). In sum, all three components of the MRN complex are linked to cancer, presumably due to the central role of this complex in ensuring genome stability.

Mutations in other components of DDR signalling downstream of ATM and MRN complex can also lead to cancer predisposition. Chk2 knockout mice don't develop tumours spontaneously, however exposure to carcinogens increases the risk of skin tumours (Hirao et al., 2002). Surprisingly, tumours originated from these individuals don't tend to lose the other Chk2 allele, suggesting a non-canonical role of Chk2 as tumour suppressor (Antoni et al., 2007). Chk1 is also considered to be a tumour suppressor gene. Chk1 knockout mice are not viable and it has been hypothesized that a haploinsufficiency of Chk1 contributes to tumourigenesis. This mechanism was studied by the conditional knockout of Chk1 in mammary epithelial cells. The disruption of Chk1 gene was found to cause inappropriate entry of cells into S phase, accumulation of damage during DNA replication and abnormal progression to mitosis (Lam et al., 2004). These observations indicate that defects in checkpoint activation caused by Chk1 or Chk2 haploinsufficiency predispose cells to cancer (Tho et al., 2012).

Inheritance of one or both mutated alleles of BRCA1 or BRCA2 is associated with high risk of developing breast and ovarian cancers (O'Donovan and Livingston, 2010). Differently from Chk1 and Chk2, BRCA1/2 is a canonical tumour suppressor, whereby loss of both alleles is observed in tumours. BRCA proteins are activated in response to DSBs by ATM, Chk2 and ATR, thus contributing to intra-S and G2/M checkpoint regulation. Specifically, BRCA proteins are necessary for HR repair during those phases of the cell cycle. Therefore, BRCA deficient tumours are characterized by HR deficiency, whereas tissue surrounding it is HR proficient (Turner et al., 2005).

However, an altered DDR response pathway is not sufficient for cancer development, implying that additional mutations are critical for promoting viability or growth and proliferation of pre-cancer cells. Hypomorphic mice for Mre11 show high level of genomic instability, but are not prone to tumourigenesis (Liu et al., 2004). However, the additional mutation of p53 helps tumour development, suggesting that the combination of genomic instability and checkpoint defects contribute significantly to tumour progression.

In conclusion, defects in DDR pathway as a result of genetic mutations of its components cause predisposition to cancer. In addition to this, alterations in proteins that regulate the

DDR pathway can also lead to a similar predisposition. Mounting evidences have shown that the PI3kinase pathway is involved in the modulation of DNA damage response and genome instability in many complex ways.

1.5 PI3K/Akt/mTOR signalling, DDR and cancer

Phosphoinositide 3-kinase (PI3K), Akt and mammalian target of rapamycin (mTOR) signalling network has a central role in several cellular processes critical for cancer progression, such as survival, growth, metabolism, differentiation, motility and genomic instability (Hanahan and Weinberg, 2011). It is recognised to be one of the most mutated pathways in cancer (Engelman et al., 2006; Samuels et al., 2004).

1.5.1 The PI3K/Akt/mTOR signalling network

PI3K belongs to the PI3K family of protein kinases, which includes ATM and ATR. There are three classes of PI3K grouped according their structure and function, of which class I^A is the most mutated one (Yuan and Cantley, 2008). PI3K is characterized by a catalytic subunit called p100 (or also PIK3CA) and a regulatory domain called p85 (or also PIK3R). PI3K is activated by numerous factors such as cytokines and growth factors that bind receptor tyrosine kinases (RTKs), G-protein coupled receptor or integrins (Kang et al., 2006; Zhao and Vogt, 2008). Following stimuli, the regulatory p85 subunit recognizes and binds the phosphotyrosine residues on RTK relieving its intermolecular inhibition of p110 the catalytic subunit (Carpenter et al., 1993). PI3K can be also activated in a receptor-independent mechanism, for example by direct binding of RAS to p110 (Shaw and Cantley, 2006). PIK3CA is the second most often mutated oncogene in human cancer, resulting in its increased enzymatic activity. However, oncogenic lesions upstream in the tyrosine kinase and/or RAS can also cause the constitutive activation of PI3K signalling (Engelman, 2009).

Activated PI3K at the plasma membrane initiates a multi-step PI3K-mediated signalling process (Figure 1.7). PI3K phosphorylates PIP_2 (phosphotidylinositol 4,5-bisphosphate) converting it to PIP_3 (phosphotidylinositol 3,4,5-trisphosphate). This reaction is antagonized by the phosphatase and tensin homolog deleted on chromosome 10 (PTEN), blocking PI3K-mediated signalling. PIP_3 acts by forming a docking site for pleckstrin homology (PH)-containing proteins, such as Akt and 3-phosphoinositide dependent protein kinase-1 (PDPK1) (Ericson et al., 2010). Akt, once translocated to the plasma membrane, is phosphorylated by PDPK1 at Threonine 308. However, additional phosphorylation at a

secondary site Serine 473 is required for its full activation. This can be catalysed by target of rapamycin complex 2 (mTORC2), however other molecules like Integrin kinase (ILK) and Mitogen-Activated protein Kinase Activated protein kinase-2 (MAPKAPK2) are also able to phosphorylate this residue of Akt. Akt is the central protein kinase in mediating PI3K-signalling and active Akt modulates the function of numerous substrates to regulate multiple cellular events.

1.5.1.1 Akt, a multifunction protein kinase

Akt (also known as PKB) belongs to a family of Serine/Threonine protein kinases. Three members characterize this family, which are tissue-specific: Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ) (Brodbeck et al., 1999; Cheng et al., 1992). Despite being produced by three different genes, they share 80% homology of amino acid sequence, suggesting similar substrates specificities (Vanhaesebroeck and Alessi, 2000). All three isoforms have the same protein structure that contains a pleckstrin homology domain (PH) domain, which mediates Akt binding to the plasma membrane, a central kinase domain, and a regulatory domain typical of the cAMP-dependent protein kinase A/G superfamily of protein kinases (Song et al., 2005).

Activated Akt regulates many cellular events controlling many targets by either direct phosphorylation, or indirectly by regulating their expression levels. For example, Akt promotes cell survival through the phosphorylation-mediated inhibition of pro-apoptotic Bcl2-family proteins BAD and BAX (Engelman et al., 2006). This pro-survival effect is reinforced by Akt-mediated MDM2 activation and then p53 inhibition. In addition to this, Akt also negatively regulates the pro-apoptotic forkhead box O (FOXO) transcription factors (Majumder and Sellers, 2005).

mTORC1 is one of the most important Akt targets and its activity relies on the nutrient status of the cells. Under nutrient sufficiency, it regulates cell growth by promoting metabolic processes such as anabolism and biosynthesis, whereas it suppress catabolic processes such as autophagy (Laplante and Sabatini, 2012). Akt inhibits tuberous sclerosis complex (TSC1/2) by phosphorylation of TSC2, thus relieving the inhibition of Rheb GTPase, which is then free to activate mTORC1. Akt can also inhibit the glycogen synthase kinase 3 (GSK3), which phosphorylates TSC2, reinforcing its inhibitory effect on TSC complex. Moreover, mTORC1 activation can also be fine tuned by other inputs such as growth factors, energy sensors and cellular stresses (Laplante and Sabatini, 2012).

mTORC1 activates p70-S6 kinase that then phosphorylates S6 protein and 4E-BP1, thus inducing protein synthesis (Engelman et al., 2006). It also inhibits autophagy by preventing the formation of the autophagosome by inhibiting phosphorylation of its components ULK1 and ATG13 (Chan, 2009).

Lastly, Akt signalling pathway also contributes to regulating cellular progression through an unperturbed cell cycle. Akt controls G1/S transition by positive regulation of cyclin-CDK complexes by phosphorylation and inactivation of CKIs such as p21 and p27 (Xia et al., 2011). Akt also protects cells from apoptosis during G2/M transition by phosphorylation of CDK1 activators and inhibitors, promoting entry into mitosis (Baldin et al., 2003; Karlsson et al., 1999). In summary, Akt is a fundamental protein for promoting survival and cell cycle progression, thus its overexpression or overactivation may play a central role in cancer.

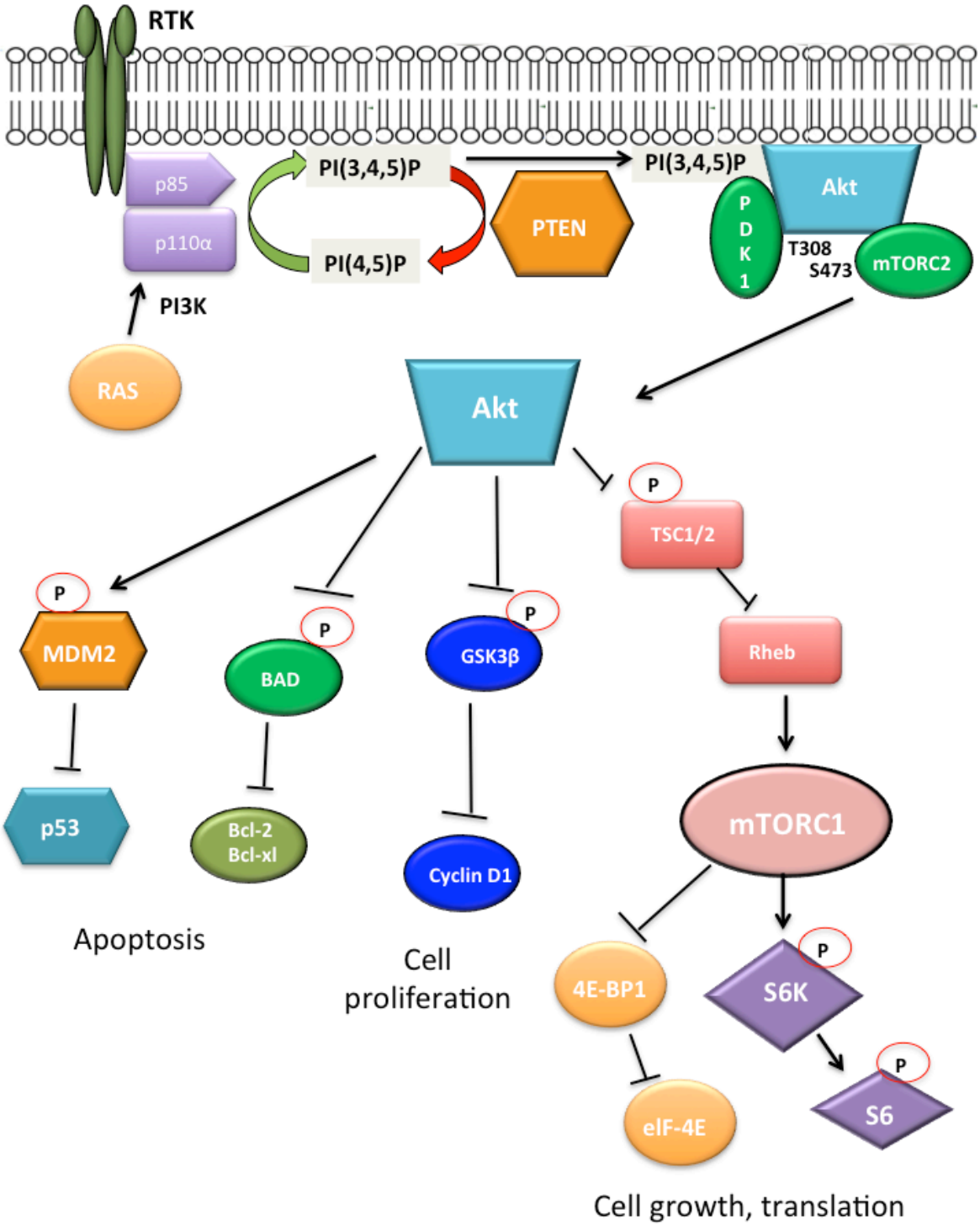


Figure 1.7: PI3K activation and its signalling pathway. A schematic showing the PI3K signalling pathway, the proteins involved and achieved cellular outcome [Figure adapted from Yap et al., 2008].

1.5.1.2 Akt, DDR and cancer

It has been shown that several components of PI3K/Akt/mTOR pathway can also be phosphorylated in response to DNA damage (Matsuoka et al., 2007). PIKK family members can mediate the phosphorylation and activation of Akt, thus providing a pro-survival signal by triggering cell cycle arrest or inhibiting apoptosis (Bozulic et al., 2008; Caporali et al., 2008; Lu et al., 2006; Viniegra et al., 2005). For example, following γ-irradiation DNA-PK phosphorylates Akt at Serine 473, which then forms nuclear foci together with DNA-PK at DNA double-strand breaks. Following this, Akt induces a transcriptional program that promotes survival in response to DNA damage (Bozulic et al., 2008). Other data have shown that ATM also interacts with Akt targeting it for phosphorylation at S473 in response to stimuli such as γ-irradiation or insulin (Viniegra et al., 2005). Interestingly, it has also been reported that following treatment with temozolomide (TMZ), a therapeutic methylating agent, ATR is required for phosphorylation of Akt at S473 (Caporali et al., 2008).

Thus, Akt promotes cell survival in presence of damage induced by various genotoxic stresses through inhibition of apoptosis. Accordingly, positive regulators of Akt activity are often found upregulated and PTEN lost or inactivated by mutations in cancer. Increased Akt activity is associated with cellular resistance to radiotherapy in a broad variety of cancers such as glioblastoma, prostate, colon, breast and cervix (Brognard et al., 2001; Kim et al., 2006). However, the role of Akt in radiation resistance is not only dependent on its anti-apoptotic activity. Akt has indeed been reported to modulate DNA repair, hence affecting the efficacy of radiation therapy by several possible mechanisms (Kao et al., 2007; Toulany et al., 2008; Tran et al., 2002). Several studies have shown that activated Akt could contribute to resistance to radiotherapy by stimulating error-prone NHEJ repair and promoting cell survival by interaction with DNA-PK. This seems to be a reciprocal interaction, where they phosphorylate each other in a positive feedback loop, stimulating high repair efficiency (Bozulic et al., 2008; Toulany et al., 2008). In breast cancer cell lines, high levels of Akt affect BRCA1 nuclear localization at DNA foci inducing its cytoplasmatic retention (Plo et al., 2008; Tonic et al., 2010). Thus, Akt can affect DNA processing by the promotion of error-prone NHEJ and inhibition of the initiation of HR repair following DNA damage.

Akt can also promote genome instability by other mechanisms. Evidences show that hyper-activated Akt is able to abrogate DNA damage induced G2/M checkpoint upon exposure to genotoxic stresses. It has been shown that Akt can phosphorylate Chk1 at Serine 280 inhibiting its activation and its execution of the checkpoint (Kandel et al., 2002). In addition, Akt is able to reduce Chk2 recruitment at sites of DNA lesions and its activation in glioblastoma cells (Hirose et al., 2005).

In summary, alterations in PI3K pathway usually result in production of constitutively active Akt, which enhance its oncogenic activity. This seems to be strongly linked to cell transformation, as demonstrated by high frequency of hyper-activated Akt in tumours. Numerous evidences have shown that Akt may have a crucial role in negatively regulating checkpoint activation and fidelity of DNA repair, thus directly affecting genome stability. Therefore, an activated Akt signalling pathway may contribute to tumourigenesis and tumour survival by several mechanisms. However, further work clarifying the exact mechanism by which Akt exerts these functions will be particularly important for cancer therapy.

1.5.1.3 mTOR, DDR and cancer

mTOR is a Serine/Threonine kinase related to PI3K family and it exists in two complexes: mTORC1 and mTORC2 (Hara et al., 2002). Both these are large multisubunit complexes that have distinct binding partners and specific substrates. Moreover, they are differently sensitive to rapamycin and are differently regulated (Sarbassov et al., 2004; Zoncu et al., 2011).

mTORC2 is composed of the rapamycin-insensitive partner (Rictor), Sin1, GβL and Deptor. The functions of this complex are less understood compared to mTORC1. It's thought to modulate the signalling of growth factors by phosphorylation of the C-terminus of several members of the AGC subfamily of kinases such as Akt, glucocorticoid-induced protein kinase 1 (SGK1) and protein kinase C-α (PKC-α) controlling cell survival, metabolism and cytoskeletal organization (Jacinto et al., 2004; Sarbassov et al., 2004; Sarbassov et al., 2005).

The rapamycin-sensitive mTORC1 is formed by a regulatory protein Raptor, an associated protein LST8, and an mTOR-interacting protein Deptor (Efeyan et al., 2012). mTORC1 functions as integrator of signals from five different major intracellular and extracellular cues, such as nutrients, growth factors, energy levels, stress and oxygen, to regulate major

processes such as autophagy and lipid/protein synthesis. mTORC1 promotes protein synthesis by phosphorylation of the translation initiation factor 4E-BP1 (eIF4E-binding protein) and S6K1/2. 4E-BP1 phosphorylation prevents its binding to cap-binding protein eIF4E, which binds and forms the eIF4F complex stimulating the initiation of the translation. Instead through S6K activation, mTORC1 controls the biogenesis of a variety of mRNAs, as well as the initiation and elongation of the translation (Ma and Blenis, 2009; Ruggero et al., 2004).

Mounting evidences have shown that mTOR is a critical mediator of the cellular responses to many stresses including DNA damage. In normal conditions p53 is the central regulator of DNA damage response (Riley et al., 2008; Vousden and Ryan, 2009). It has been reported that p53 in these conditions mediates mTORC1 repression by different mechanisms (Feng et al., 2005). p53 induces transcription of PTEN, TSCs and Redd1, all of which inhibit mTORC1 activity (Ellisen et al., 2002; Feng et al., 2005; Stambolic et al., 2001). It also transactivates Sestrin1 and Sestrin2 that inhibit mTORC1 by AMPK-dependent regulation of TSC1/TSC2 complex (Budanov and Karin, 2008). In addition to this, p53 also decreases mTORC1-mediated translation initiation by affecting the phosphorylation and activation of the 4E-BP1 along with the activity of the p70-S6k (Horton et al., 2002). Contrary to this, it has also been reported that mTOR/S6K1 regulates the p53-mediated DNA damage response. S6K1 is able to phosphorylate and inhibit MDM2, thus inducing p53 activation and response to DNA damage. S6K1/MDM2 forms a complex that adjusts DNA damage response to the growth conditions (Lai et al., 2010). Several evidences have shown that mTOR has a role in S phase of the cell cycle, suggesting the requirement of TORC1 function in response to DNA replication stress. Therefore, treatment of several p53 null cell lines with rapamycin induces apoptosis that coincides with the entry into S phase (Huang et al., 2001). Moreover, the use of rapamycin enhances the DNA-damaging agent cisplatin (Shi et al., 1995). These observations suggest that mTOR has an important role in cell survival in response to aberrations in DNA replication (Shen et al., 2007a).

In summary, mTOR is important for many cellular processes that are crucial for cancer cell growth, survival and proliferation. Therefore, many components upstream and downstream of mTOR are found mutated in cancer. mTOR activity is altered in nearly 100% of advanced human prostate cancer (Taylor et al., 2010). Constitutive activation of mTOR by deficiency of TSC1 and TSC2 causes the rare genetic disease tuberous sclerosis, in which patients develop benign tumours in many organs including the brain (Guertin and Sabatini,

2007). Therefore, diverse cancer therapies use rapamycin and its analogues targeting the activated mTOR pathway. Interestingly, it has been reported that the inhibition of mTOR signalling by rapamycin or S6K deficiency significantly extends lifespan in mice suggesting the crucial role of mTOR/S6K signalling in ageing (Harrison et al., 2009; Selman et al., 2009). In conclusion, the correct regulation of mTOR pathway is pivotal for protecting cells from both tumourigenesis and ageing-associated disease.

1.6 Senescence

Senescence is an irreversible growth arrest that is associated with morphological and gene expression changes which cause resistance to apoptosis (Beausejour et al., 2003; Cristofalo et al., 2004; Seluanov et al., 2001). It is a potent natural barrier to tumourigenesis in response to not just DNA damage, but also to many insults like oncogene activation, telomere dysfunctions, oxidative stress and to drug treatments. However, depending on the cellular or extracellular context, cellular senescence can have detrimental effects (Campisi, 1997; Ohtani et al., 2012). Senescent cells seem to recapitulate ageing or loss of regenerative capacity of cells *in vivo*. In this case, senescence is considered to be deleterious, because it affects tissue renewal and function. Thus, senescence is a "double edged-sword" that prevent tomourigenesis, however contributes to ageing (Campisi, 1997).

1.6.1 Senescence triggers

A variety of stimuli can trigger differential senescence responses (Figure 1.8). These responses can be categorized as replicative senescence and premature senescence, the latter of which is induced by oncogene-activation or the loss of tumour suppressor genes such as PTEN (PICS) (Chen et al., 2005b; Di Micco et al., 2007; Gewinner et al., 2009; Moiseeva et al., 2006; Shamma et al., 2009).

Replicative senescence is the result of a combination of events, the first of which is telomere erosion. Telomeres are constituted by G-rich sequence repeats and associated proteins that cap the ends of linear chromosomes to protect them from degradation or end to end fusion by DNA repair processes (Allshire et al., 1989; Moyzis et al., 1988). Cell cycle after cell cycle, cells undergo a progressive shortening of these repeats, because DNA polymerase can't completely replicate DNA ends in S phase (the end-replication problem), resulting in "critically short" or "uncapped telomeres" (Campisi and d'Adda di Fagagna, 2007; Olovnikov, 1973). These eroded telomeres are sensed by the cells as persistent DSBs, resulting in the formation of γ-H2Ax-positive associated DNA damage

foci (SDF), which trigger ATM/ATR-mediated DDR. The unrestrained DDR signalling activates p53 in manner to execute senescence, mediating cell cycle progression arrest and DNA repair if it is possible (d'Adda di Fagagna et al., 2003). In addition to this, the increase in CDKIs such as p16 and Rb tumour suppressor also contribute to the replicative senescence functioning as second barrier to growth arrest of those cells with severely damaged DNA (Campisi and d'Adda di Fagagna, 2007; Kiyono et al., 1998; Kuilman et al., 2010). Several mouse models have addressed the importance of healthy telomeres in tumour development (Artandi et al., 2000; Deng et al., 2008). Mice deficient for the RNA component of telomerase (Terc) (the enzyme required for the maintenance of telomere length) develop significantly less tumours by activating a p53-dependent cellular senescence. Thus, the loss of p53 in these mice significantly increases tumour formation (Cosme-Blanco et al., 2007; Feldser and Greider, 2007).

The mutation of certain genes in certain circumstances can potentially induce the transformation of normal cells into cancerous tumour cells. These are called oncogenes, and normal cells respond to it by undergoing senescence, thus preventing oncogenic transformation (Campisi and d'Adda di Fagagna, 2007; Serrano et al., 1997). This is called oncogene-induced senescence. In human fibroblasts, overexpression of the oncogenic form of RAS ($HRAS^{G12V}$), a cytoplasmic transducer of mitogenic signals, induces OIS due to DNA hyper-replication and hyperproliferation (Bartkova et al., 2006; Di Micco et al., 2006; Moiseeva et al., 2006). Moreover, the overexpression of other proteins in the RAS pathway (such as RAF, MEK and BRAF) has also been shown to induce OIS (Lin et al., 1998; Michaloglou et al., 2005; Zhu et al., 1998). OIS causes the activation of a S-phase specific DDR pathway. Although, in this case DDR is initiated by a different pathway from that one of replicative senescence, it still forms SDF and shares the activation of the same pathways and effectors downstream culminating in the activation of p53 (d'Adda di Fagagna, 2008). Moreover, OIS-induced DNA damage leads to ROS-mediated p16 expression, which significantly contributes to the maintenance of OIS (Yamakoshi et al., 2009). OIS is entirely dependent on p53 and p16 functions, and therefore their inactivation by E1A protein (viral oncoprotein) bypasses senescence (Serrano et al., 1997). Numerous mouse models support the physiological role of OIS *in vivo* and its relevance to cancer progression. Activated oncogenes or loss of tumour suppressor genes cause the formation of benign lesions in mice, which contain senescent like-cells. An example of it is the benign naevi in human skin, which shows the presence of cells expressing oncogenic BRAF and are senescent at the same time (Michaloglou et al., 2005). However, DDR can

be also triggered directly by γ-irradiation or UV-light or chemotheraupetic drugs inducing a p53/p21 and p16-dependent senescent phenotype (Borodkina et al., 2014).

Senescence can also occur in a DDR-independent way. PTEN-induced cellular senescence (PICS) is a form of senescence caused by PTEN deletion. In this case, senescence doesn't involve OIS-like hyper-replication or DDR. It manifests when cells are treated with aphidicolin, arresting them before entry into S phase and then preventing DNA replication (Alimonti et al., 2010). Also in this case, the main effector of PICS is p53, whose activation is mediated by mTOR translation (Alimonti et al., 2010; Nardella et al., 2009). PTEN deletion also induces p16, resulting in disruption of the anaphase-promoting complex APC/C-CDH1 and high levels of ETS2, interrupting the completion of mitosis (Song et al., 2011). The inactivation of p53 with PTEN loss in mice prostate demonstrated the relevance of PICS for preventing the progression to aggressive form of cancers (Chen et al., 2005b).

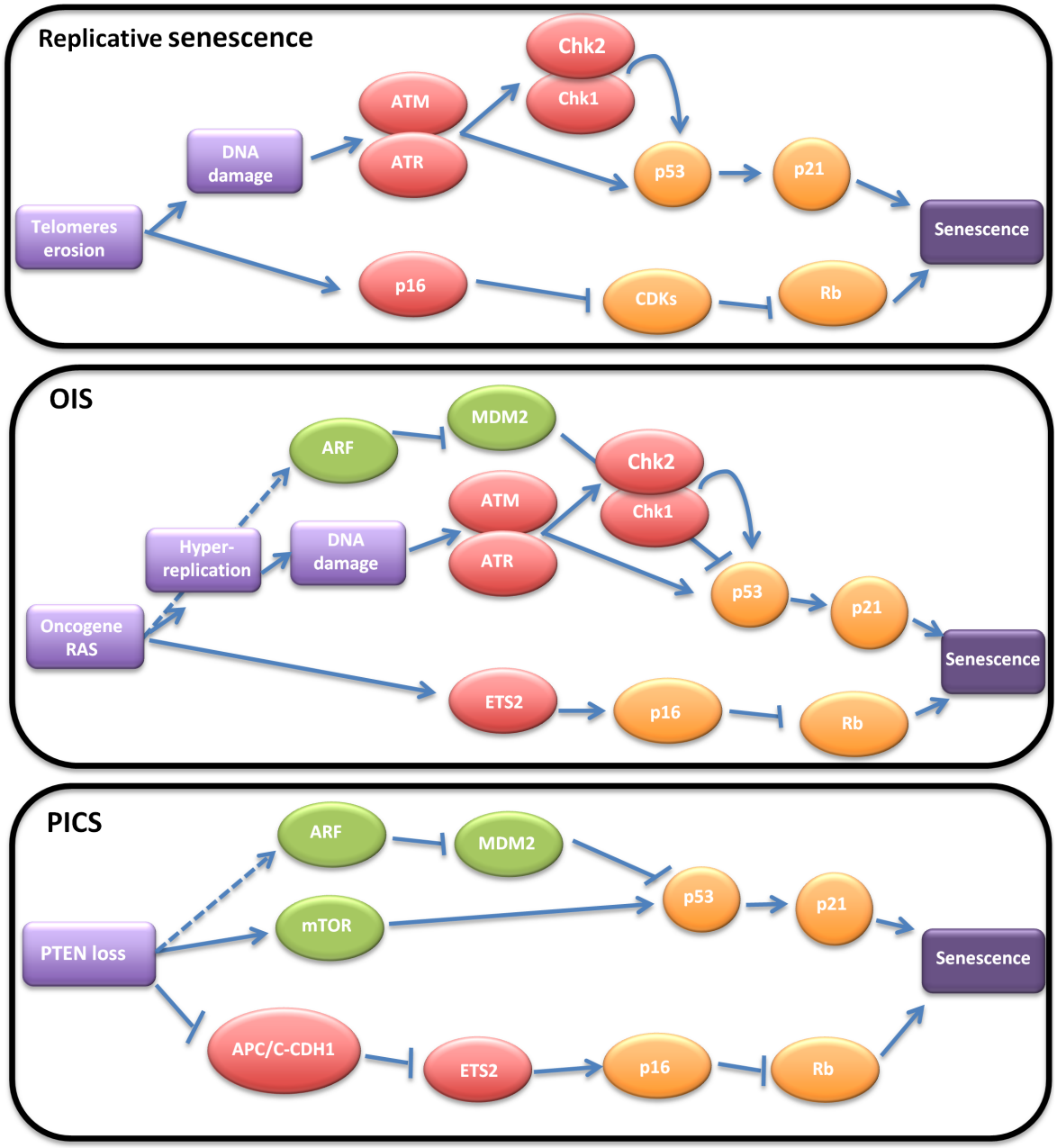


Figure 1.8: Cellular senescence and the associated pathways. A schematic representation of the various causes and effectors leading to cellular senescence [Figure adapted from Nardella et al., 2011].

1.6.2 Effectors of senescence

As mentioned previously, the most important pathways that mediate senescence growth arrest are the p53-p21 and p16-pRb pathways. They affect the cell cycle independently and by different mechanisms. The activation of one or the other or both pathways, and their ability to induce senescence depends on various conditions that are cell-type and species-specific. However, it appears that there are also cases of senescence driven by pathways other than p53 and pRb (Michaloglou et al., 2005; Olsen et al., 2002).

1.6.2.1 The p53 pathway

p53 is a key tumour suppressor protein, integrating many signals and mediating many physiological processes, such as cell cycle arrest, differentiation and also senescence following DDR. p53 activity is regulated at different levels by several mechanisms. As previously mentioned, the ATM/Chk1 and ATR/Chk2 pathways induce p53 activation by enhancing its stabilization through its direct phosphorylation or phosphorylation and inhibition of its negative regulator MDM2 (Ou et al., 2005; Sancar et al., 2004). MDM2 is also negatively regulated by $p19^{ARF}$ resulting in p53 activation (Sherr and McCormick, 2002). In addition to this, many other proteins or modification can also regulate p53 activity. p53 activation results in transcriptional regulation of many genes, most importantly, that of p21, which is responsible for both transient and permanent DNA damage-induced arrest. The choice between transient arrest of the cell cycle or permanent arrest leading to senescence, depends on the severity of the DNA damage. It has been shown that senescent cells present irreparable DNA damage, which is caused by the accumulation of DNA lesions at telomeric regions, causing a persistent DDR activation p53-mediated (Fumagalli et al., 2012; Hewitt et al., 2012; Suram et al., 2012).

Experimental reduction in p53, p21 or any of the DDR proteins (such as ATM and Chk2) in mice and human cells, restrains the induction of DNA damaged-induced senescence. Moreover, in some cells that don't express p16 or RAS, this can even reverse senescence growth arrest (Baus et al., 2003; Beausejour et al., 2003; Brown et al., 1997; d'Adda di Fagagna et al., 2003; Di Micco et al., 2006). Overall, p53 and the DDR pathway function as quick and powerful barriers for arresting cells with severely damaged DNA, thus

preventing the accumulation and propagation of oncogenic mutations (Bartkova et al., 2005; Gorgoulis et al., 2005).

1.6.2.2 The p16-pRb pathway

Several stimuli can induce the activation of pRB pathway. As mentioned, p16 can activate pRb by the inhibition of cyclin/CDK complexes, inducing inhibition of the E2F-mediated regulation of the cell proliferation and control genes (Sherr, 1996). p16-pRb pathway contributes to induce senescence, sometimes as a secondary effect after the activation of p53 (Jacobs and de Lange, 2004; Stein et al., 1999). However, senescence can also be directly induced via p16-pRb pathway in a cell type dependent and species-specific manner. For example, epithelial cells, contrary to fibroblast, engage senescence by a p16-mediated proliferation arrest (Overhoff et al., 2014). The decision to engage one or both pathways not only depends on the cell type, but also on the species. For example, telomere disruption in human cells induces senescence by both p53 and p16-pRb pathways, whereas in mice it induces senescence just by p53 activation (Nalapareddy et al., 2010; Smogorzewska and de Lange, 2002).

p16 expression can be regulated by different factors. p16 expression is induced by the oncogenic RAS through the activation of the transcriptional factor ET2 (E26 transformation-specific). The ID family of helix-loop-helix (HLH) proteins generally counteract ET2 activity, however they are downregulated upon induction of senescence (Ohtani et al., 2001). Another class of proteins called Polycomb repressor complexes 1 and 2 (PRC1/PRC2) regulate growth arrest by regulating transcription from the INK4A-ARF locus, which encodes for p16 and p14 (Bracken et al., 2007). In normal proliferating cells, p16 and p14 expression is repressed by promoter hypermethylation mediated by Ezh2 factor of the PRC2 complex. This repression is maintained by the factors of the PRC1 complex, such as Bmi-1, CBX8 and CBX7. In senescent cells, the reduction in the level of Ezh2 decrease the trimethylation of the promoter, causing the dissociation of PRC1 proteins from chromatin and the transcription of the INK4A-ARF genes (Bracken et al., 2007; Maertens et al., 2009). Moreover, p16 expression can be also regulated in a ROS-dependent manner (Yamakoshi et al., 2009). As previously mentioned, the expression of oncogenic RAS induces E2F mediated transcription of DNMT1, a methyl-transferase involved in the methylation-mediated repression of the p16 promoter. However, RAS-induced hyperproliferation causes DNA damage and production of ROS, which in-turn

inhibits E2F activity, thereby supressing DNMT1 expression and allowing p16 transcription.

Despite p21 and p16 both being CDK inhibitors involved in keeping pRb in the active form, they are not equivalent and the senescence caused by them seems qualitatively different. In case of p53-p21 mediated senescence, growth arrest can be resumed after deletion of p53 (Beausejour et al., 2003; d'Adda di Fagagna et al., 2003; Gire et al., 2004; Won et al., 2006). Instead, once the cells completely engage p16-pRB pathway for several days, growth cannot be resumed even after deletion of p53, pRb or p16 (Beausejour et al., 2003). In addition to this, the loss of pRb-p16 pathway causes upregulation of p53 and p21, because E2F induces ARF expression (Bates et al., 1998; Zhang et al., 2006). In conclusion, p53 and pRb are the master regulators of senescence and they reciprocally regulate each other. However, the outcome of the activation of one or the other, and nature of senescence achieved are different.

1.6.3 Features of senescent cells

Senescent cells can be identified by several characteristic features, which all together define the state of senescence. These features are discussed below.

1.6.3.1 Growth arrest

Senescent cells are characterised by their inability to progress through the cell cycle. The genetic background and the species of a cell define the severity and the features associated with the senescence growth arrest (Di Micco et al., 2006; Olsen et al., 2002; Zhu et al., 1998). In most cases, senescence induces a G1 arrest, thus preventing cells from entering the S phase and replicating their DNA. In this case the cell remains metabolically active (Di Leonardo et al., 1994; Herbig et al., 2004; Ogryzko et al., 1996; Serrano et al., 1997). High levels of cell cycle inhibitors such as p21 and p16 mediate this permanent arrest, which is different from quiescence and cannot be reversed by any physiological stimulus. It has been shown that oncogene activation can also induce a fraction of cells to arrest in the G2 phase of the cell cycle (Di Micco et al., 2006; Olsen et al., 2002; Zhu et al., 1998).

1.6.3.2 Senescence-Associated Secretory phenotype

Various transcriptional profiling experiments have revealed that senescent cells compared to proliferating cells demonstrate a pronounced change in gene expression (Shelton et al.,

1999; Yoon et al., 2004; Zhang et al., 2003). As expected, many genes associated with proliferation and cell cycle progression are significantly downregulated. However, a surprising number of gene expression alterations involve genes not correlated with growth arrest. Many of them encode for secreted factors, such as pro-inflammatory cytokines and chemokines, or proteases and various growth factors, upregulation in which affect the surrounding cells and the tissue microenvironment (Chang et al., 2002; Mason et al., 2004; Rodier and Campisi, 2011; Shelton et al., 1999; Zhang et al., 2003). This feature of senescent cells is called senescence-associated secretory phenotype (SASP). SASP is a key feature of senescent cells that distinguishes them from other non-proliferative cells (Coppe et al., 2008). SASP-produced factors and their composition vary depending on the cell context and senescence trigger, suggesting that SASP is plastic and versatile. Pro-inflammatory cytokines and chemokines are the most conserved SASP-secreted factors, indicating that most senescent cells share the characteristic to attract immune cells to induce local inflammation (Coppe et al., 2008). However, there are exceptions such as the human nevi where the accumulation of senescent cells doesn't involve any immune response or inflammation (Benz et al., 1991).

Although it is considered a typical hallmark of senescent cells, SASP is also important effector mechanism involved in the establishment of senescence. Some of its components have indeed been shown to reinforce the senescence-mediated proliferation arrest by an autocrine feedback mechanism. For example, the expression of oncogenic RAS in human ovarian fibroblasts has been reported to induce the expression and secretion of the factor GRO-1. Pre-conditioned medium with GRO-1 was found to be able to induce senescence in wild-type fibroblasts. (Yang et al., 2006). Similarly, the expression of BRAF in human cells causes senescence through the expression and secretion of the IGFB7, which inhibits the RAF-MEK-ERK pathway (Wajapeyee et al., 2008). Moreover, other works have shown that the cytokine IL6 and the chemokine IL8 significantly contributes to establish and maintain senescence by expression of a C/EBPβ- and NF-κB-dependent network of inflammatory factors (Acosta et al., 2008; Kuilman et al., 2008). These two transcription factors C/EBPβ- and NF-κB in particular are crucial for the regulation of the secretion of several SAPS factors to reinforce senescence. The depletion of C/EBPβ- in BRAFV600E expressing cells has shown indeed to abolish the expression of IL6 and IL8 bypassing senescence (Kuilman et al., 2008). Another factor that contributes in regulating SASP-secreted factors is DDR. The silencing of several components of DDR decreases the expression of diverse SASP factors (Rodier et al., 2009). Altogether, these evidences

demonstrate that SASP factors can reinforce senescence, but its complexity warrants additional investigations.

1.6.3.3 Apoptosis resistance

Many senescent cells acquire resistance to certain apoptotic signals. Senescent human fibroblasts are much more resistant to ceramide-induced apoptosis, compared to endothelial senescent cells (Hampel et al., 2004). Senescent human fibroblasts are also resistant to apoptosis induced by growth factor deprivation and oxidative stress, but they are not resistant to apoptosis induced by Fas death receptors (Chen et al., 2000; Tepper et al., 2000). The capacity of these cells to be stable in culture and resist apoptosis could explain why the number of senescent cells increases with age. However, how a cell acquires resistance to apoptosis is still not fully understood, but it is clear that p53 plays a crucial role in this process. In some cells, resistance is associated by p53-mediated regulation of expression of proteins that inhibit, promote or implement apoptotic cell death (Marcotte et al., 2004; Murata et al., 2006). In some others, p53 can specifically activate genes to mediate cell proliferation arrest, instead of activating pro-apoptotic factors (Jackson and Pereira-Smith, 2006).

1.6.4 Senescence markers

Several biomarkers can be used to identify senescent cells in culture and *in vivo*. However, it must be noted that none of these markers is present exclusively in the state of senescence, and they require several days to develop. Amongst these, the most striking marker is the lack of DNA replication due to cell cycle arrest. This phenotype can be easily identified by incorporation of 5-bromo-2'-deoxyuridine (BrdU) or [^{3}H]-thymidine, or by immunostaining for proteins like PCNA or Ki-67. However, this marker can't discern a senescent cell from one that may share the features of the terminal arrest but it still retains the ability to re-enter the cell cycle, i.e. quiescent or differentiated post-mitotic cells (Campisi and d'Adda di Fagagna, 2007; Schmitt, 2007).

Another marker that can help recognize senescent cells is the presence of senescence-associated β-galactosidase (SA-βgal). It is known that SA-βgal derives from the lysosomal β-galactosidase due to an increase of lysosomes biogenesis in senescent cells (Lee et al., 2006), and it can be detected by the use of the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside). Organs from aged animals show accumulation of

senescent cells and high level of SA-βgal activity (Kurz et al., 2000). However, this marker can also present in cells in a condition of stresses such as prolonged confluence in culture.

Senescent cells can also be directly identified by their characteristic morphological changes. They appear to have a flattened and enlarged cell shape with a vacuole-rich cytoplasm. However, this phenotype doesn't present in all senescent cells. For example, BRAF induced senescence or p400 silencing-induced senescence result in a spindle-shaped morphology of the cells (Chan et al., 2005; Michaloglou et al., 2005). Thus, morphological features of senescence can be different according to cell type and context.

Senescent cells undergo a profound change in chromatin structure, and one the most characteristic one is the appearance of senescence-associated heterochromatin foci (SAHF). SAHFs are described as structures of facultative heterochromatin that can be detected by the use of dyes such as 4',6-diamidino-2-phenylindole (DAPI) as a distinct punctuate foci (Narita et al., 2003). Studies on SAHF composition have shown that these heterochromatic foci are enriched in HP1 (Heterochromatin protein 1), histone marks associated with gene repression, such as H3K9me3, and the histone variant macro-H2A (Narita et al., 2003). Therefore, the function of these SAHF was thought to facilitate the silencing of genes involved in cell-cycle control to reinforce the growth arrest during senescence (Kosar et al., 2011). Consistent with this concept, the expression of cyclin A2 gene in senescent cells is inhibited by H3K9 methylation histone and co-localization with SAHF foci (Funayama et al., 2006; Narita et al., 2003). However, a recent study has described the presence of two distinct forms of heterochromatin in senescent cells. ATM or p53-dependent heterochromatin that is required for the repression of gene expression and DNA replication/ATR-dependent SAHF heterochromatin, which dampens DDR following OIS-induced replication stress (Di Micco et al., 2011). Interestingly, loss of p53 or ATM in cells expressing oncogenic RAS was found to allow cell proliferation, even in presence of SAHF, suggesting that SAHFs are not associated with cell cycle arrest.

1.7 Physiological consequences of senescence

The concept of senescence was historically restricted to a cellular context. However, during the past decade the role of senescence in a number of physiological processes such as embryogenesis, development, tissue repair, wound healing and tumour suppression has been highlighted.

1.7.1 Role of senescence in wound healing

Several evidences have shown that senescence contributes to wound healing. Wound healing is a coordinated process involved in restoring tissue integrity following damage or injury. Many cellular components and pathways are involved in this process, which can be broken down into three different stages: inflammation, re-epithelialization, and tissue remodeling (Gurtner et al., 2008). Wound repair in adults causes the formation of scars, which are constituted by regions of non-functional fibrotic tissue. It has been shown that senescent cells accumulate in these regions restricting the formation of this fibrotic tissue by secretion of extracellular matrix enzymes. Experiments on a mouse model of chemical-induced liver fibrosis have shown that the treatment of hepatic stellate cells (HSCs) with carbon tetrachloride (CCl_4) induced their initial proliferation and production of extracellular matrix components (ECM) (Krizhanovsky et al., 2008). These HSCs then senesce, downregulate the production of ECM and upregulate the expression of ECM degrading enzymes and immune modulators, thus reducing the fibrotic scarring. However, when the treatment with CCl_4 was stopped, the hepatic fibrosis was reversed and HSCs eliminated by immune clearance. Conversely, in $p53^{-/-}$ and INK4a/ARF-senescence deficient mice, hepatic fibrosis and activated HSCs were present even after withdrawal of CCl_4. Therefore, senescent cells are able to limit the extent of chemical-induced hepatic fibrosis by secretion of ECM degrading enzymes and by regulating the immune clearance of activated HSCs.

Another study, performed in mouse model of cutaneous wound healing has showed that senescent fibroblasts accumulate in granulating tissue during the proliferating phase of the wound repair process. The expression of CCN1, a matricellular protein expressed in granulating tissue of transgenic mice, induces senescence in fibroblasts by p53 and ROS-mediated activation of p16 (Jun and Lau, 2010). The accumulation of these senescent fibroblasts causes a reduction in type I collagen by secretion of ECM degrading enzymes

MMP1 and MMP3. Therefore, senescent cells are able to restrict the formation of fibrotic tissue during cutaneous wound healing.

A recent work from the Campisi lab has used mice models where the precocious appearance of senescent cells in the wound was found to be beneficial for the wound repair. This study showed that by the secretion of platelet-derived growth factor (PDGF-AA), senescent cells induce the differentiation of myofibroblasts to accelerate the closure of the wound (Demaria et al., 2014). In conclusion, all these evidences highlight that crucial role of senescent cells, in particular of SASP, in tissue repair.

1.7.2 Role of senescence in tumour suppression

For years, senescence has been considered a powerful tumour suppression mechanism that limits proliferation of pre-malignant cells, thus severely hindering the development of cancer (Mathon and Lloyd, 2001). Numerous evidences have shown the presence of senescent cells in premalignant neoplastic lesions such as benign melanocytic nevi, dermal neurofibroma, prostate and pancreatic intraepithelial neoplasia, colon adenoma and early-stage of thyrods tumours (Braig et al., 2005; Caldwell et al., 2012; Chen et al., 2005b; Collado et al., 2005; Courtois-Cox et al., 2006; Michaloglou et al., 2005; Morton et al., 2010). In addition to this, the loss of function of key senescence pathway proteins has been shown to enable the progression of early stage pre-malignant lesions to more advanced tumour stages. Several studies in various mice models have shown that loss of p53 or INK4/ARF accelerates tumourigenesis (Braig et al., 2005; Chen et al., 2005b). All these evidences suggest that senescence functions a barrier to tumourigenesis by its anti-proliferative property. However, other studies have shown that senescence restrains tumour formation also by promoting immune-clearance of damaged cells.

An elegant study by Scott Lowe and colleagues has shown that the reactivation of p53 in HRAS-induced hepatocarcinomas in mice induces senescence, tumour regression and immune-mediated tumour clearance (Xue et al., 2007). Interestingly, the tumour regression was facilitated by the active clearance of senescent cells by natural killer (NK) cells. Following studies have confirmed that NK cells preferentially eliminate senescent cells *in vitro* (Krizhanovsky et al., 2008). Moreover, RAS-induced senescent hepatocytes secrete a wide array of chemokines and cytokines, which attract the innate and adaptive immune response elements such as monocyte/macrophage and $CD4^+$ T cells (Kang et al., 2011). Thus, SASP secreted factors are important for the immune clearance of senescent cells *in*

vivo and fulfil a crucial role in tumour suppressive potential of senescence (Acosta et al., 2013). In conclusion, senescence is a potent barrier to tumourigenesis due to the permanent proliferation arrest and SASP.

1.8 Pathological consequences of senescence

Senescence has also been associated with multiple pathological processes, such as ageing and tumour promotion (Munoz-Espin and Serrano, 2014). However, the kinetics of senescence induction and functionality differ amongst these different processes, regarding acute or chronic senescence (Figure 1.9). For example, acute senescence occurs during tissue repair and embryogenesis, where specific cell-extrinsic stimuli target a specific population of cells to senesce in a strictly programmed manner. Those senescent cells are then eliminated efficiently by the immune system that targets the secreted SASP components. Conversely, ageing and age-related diseases are caused by chronic senescence. In this case, senescence is a stochastic and unscheduled event that can affect any cell and occurs after a period of accumulation of macromolecular damage and cellular stress, resulting a permanent arrest of the cell cycle (Munoz-Espin et al., 2013; Storer et al., 2013; van Deursen, 2014). This type of senescence is more persistent probably due to the age-related decline in the efficiency of immune system or reduced production of SASP factors (Nikolich-Zugich, 2008; Wang et al., 2011). Cancer therapies, for example, can induce acute and then chronic senescence affecting the integrity of tissues and organs in cancer survivors (Allan and Travis, 2005).

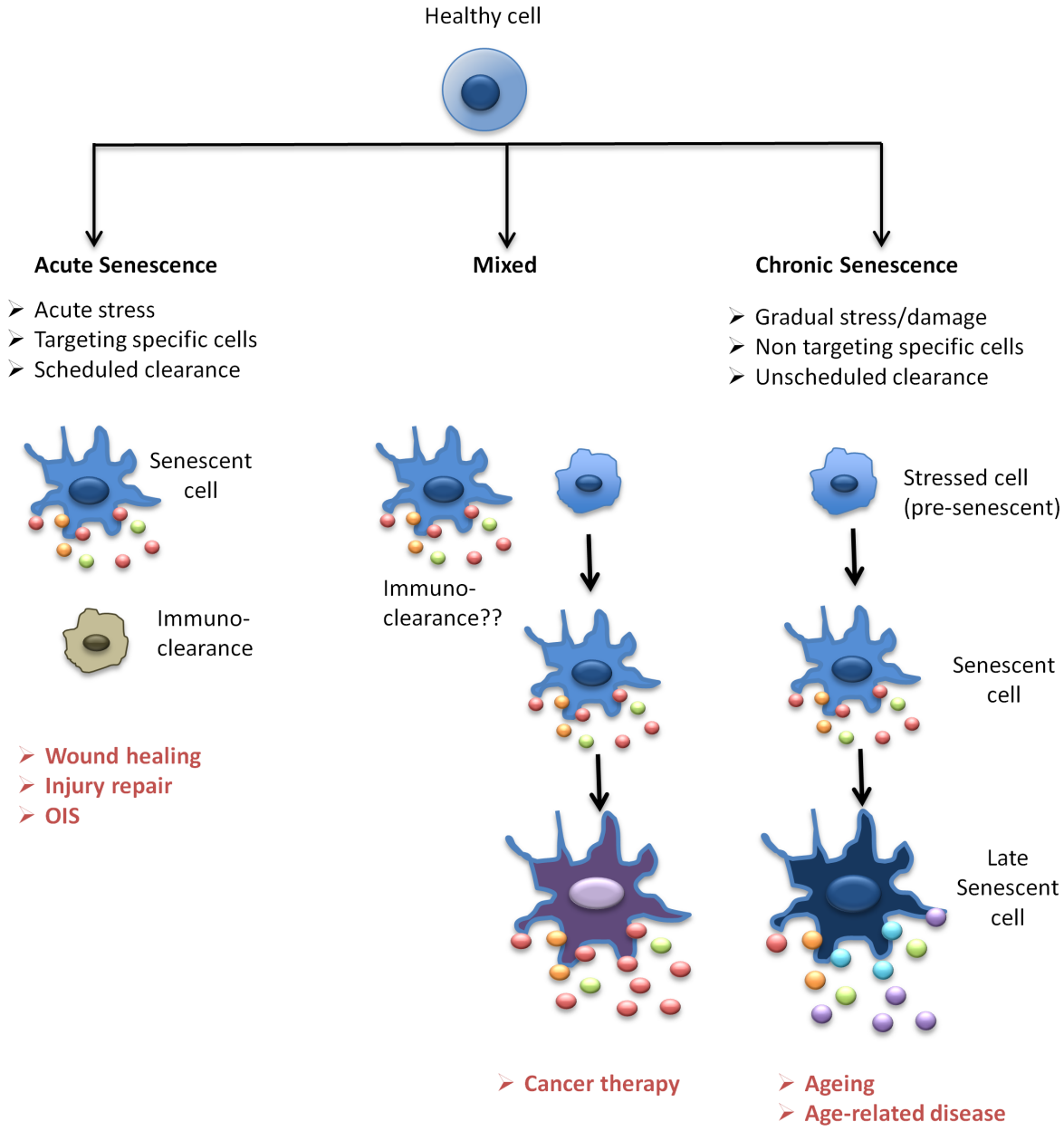


Figure 1.9: Acute and chronic senescence. A model depicting the differences between acute and chronic senescence along with related senescence triggers and the associated physiological processes [Figure adapted from Van Deursen, 2014].

1.8.1 Role of senescence in ageing

The correlation between ageing and senescence is demonstrated by the increase in the number of senescent cells in the tissue of a number of different species such as rodents, monkeys and humans. This increase is most marked in the liver, spleen, skin and lung (Dimri et al., 1995; Herbig et al., 2006; Wang et al., 2009). The number of senescent cells is also found elevated in tissue associated with age-related diseases such as atherosclerosis, osteoarthritis, degenerating intervertebral discs, renal tubuleinterstitial fibrosis, glomerulosclerosis and slow healing venous ulcers (Ding et al., 2001; Erusalimsky and Kurz, 2005; Stanley and Osler, 2001). Therefore, it is important to address why ageing leads to accumulation of senescent cells. Various different factors are found to influence this phenomenon. It has been shown that the number of stimuli inducing senescence increases with age (Baker et al., 2004; Faggioli et al., 2012; Garinis et al., 2008), which is further helped by the reduced efficiency in the immune clearance of senescent cells. In both rodents and humans, the immune system goes through a series of changes in innate and adaptive immunity leading to age-associated immunodeficiency (Nikolich-Zugich, 2008).

It has been shown that senescence contributes to all these age-related diseases and organs/tissues dysfunction through different mechanisms (Munoz-Espin and Serrano, 2014). A clear example is provided by studies in mice showing the relevance of p16-dependent senescence in haematopoiesis, neurogenesis and pancreatic functions (Janzen et al., 2006; Krishnamurthy et al., 2006; Molofsky et al., 2006). In middle-age mice, the expression of p16 in stem and progenitor cells of brain, bone marrow and pancreas is high. This rise in p16 levels is associated with the suppression of stem-cell proliferation and tissue regeneration. Thus, as expected, p16-knockout mice present a retarded decline in stem-cell growth and tissue regeneration, however they die prematurely of cancer (Janzen et al., 2006; Krishnamurthy et al., 2006; Molofsky et al., 2006). This suggests that p16 pro-ageing effect is strictly linked to its tumour suppressive function. Thus, senescence contributes to the decline of tissue homeostasis and regeneration in a cell-autonomous manner by depleting the organism of cycling cells, including progenitor and stem cells, and in a cell-non-autonomous manner by disrupting the local stem-cell niche.

Moreover, ageing is also severely influenced by the senescence-associated alterations in gene expression that may not only affect the function of cell itself, but also of neighbouring cells, and in some cases the morphological structure of distal tissues. For

example, the altered expression and secretion of proteases in the extracellular matrix can induce the cleavage of extracellular proteins, membrane-bound receptors, signalling ligands and other components, thus affecting the tissue structure and organization (Coppe et al., 2008; Parrinello et al., 2005). SASP components such as IL-6 and IL-8 can induce fibrosis in epithelial tissues by inducing epithelial-mesenchymal transition (EMT) (Laberge et al., 2012; Parrinello et al., 2005). Therefore, SASP is thought to drastically affect the tissue architecture amplifying the age-related tissue deterioration. Furthermore, it has been shown that senescent cells can spread their phenotype to nearby cells by secretion of IL-1β, TGFβ and other chemokine ligands (Acosta et al., 2013; Nelson et al., 2012), so called paracrine or secondary senescence.

In addition to this, the accumulation of senescent cells induces chronic tissue inflammation by infiltration of lymphocytes, macrophages, cell death and fibrosis, which are the causes of further ageing-related diseases. This inflammation is caused by secretion of pro-inflammatory SASP factors such as GM-CSF, GROα and macrophage inflammatory proteins (MIPs), that altogether with matrix metalloproteinases to create a tissue microenvironment that stimulates survival, proliferation, dissemination and angiogenesis of nearby premalignant cells (Bavik et al., 2006; Campisi, 2013; Coppe et al., 2006; Dilley et al., 2003; Krtolica et al., 2001; Martens et al., 2003). This may in part explain why the risk of cancer increases with age (Campisi, 2011, 2013; Urassa et al., 2006). Therefore, senescent cells protect themselves from transformation, however, ironically can promote cancer progression in nearby premalignant cells in ageing organisms.

Some post-mitotic cells show key features of senescent cells. Post-mitotic cells constitute most of the cells in mammalian organisms. It has been shown that post-mitotic neurons in human and mice brains have a typical p21-dependent senescence phenotype caused by accumulation of high amounts of DNA damage. This phenotype is characterized by heterochromatinization, synthesis of pro-inflammatory cytokines and β-galactosidase activity, directly linking them with senescence (Jurk et al., 2012; Sedelnikova et al., 2004). Further investigations have shown that adipocytes in mice under high-fat diet also present senescence-like features, suggesting that this could be a broader phenomenon (Minamino et al., 2009; van Deursen, 2014). Thus, also terminally differentiated cells can undergo senescence contributing to the deterioration of the tissues that characterizes ageing.

1.8.2 Role of senescence in tumour promotion

Contrary to the tumour suppressive role of senescence, mounting evidences have now shown that senescence may also contribute to hyperplastic pathologies. Numerous xenograft studies have demonstrated that the injection of senescent fibroblasts into immune-deficient mice stimulates the proliferation of mouse and human epithelial tumour cells, but has no effect on normal epithelial cells (Coppe et al., 2006; Krtolica et al., 2001; Liu and Hornsby, 2007). This tumour promoting effect of senescent cells is partly due to the secretion of SASP factors. Several SASP components have been shown to promote tumourigenesis: MMP3 (stromelysin) promotes tumour cell invasion, VEGF is involved in tumour-driven angiogenesis, GROs and amphiregulin stimulate tumour growth (Bavik et al., 2006; Coppe et al., 2006; Coppe et al., 2010; Liu and Hornsby, 2007; Yang et al., 2006). Furthermore, other SASP factors can also fuel the malignant phenotypes. SASP secreted IL-6 and IL-8 can facilitate the EMT of pre-malignant epithelial cells and nonaggressive cancer epithelial cells (Coppe et al., 2008; Laberge et al., 2012; Parrinello et al., 2005). A reduction in secretion of IL-6 and IL-8 by depletion of IL-1α, an upstream regulator, drastically reduces the pro-malignant properties of the SASP (Orjalo et al., 2009). Moreover, senescent cells, in particular ones that engage senescence due to DNA-damaging radiation or chemotherapeutic agents, can produce SASP factors (IL-6, WNT6B, TIMP-1) that have been shown to protect neighbouring tumour cells from being killed by the chemotherapeutic agents (Lujambio et al., 2013).

In conclusion, depending on the physiological context, senescent cells play an important role in creating a tissue microenvironment that is permissive for the development or progression of cancer. Therefore, it will be important to consider combination therapies that not only kill tumour cells, but also prevent the formation of a malignant chemoresistant senescent niche.

1.9 General Aims

The aims of this study were to investigate the effect of activated Akt on the DNA damage response and DNA repair. In addition to this, the impact of Akt signalling on the establishment of oncogene-induced senescence was evaluated.

Chapter 2: Materials & Methods

2.1 Tissue Culture

2.1.1 Cell culture of human cell lines

Human colorectal carcinoma cells line DLD1WT, DLD1 PTEN$^{-/-}$, HCT116 WT and HCT116 PTEN$^{-/-}$ were kindly provided by Todd Waldman's lab. They were all cultured in DMEM (Dulbecco's modified Eagle medium) supplemented with 10% Foetal Bovine Serum (FBS), 2mM L-Glutamine, 50U/ml Penicillin G and 50µg/ml Streptomycin. The cells were grown in a humidified incubator at 37°C and 5% CO2.

Phoenix-AMPHO embryonic kidney cells (SD-3443) were used for the generation of helper-free ecotropic and amphotropic retroviruses. This cell line was obtained from the American Type Culture Collection. They were cultured in DMEM supplemented with 10% FBS, 2mM L-Glutamine, 50U/ml Penicillin and 50µg/ml Streptomycin at 37°C in a humidified atmosphere containing 5% CO2.

Human foetal lung primary fibroblasts IMR90 were obtained from Coriell Cell Repositories. They were cultured in DMEM supplemented with 20% FBS, 2mM L-Glutamine, 50U/ml penicillin G and 50µg/ml Streptomycin. The cells were grown in a humidified incubator at 37°C with 5% of CO_2 and 3% O_2.

ER-RAS cells containing the conditional inducible system with the estrogen receptor ER fused to the RAS protein were cultured in DMEM supplemented with 20% FBS, 2mM L-Glutamine, 50U/ml penicillin G and 50µg/ml Streptomycin. The cells were grown in a humidified incubator at 37°C with 5% of CO_2 and 3% O_2. RAS induction was achieved by the addition of 4-hydroxytamoxifen (4-OHT) to the growth medium.

Cells were passaged at 70-80% confluency in order to allow continued growth of the culture. The media was aspirated and the adherents cells were washed twice with 5 ml of buffered 0.05% EDTA. After the wash, 1ml of buffered trypsin-EDTA (0.05% trypsin, 0.02% EDTA) was added and the plate was left at 37°C for 3mins. Following trypsinisation, fresh medium containing FBS was immediately added to the dissociated cells in order to neutralise the trypsin, and a proportion of this cell suspension was transferred to a new plate containing fresh media. The cells were then returned to the incubator.

2.1.2 Cryo-freezing and recovery

Cryo-freezing was used for long-term storage of all cell lines. Healthy cells in log phase of growth were trypsinised as described, pelleted by centrifugation at 1000 rpm for 5mins, resuspended in 90% FBS and 10% of dymethil sulphoxide (DMSO, sigma) and divided into 1ml aliquots in 1.5 ml cyovials. Initial freezing was carried out in a Mr Frosty container (containing isopropanol) at -80°C to give a cooling rate of 1°C/minute. The cells were then transferred to liquid nitrogen storage at -180°C. For cell recovery after liquid nitrogen storage, the cryovials were quickly thawed at 37°C by placing in a container of warm water and washed in media at 37°C to remove DMSO. The following day the cells were passaged or media was changed depending on the confluence of the cells.

2.1.3 Cell counting

The automated Casy® cell counter and analyser system was used to count cells. Following trypsinisation, cells were resuspended with media at 37°C, and 400µl of cell suspension was added to 20ml PBS and counted using cell size cut-off settings that exclude debris from the calculation. For growth curve analysis measurements were taken in triplicate.

2.2 Transfection/Transduction

2.2.1 Transient transfection

Transient transfection was performed using the reagent Lipofectamine® 2000 (Invitrogen) following the manufacturer's instruction. This method was used to introduce plasmid DNA and siRNA into cells (Table 2.1). The cells were seeded in antibiotic free medium the day before transfection in order to be 60-70% confluent. The transfection reaction was prepared with Lipofectamine® 2000 and plasmid were diluted separately in Opti-MEM® (Eagle's Minimum Essential Media) in a ratio of 6µl:1µg per plate of HCT116 cell line and a ratio of 5µl:3µg per phoenix cells. 10ul Lipofectamine® 2000 was used with siRNAs to give 200nM final concentration of siRNA per plate. Following incubation of 5mins, Lipofectamine® 2000 containing Opti-MEM® and plasmid containing Opti-MEM® were mixed and incubated at room temperature for 20mins. The resulting lipid-plasmid complexes were added to the cells and the dish rocked gently to ensure an even coverage of the plate. Cells were returned to the incubator and harvested at the appropriate time point to check expression.

Table 2.1: Plasmids or siRNA used.

Plasmid/siRNA	Details	Source
pBabe-neo	Empty vector	Peter Adams Lab
pBabe-puro	Empty vector	Peter Adams Lab
pBabe-neo-H-RASG12V	Activated RAS	Peter Adams Lab
pBabe-puro-Myr-Akt	Activated Akt	Peter Adams Lab
pCMV6	Empty vector	Origene PS100001
pCMV6-Mre11A	Mre11A	Origene RC209414
pcDNA5/FRT/TO	Empty vector	Dave Gillespie Lab
pcDNA5/FRT/TO/GAG-Akt	Activated Akt	Cloning
ON-TARGET plus Non-targeting pool	Negative control	Dharmacon D001810
ON-TARGET plus SMARTpool	Mre11A	Dharmacon L009271

2.2.2 Viral transduction

Phoenix-AMPHO cells was used to package retroviruses for transduction. The cells were transfected with the appropriate plasmid as described above. The day after transfection, the cells were replenished with 6ml of fresh media and incubated at 32°C for 24 hours allowing the production of the recombinant viruses. The target cells IMR90 were seeded in order to reach 50% of confluency the day after. Two hours prior infection, IMR90 cells were incubated with 3ml of fresh media containing 8μg/ml of Polybrene. Media from the Phoenix cells was collected 2 days after transfection and passed through a 0.45μM filter in order to remove cellular material from the retroviral supernatant. 3ml of the retroviral supernatant was added to IMR90 cells. The plate of Phoenix cells was replenished with 6ml of fresh media for second viral harvest and incubated at 32°C overnight. A second round of infection was performed as outlined 24 hours later. Infected IMR-90 cells were then selected for Puromycin (1mg/ml) or Neomycin (0.5mg/ml) resistance.

2.3 Irradiation

In order to induce DNA-Double Strand Breaks (DSBs) the cells were exposed to Ionising Irradiation (IR). Cells were treated with x-ray using the Xstrahl RS225 unit. Radiation doses used were between 1-10 Gy min^{-1}. Cells were irradiated directly in the culture plates. Control cells were left outside the incubator for the same period time as the treated ones.

2.4 Drug treatments

HCT116 cells were treated for varying times with different doses of Mirin (Sigma), an inhibitor of the nuclease activity of Mre11 disrupting its ability to repair DNA double strand breaks. HCT116 cells were treated with 2μM of AKT1/2 kinase inhibitor (Sigma), 500nM of Everolimus (Sigma) and 10μM of S6K inhibitor (Tocris Bioscence) for 72 hours to inhibit Akt/mTOR pathway. Protein translation in HCT116 cells was inhibited with 20 μM Cycloheximide (CHX) (Sigma) treatment for varying lengths of time.

2.5 Clonogenic cell survival

Clonogenic cell survival was performed to determine the ability of cells to proliferate indefinitely after exposure to an insult. Cells were trypsinised and 600 cells were seeded per well of a six well plate or 5000 cells per 10cm plate. The cells were left in incubator for 12 days until colony formation was visible. The plates were washed twice with PBS and stained for 5mins with diluted (1:30 in PBS) Crystal Violet (Sigma). Once the colonies were stained, the plates were washed abundantly until the water is clear. The plates were left to air dry. Colony number was counted with GelCount™.

2.6 Protein analysis

2.6.1 Preparation of whole cell extracts

Cells were harvested at 70-80% confluency. The media was aspirated and the cells were washed twice with PBS. Cells were then scraped into 1X sample buffer (62.5mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.1M DTT, 0.01% bromophenol blue) and transferred to eppendorfs. The amount of buffer used depends on the cell type and plate size. The lysates were then vortexed for 1 min, boiled for 5 mins at 95°C, snap-frozen on dry ice and stored at -80°C until analysis.

2.6.2 Protein quantification

The protein concentration of the lysates was determined by Bradford assay. The Bradford Assay relies on the blue colour generated when Coomassie Brilliant Blue reagent binds to protein side chains. 1µl of protein sample was added to 700µl of 1X Bradford reagent was in a cuvette. Standard measurements were generated for each experiment using 5µg and 10µg of Bovine Serum Albumin (BSA). The absorbance of the protein samples and standards were then read at 595nm using a Biophotometer. The protein concentration of the samples was determined by comparing the reading to the standard readings.

2.6.3 SDS-PAGE electrophoresis

SDS-PAGE is the most widely gel system used to separate a broad range of protein. It comprises a stacking gel component to allow the proteins to focus into sharp bands at the beginning of the electrophoresis, and a resolving gel component where varying acrylamide gel percentage separates proteins according to their mass weight. The following tables were used as guide for casting the gel.

Table 2.2: Resolving and stacking gel recipes.

	Resolving Gel					Stacking Gel
Acrylamide %	**5%**	**7.5%**	**8.75%**	**10%**	**15%**	**5%**
Distilled H_2O	21.21ml	19.35ml	17.68ml	16.12ml	9.42ml	6.34ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	8.35ml	10ml	11.7ml	13.3ml	20ml	1.3ml
4x TRIS buffer (pH=8.8 / 6.8)	10ml	10ml	10ml	10ml	10ml	2.5ml
10% w/v Ammonium Persulfate (APS)	400µl	400µl	400µl	400µl	400µl	100µl
Temed	40µl	40µl	40µl	40µl	40µl	10µl

The resolving gel mix was placed in the gel casting apparatus and over-laid with water-satured Butanol to avoid drying. Once the gel was polymerised, the stacking gel was layered on top and a comb was inserted to form the wells for loading the protein samples into the gel. SDS-PAGE running buffer (25mM Tris pH8.3, 192mM Glycine, 0.1% SDS) was added to the lower and upper chambers of the electrophoresis tank. Protein samples were prepared to have equal concentration. They were boiled for 5 mins, briefly centrifuged and loaded into the wells. A protein ladder such as the Spectra multicolor broad range (Themo Scientific) was also loaded to aid the identification of proteins by molecular weight. Depending on the percentage of the gel, the samples were resolved using 80 – 160 volts and the gel was run until the dye front had just entered the running buffer.

2.6.4 Western blot analysis

Once the SDS-PAGE electrophoresis was completed, the protein samples were from the gel transferred into a PVDF membrane in order to identify proteins using with specific antibodies. The PVDF membrane was activated with 100% methanol prior to use, following which it was washed thoroughly with dH_2O to remove all greasiness and then equilibrated in transfer buffer (25mM Tris, 192mM Glycine, 0.01% SDS, 20% methanol). The method involves in making a sandwich comprising of sponge/3MM paper/gel/membrane/3MM paper/sponge in that order. The entire sandwich is then placed into the transfer apparatus in a way that the gel is towards the negative electrode and the membrane towards the positive. The transfer was performed at 80V for 1 hour and 20mins. Following transfer, the membrane was air dry and re-hydrated with 100% methanol. This allows the proteins to be fixed onto the membrane. The membrane was then stained with Ponceau S (Sigma-Aldrich) to ensure even transfer of the proteins onto the membrane. It was then washed and incubated in blocking buffer (5% Marvel (non-fat dried milk powder) solution in 1X TBS-Tween) for 1 hour at RT with gentle agitation. Following blocking, the membrane was incubated with the appropriate dilution of the primary antibody (Table 2.3) in blocking buffer either for 1 hour at RT or overnight at 4°C. The membrane was then washed three times in 1 x TBS-Tween for 10mins with gentle agitation, and then incubated with the appropriate HRP conjugated secondary antibody diluted 1:5000 in blocking for 1 hour. The membrane was again washed three times in 1X TSB-Tween and the bound secondary antibody was detected using Enhanced Chemiluminescence by autoradiography. The film was developed in a Kodak X-Omat 3000RA automatic film processor.

Table 2.3: List of antibodies and the appropriate dilutions used for Western blotting.

Antigen	Host	Dilution	Source
Anti-Flag® M2	Mouse	1:10000	Sigma F1804
Akt (pan)(40D4)	Mouse	1:1000	Cell signalling 2920
Phospho-Akt (Ser473)(D9E) XP®	Rabbit	1:1000	Cell signalling 4060
Beta-actin	Mouse	1:5000	Sigma A1978
Chk1 (2G1D5)	Mouse	1:1000	Cell signalling 2360
Phospho-Chk1 (Ser345)	Rabbit	1:1000	Cell signalling 2341
Chk2 (1C12)	Mouse	1:1000	Cell signalling 3440
Phospho-Chk2 (Thr68)	Rabbit	1:1000	Cell signalling 2661
Cyclin A	Rabbit	1:1000	Santa Cruz SC-751
Phospho-GSK-3α/β (Ser21/9)	Rabbit	1:1000	Cell signalling 9331
MYC tag	Mouse	1:1000	Proteintech 60002-2-Ig
Mre11 (C16)	Goat	1:1000	Santa-Cruz 5859
Nibrin (Nbs1)(H300)	Rabbit	1:1000	Santa Cruz 11431
PTEN (D4.3) XP®	Rabbit	1:1000	Cell signalling 9188
Rb (4H1)	Mouse	1:1000	Cell signalling 9309
Phospho-Rb (Ser807/811)	Rabbit	1:1000	Cell signalling 9308
p21	Mouse	1:1000	Santa Cruz SC-817
p53 (1C12)	Mouse	1:1000	Cell signalling 2524
p53 (Ser15)	Rabbit	1:1000	Cell signalling 9284
p16 (G175-405)	Mouse	1:1000	BD 51-1325GR
Rad50 (13B3/2C6)	Mouse	1:1000	Abcam 89
RAS	Mouse	1:1000	BD 610001
p70-S6K (49D7)	Rabbit	1:1000	Cell signalling 2708
p70-S6K (Thr389)(1A5)	Mouse	1:1000	Cell signalling 9206
S6 Ribosomal protein (54D2)	Mouse	1:1000	Cell signalling 2317
S6 Ribosomal protein (Ser240/244)(D68F8) XP®	Rabbit	1:1000	Cell signalling 5364

2.7 Gene expression analysis

2.7.1 RNA extraction

RNeasy mini kit was used according manufacturer's (QIAGEN) specifications to extract total RNA. The medium was aspirated and the cells were washed twice with PBS. Cells were lysed in 600µl of RLT buffer per 10cm plate and samples were harvested with a cell scraper. The lysate was vortex for few minutes to remove any clumps and then homogenized by blunt 20-gauge needle. One volume of 70% ethanol was added to each homogenized lysate, mixed and transferred to an RNeasy spin column. The samples were centrifuged at 8000g for 15 sec at 4°C to allow the RNA to bind the spin column resin. The resin was then washed few times with RW1 and RPE buffers. The RNA eluted from the column using 30-50µl of RNase-free water. The RNA concentration was determined by reading the absorbance at 260nm with a NanoVue spectrophotometer (GE Healthcare).

2.7.2 cDNA synthesis

The SuperScriptIII® reverse transcriptase (Invitrogen) was used to synthesise cDNA. 0.4µg of RNA was mixed with 2µl of 1X hexanucleotide mix (Roche) and made up to a final volume of 25µl with RNase-free water. The mix was then incubated at 80°C for 10mins and cooled down quickly to allow primer annealing. 14µl of reaction mixture with 8µl First Strand Buffer (Invitrogen), 4µl 0.1M DTT, 2µl 10mM dNTP mix and 1µl of SuperScriptIII was added to the random primed RNA mix. The mix was then incubated at 42°C for 10mins to allow the cDNA synthesis and the reactions stopped by boiling the samples at 70°C for 15mins. cDNA was stored at -20°C. A duplicate reaction without SuperScriptIII was also performed in order to rule out any DNA contamination.

2.7.3 Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR was performed using the C1000™ Thermal Cycler (BIO-RAD). The RT-PCR mix consisted of 0.5µg of cDNA, 2X PerfeCTa™ SYBR® Green FastMix™ and 0.25µM of appropriate primers (Table 2.4) in a final volume of 10µl was prepared in white walled 96 well qPCR plates. Each reaction mix was performed in duplicate to account for pipetting errors. An appropriate standard curve encompassing the samples was constructed for each RT-PCR to ensure that the samples were within the linear range of detection. The cycling protocol consisted of initial denaturation at 95◦C for 15mins, 40 cycles of 95◦C for 20sec, annealing at 60◦C for 20sec and 72◦C for 20sec. A final extension of 5mins at 72◦C

was carried out followed by melt curve analysis, whereby the reaction is heated from 65◦C to 95◦C in 0.5◦C increments for 5sec each. The melt curves were analysed to ensure that a unique product was obtained from each primer set. The average of the ΔΔC(t) of duplicate samples was calculated using Actin as a lading control. Overall average and standard deviation values were calculated from multiple experiments.

Table 2.4: Sequences for the primers used in the qRT-PCR analysis.

Gene name [Species]		Primers
Mre11	F R	GCCTTCCCGAAATGTCACTA TTCAAAATCAACCCCTTTCG
Rad50	F R	CTTGGATATGCGAGGACGAT CCAGAAGCTGGAAGTTACGC
Nsb1	F R	TTGGTTGCATGCTCTTCTTG GGCTGCTTCTTGGACTCAAC
PTEN	F R	GCAGAAAGACTTGAAGGCGTA AGCTGTGGTGGGTTATGGTC

2.8 Flow Cytometry

2.8.1 Fixing cells

Cells were resuspended in warm media after trypsinisation and pelleted at 1000 rpm for 5 mins. The resulting pellet was resuspended in 200µl of phosphate buffered saline (PBS) and 2ml of ice cold 70% Ethanol was added slowly while vortexing the cells in order to ensure uniform fixation. The samples were stored at 4°C or at -20°C before further analysis.

2.8.2 DNA content

Cells were fixed as described and kept at 4°C for at least 30 mins. Cells were washed with PBS for a few times by centrifugation at 1000rpm in order to remove the Ethanol and the pellet resuspended with 400µl of PBS containing 10µg/ml Propidium Iodide (PI) and 250µg/ml RNAse A. The cells were stored in the darkness for 30 mins prior to analysis using Fluorescence-Activated Cell Sorting (FACS).

2.8.3 S-phase analysis

To analyse the proliferation status of a culture, cells were incubated with 25μM of 5-Bromo-2′-Deoxyuridine (BrdU) for 4 to 24 hours, then fixed as described and kept at 4°C for at least 30 mins. BrdU is a Uridine derivative and a structural Thymidine analog, thus it is selectively incorporated into the newly synthesized DNA of replicating cells (during the S phase of the cell cycle) by substituting Thymidine. BrdU incorporation thereby serves as a marker for proliferation.

Fixed cells were resuspended in 1ml of PBS and then 1ml of 4N HCl was added while vortexing. The samples were incubated at room temperature (RT) for 15 minutes. The HCl treatment denatures the DNA and exposes the BrdU epitopes that can then be recognized by the anti-BrdU antibody. The cells were then washed once with PBS and once with PBS containing 0.5% BSA, 0.1% Tween 20 (PBT). The pellet was then resuspended in 200μl of PBT containing a 1:40 dilution of the anti-BrdU antibody (BD Bioscience) and incubated at RT for one hour. The cells were then washed with 1ml of PBT and resuspended in 200μl of PBT containing a 1:40 dilution of the Alexa Fluor® 488 Goat anti-mouse antibody (Life technologies). The cells were incubated for 30 minutes in the dark and then washed with 1ml of PBT. The cell pellet was then resuspended in 400μl of PBS containing 10μg/ml PI and 250μg/ml RNase A. The cells were stored in the dark and after 30 minutes were analyzed by FACS. The data analyzed using WinMDI software.

2.8.4 M-phase analysis

To estimate the number of cells undergoing mitosis in a culture, cells were analysed for the phosphorylation of Histone H3 on Serine 10 (pS10 H3) by Flow Cytometry. This modification is tightly correlated with the chromatin condensation during mitosis, thereby is specific mitotic marker.

Cells were fixed as above and stored at. The cells were permeabilised by incubation in 1ml PBS containing 0.25% Triton X100 for 15 minutes on ice. The cells were then incubated in 200μl of PBT containing a 1:50 dilution of the anti-pS10 H3 antibody for one hour at RT. The cells were washed once with PBT and then resuspended in 100μl of PBT containing 1:100 dilution of the Alexa Fluor® 488 Goat anti-rabbit antibody (Life technologies). Following incubation for 30 minutes in the dark, the cells were washed with 1ml of PBS. The cell pellet was then resuspended in 1ml of PBS containing 10μg/ml PI and

250µg/ml RNase A and stored in the dark for 30 minutes before FACSCalibur analysis. The data analyzed using WinMDI software.

2.8.5 Reporter assay of NHEJ and HR repair

The NHEJ and HR reporter vectors were obtained from Vera Gorbunova's lab (Seluanov A., 2010). The constructs were digested with I-SceI enzyme *in vitro* to induce DSBs, and then transfected as linear DNA into cells. In the extrachromosomal assay 0.5µg of NHEJ and 2µg of HR construct were co-trasfected with 0.1µg of pDs-Red2-N1 as transfection control. Cells transfected with 1µg of GFP-expressing plasmid, 1µg of pDs-Red2-N1 and 1µg of a control plasmid that does not express a fluorescent protein were used as calibration controls for the FACS. Cells were trypsinised and resuspended in ice-cold PBS for FACS analysis. Prior to the analysis the FACS machine was calibrated by the acquisition of GFP, DsRed and control samples. The voltage and color compensation was adjusted in order to include all the fluorescent cells. The samples were acquired counting 100.000 events. The efficiency of DNA DSB repair was calculated as the ratio of GFP+ to DsRed+ cells.

Table 2.5: Primary and secondary antibodies used for FACS analysis.

Antigen	**Host**	**Dilution**	**Source**
Alexafluor 488 goat (secondary)	Mouse	1:40	Life Technologies A11011
Alexafluor 488 goat (secondary)	Rabbit	1:40	Life Technologies A11008
BrdU (CloneBu20a)	Mouse	1:40	Dako M0744
Phospho-Histone H3 (Ser10)	Rabbit	1:50	Santa Cruz sc-8656-R

2.9 Immunofluorescence

Cells were seeded on glass coverslip and treated as required before being harvested. For RPA staining, coverslips were pre-treated with an extraction buffer (25mM HEPES pH 7.4, 50mM NaCl, 1mM EDTA, 3mM $MgCl_2$, 300mM Sucrose, 0.5% Triton x-100 in ddH_2O) on ice for 5mins to extract soluble RPA, so that the retained RPA fraction is primarily chromatin-bound. The cells were then fixed with 4% of Paraformaldehyde for 15mins at RT, washed three times with PBS and blocked with 3% of BSA in PBS for 30mins. The cells were then incubated with the appropriate dilution of the primary antibody (Table 2.6) in 3% BSA in PBS for 1hour at RT or overnight at 4°C. The coverslips were washed few times with PBS and incubated with the appropriate dilution of secondary antibody (Table 2.6) for 40mins at RT in the dark. Following few washes in PBS, the cells were stained with a solution of DAPI (Sigma) diluted in PBS for 10mins. The coverslips were then mounted on glass slides and stored in the dark at 4°C. Fluorescent microscopic images were acquired with a 60x oil immersion lens on a Nikon A1R laser scanning confocal microscope.

Table 2.6: Primary and secondary antibodies used for immunofluorescence analysis.

Antigen	**Host**	**Dilution**	**Source**
Alexafluor 594 goat (secondary)	Mouse	1:300	Life Technologies A11005
Alexafluor 488 donkey (secondary)	Rat	1:200	Life Technologies A21208
Phospho-Histone H2A.X (Ser139)	Mouse	1:300	Millipore 05-636
RPA32/RPA2 (4E4)	Rat	1:200	Cell signalling 2208

2.10 β-Galactosidase staining

Prior to the onset of senescence, $3x10^5$ cells were seeded onto sterile glass coverslips, allowed to adhere and transferred to 6-well tissue culture dishes. When the cells were senescent, the coverslips were rinsed twice with 1X PBS, and incubated with 2ml of freshly prepared fixing solution (1X PBS containing 2% formaldehyde, 0.2% glutaraldehyde) for 5 mins at room temperature. After two washes in 1X PBS, the cells were incubated with 2ml of freshly prepared staining solution (40mM Na_2HPO_4 pH 6, 150mM NaCl, 2mM $MgCl_2$, 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, 1mg/mL X-gal (in DMSO)) and incubated for 12-16 hours at 37° C in a non-CO2 incubator. The coverslips were then washed twice with 1X PBS and twice with dH_2O to remove any residual salt. The coverslips were air dried at room temperature, mounted on glass slides and visualized using conventional bright field microscopy.

2.11 CometAssay®, single cell gel electrophoresis

Alkaline CometAssay® was used to quantify single and double DNA strand breaks in single cells after exposure to DNA damage agents. $2.5x10^5$ cells were resuspended with 1ml of 1X Ca^{2+} Mg^{2+} free PBS (to inhibit endonuclease activity). 50µl of this cell suspension was then mixed with 500µl of molten low melting Agarose (at 37°C) and spread in a CometSlide™ (Trevigen). The slides were then placed in the fridge for 10mins to allow the agarose to solidify. Once the cells were immobilized on the slides, they were covered in lysis buffer (Trevigen) and incubated overnight at 4°C. The slides were immerged in Alkaline unwinding solution (200mM NaOH, 1mM EDTA pH 13) for 20mins at RT. The cells were then placed in the slide tray on the CometAssay® ES unit, covered in Alkaline Electrophoresis solution (200mM NaOH, 1mM EDTA pH 13) and run at 21Volts for 40mins. After washing the slides twice with ddH_2O, the cells were fixed with 70% ethanol for 5mins and then dried at 37°C for 15-30mins. DNA was stained using diluted SYBR® Gold and visualized by fluorescent microscopy.

2.12 Quantification of CometAssay® with ImageJ

In healthy cells the fluorescence is confined to the nucleoid (comet head), which is constituted of undamaged DNA. Undamaged DNA is supercoiled and thus, does not migrate far out of the nucleoid under the influence of an electric current. Whereas in cells with accrued DNA damage, the presence of DNA fragments migrating from the nucleoid is observed forming the comet tail. The negatively charged DNA migrates toward the anode,

and the extraction length reflects increasing relaxation of supercoiling, which is indicative of damage. OpenComet, a plug-in for ImageJ, was used to identify comets and quantify their parameters. The head-finding function identifies the head of the comet and quantifies the intensity of fluorescence. Another function is used to identify the tail and quantify its fluorescence. The measurement results are saved in an excel spreadsheet, which includes measurement obtained for each individual comet, as well as the statistics for the population of comets extracted from all input images. The extent of DNA damage is a normalized measure of the DNA found in the tail as a percentage of total DNA in the cell (i.e. head + tail).

2.13 *In vitro* kinase assay

A kinase assay is used to verify if a specific protein is substrate of a protein kinase by measuring the incorporation of radiolabeled phosphate from [γ-^{32}P]-ATP. Each reaction contained 500ng of Mre11A (Origene) and a range of 2-400 ng of S6k1 (Abnova). The kinase and the substrate were incubated at 30°C for 30mins along with 2mM DTT, 20mM Tris, 1mM EGTA, 1mM $MgCl_2$ and 1µl of [γ-^{32}P]-ATP (2µCi/µl) made up to a final volume of 20µl with ddH_2O. The reaction was terminated by adding 6µl of 4x NuPAGE® LDS Sample Buffer at 95°C for 5mins. The samples were resolved on a SDS-PAGE gel, which were coomassie stained. The gel was then incubated with 10% Glycerol for 30mins, placed on two-sheet wet paper and covered with cling film. The gel was then dried in a GelAir dryer for 1hour at 80°C.

The gel was exposed to x-ray film for 1-24hrs in an autoradiography cassette and developed in a Kodak X-Omat 3000RA automatic film processor. The proteins bands of interest were then excised, transferred to microcentrifuge tubes and ^{32}P incorporation was quantified by Cerenkov counting in a scintillation counter.

2.14 Cell viability assay

The CellTiter-Glo® Luminescent Cell Viability Assay is a method to determine the number of viable cells in culture based on quantitation of the ATP present, which signals the presence of metabolically active cells. The cells were seeded in an opaque-walled 96-well plate. They were then incubated with a concentration range 0-100µM of drug Mirin for one day. The following day, the cells were replenished with 100µl of fresh medium. An empty well just containing medium was also filled with fresh medium. This was used obtain a

value for the background luminescence. 100µl of CellTiter-Glo® Reagent was added to each well, and the plate was placed in an orbital shaker for 2mins to mix the content in the wells and cause cell lysis. The plate was then incubated for 10mins at RT to stabilize the luminescence signal, which was then recorded with a Luminometer.

Chapter 3: PTEN deletion suppresses G2/M checkpoint activation and HR repair via inhibition of MRN complex to promote genomic instability

3.1 Introduction

The DNA damage response is a crucial and complex mechanism aimed at safeguarding cells from accumulating damage during cell divisions that can affect the stability and integrity of the genome. DSBs are one of the most deleterious of form of DNA damage, which arise from the exposure to various genotoxic stresses as IR, some chemicals, and also from cellular processes as replication, meiosis and V(D)J recombination (Bierne et al., 1997; Michel et al., 1997; Sun et al., 1989; Ward, 1988). Failure to properly process such damage can result in a series of chromosomal rearrangements causing various immunological, developmental and neurological disorders, and even cancer (Hoeijmakers, 2001; Khanna and Jackson, 2001). Therefore, it is essential to protect from these diseases by activating the cell cycle checkpoints and coordinating the appropriate repair pathways in response to DSBs.

MRN complex is a multifaceted molecular machine critical for DNA repair and checkpoint responses, as well as for the appropriate induction of apoptosis (Bakkenist and Kastan, 2003; D'Amours and Jackson, 2002; Stracker et al., 2004). Mre11 is a sensor for damage and one of the first to be recruited to DSBs in the S and G2 phases of the cell cycle. It then recruits the ATM machinery, which in turns phosphorylates Nbs1 for the full activation of MRN complex (Dupre et al., 2006; You et al., 2005), and triggers the activation of DDR pathway, by phosphorylating Chk2. Mre11 also activates the other two components of the MRN complex, Rad50 and Nbs1, whose binding in turn stimulates its nuclease activity that is crucial for the repair process. Ultimately, the combined actions of Mre11 and Ctip proteins resect the DNA near the DSB to generate a 3' ssDNA tail, which is critical for repair by HR (Paull and Gellert, 1998). The ssDNA is then covered by the high affinity ssDNA binding protein, replication protein A (RPA) (Biankin et al.), which mediates the recruitment and activation of ATR/Chk1 pathway components.

The initial step in processing a DSB is important in determining the choice of DNA repair mechanism that is used to accomplish the repair. This choice in turn is crucial for the maintenance of genome stability (Grabarz et al., 2012). This choice is strongly influenced by the cell cycle phase in which the cells are at the moment of the damage. Cells in S and G2 phase are likely to be arrested by the G2/M checkpoint. The G2/M checkpoint prevents cells from entering mitosis, providing them an opportunity to stop proliferating and repair the damage, thus avoiding the transmission of the damage to daughter cells (Stark and Taylor, 2004). The proteins responsible for mediating the G2/M checkpoint activation are the two checkpoint kinases Chk1 and Chk2. Chk2 is been found mutated in many cancers, however Chk1 expression and function are found to be quite conserved in cancer cells (Feijoo et al., 2001; Hangaishi et al., 2002; Ingvarsson et al., 2002; Papp et al., 2007). These two kinases cooperate in the phosphorylation and inhibition of phosphatase CDC25C, stimulating its binding to 14-3-3 proteins. 14-3-3/CDC25C complexes sequester CDC25C in the cytosol, blocking its ability to dephosphorylate and activate CDK1 in the nuclei. The CDK1-cyclin B complex is therefore kept phosphorylated and inhibited by Myt1 and Wee1 kinase, thus inhibiting cellular progression into mitosis (Furnari et al., 1997; Peng et al., 1997; Sanchez et al., 1997).

Another important protein involved in DDR signalling is Akt. PIKK family members such ATM, ATR and DNA-PK are able to activate Akt in response to genotoxic stress, thus providing a pro-survival signal by triggering cell cycle arrest and inhibiting apoptosis (Bozulic et al., 2008; Caporali et al., 2008; Lu et al., 2006; Viniegra et al., 2005). However, the permanent activation of Akt enhances its oncogenic activity and has been shown to have detrimental effect on cells. Hyperactivation, overexpression or amplification of Akt is commonly found in human cancers (Calvo et al., 2009; Hennessy et al., 2005). Activation of Akt signalling also results from mutation in upstream regulators such as PI3K and PTEN. Loss of PTEN expression or function is frequently observed in primary and metastatic cancer (Carnero, 2010; Parsons et al., 2005; Vivanco and Sawyers, 2002). PIK3CA encodes for one of the p110α subunit of the PI3K, and many activating mutations in this gene are found in human colon, breast, glioblastoma and lung cancers (Knobbe and Reifenberger, 2003; Samuels et al., 2004; Woenckhaus et al., 2002). Therefore, Akt activity is crucial for cancer development and progression, because it regulates many cellular processes such as survival, proliferation and metabolism that are crucial for tumourigenesis.

However, mounting evidences have shown that both hyperactivation of Akt and PTEN deletion can affect DDR signalling in presence of genotoxic stresses (Henry et al., 2001; Kandel et al., 2002; King et al., 2004; Puc and Parsons, 2005; Tonic et al., 2010). Therefore, one could hypothesise that Akt promotes tomourigenesis by allowing cells to escape the deleterious consequences of DNA damage. Early stage neoplasia often present high levels of DNA damage, which leads to tumour suppressive responses such as senescence or apoptosis. Therefore, any mutations which affect DDR signalling would help cells to evade such cellular responses, enabling proliferating despite the presence of damage. If the damage is not repaired, this could result in elevated mutation rate and genomic instability, which are major contributors to carcinogenesis. Therefore, it is important to define the effects of active Akt on DDR signalling and clarify the role of Akt deregulation in tumour cell survival and resistance to cancer therapy.

3.2 Results

3.2.1 PTEN deletion suppresses DDR signalling

The initial experiments were designed to analyze how deletion of PTEN, one of the most common mutations in tumours and a cause of Akt activation, affects the activation and execution of DDR signalling following treatment with a DNA damaging agent.

To address these questions, several analyses were performed using two cell lines derived from colon carcinoma DLD1 and HCT116 cells, either "wild type" or harboring a targeted genetic deletion of exon II in both PTEN alleles (Figure 3.1A) (Lee et al., 2004). These cells were treated with ionizing radiation (IR) (10Gy) to induce DSBs, and an hour later the expression and activation of key DDR proteins was analyzed by Western blotting. The absence of PTEN in the knockout cells confirms the genetic deletion of the locus. As expected, this was found to cause elevated basal levels of activated S473-phosphorylated Akt when compared to wild-type controls (Figure 3.1B).

Interestingly, all three components of the MRN (Mre11, Rad50 and Nbs1) complex showed strikingly reduced expression in the PTEN null cells to the parental cell line (Figure 3.1B). In particular, Mre11 is the most affected component of the MRN complex, dropping to undetectable levels in HCT116 cells. The reduction in Rad50 and Nbs1 levels is not as strong as Mre11, suggesting that Mre11 could be the primary target that then affects the levels of the other two components. It is indeed well known that Mre11 is essential for the stabilization of Rad50 and Nbs1 polypeptides (Takemura et al., 2006).

As previously mentioned, the MRN complex is the sensor for DSBs and is necessary for the activation of the DDR machinery, the ATM kinase (Horejsi et al., 2004; Lavin, 2007; Lukas et al., 2003). The surprising reduction in the expression of MRN complex suggested that the loss of PTEN could potentially lead to a defective cellular response to DSBs. Indeed, PTEN-null cells didn't fully activate Chk2 in response to IR, as shown by the low levrl of phosphorylation at T68 by ATM, whereas total Chk2 levels remain unchanged (Figure 3.1B). Chk2 is responsible for the regulation of the G1, S and G2 checkpoints, and is also required for activation of several targets involved in initiating the DNA strand resection at DSBs (Xu et al., 2002), a precursor to ATR/Chk1 activation. In agreement with this, the downstream activation of the ATR/Chk1 pathway was suppressed, as indicated by reduced phosphorylation of the activating residue 345 of Chk1 in PTEN-null cells (Figure 3.1B).

Thus, the deletion of PTEN in two human colon carcinoma cell lines was found to cause hyperactivation of Akt and lead to suppression of the DNA damage response to ionizing radiation. All further analyses were performed with the HCT116 cells, since they presented with the strongest reduction in DDR signalling.

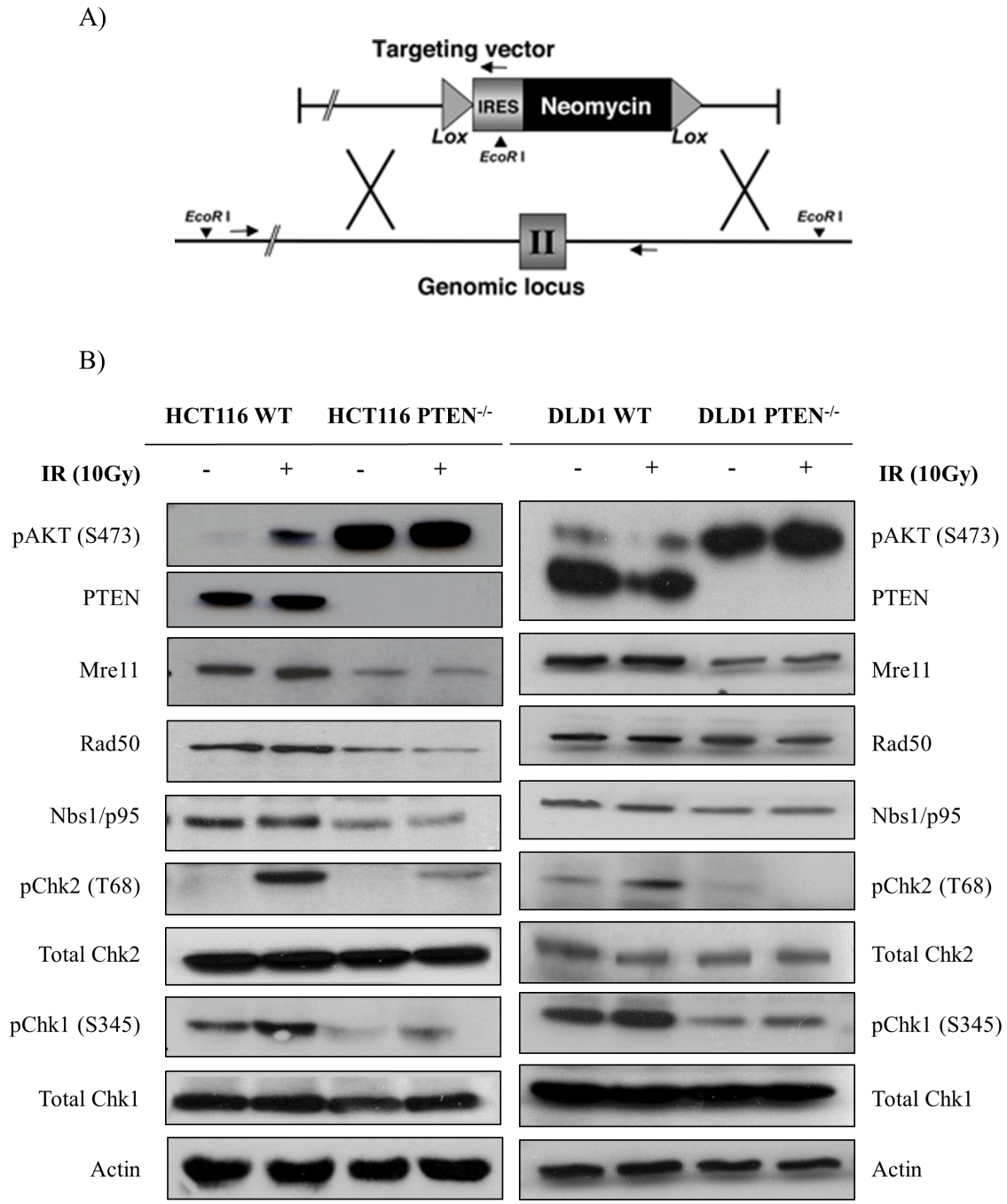


Figure 3.1: PTEN deletion affects DDR pathway activation following IR. (A) Schematic of PTEN gene targeting strategy. Exon II of PTEN was targeted by a recombination event between the genomic locus and the targeting vector, replacing it with a promoterless IRES-neoR, which is flanked by LoxP sites (Lee et al., 2004). (B) Western blots for key proteins in the DDR pathway were performed on whole cell extracts from HCT116 and DLD1 cells 1hr post irradiation with 10 Gy. Actin provides the loading control.

3.2.2. Mre11 is required for the activation of ATM/Chk2 and ATR/Chk1 pathways downstream of DSBs

In order to establish whether the reduction in expression of MRN complex components in PTEN null cells causes the reduced downstream activation of the ATM/Chk2 and ATR/Chk1 pathways, siRNA knockdowns were performed on the HCT116 WT cells. Mre11 levels were depleted by transfection with a smart pool of siRNA against Mre11 and and a non-targeting siRNA pool provided a negative control. The expression of MRN complex and activation of DDR proteins in response to IR was analysed by WB 48 hours post transfection. Mre11 expression was reduced by 60% when compared to control siRNA and as expected the expression of Rad50 was affected in a similar manner (Figure 3.2). Moreover, the reduction in expression of the MRN complex affects Chk1 and Chk2 activation one hour after exposure to IR, as evident by the reduced T68 and S345 phosphorylation of Chk1 and Chk2 respectively.

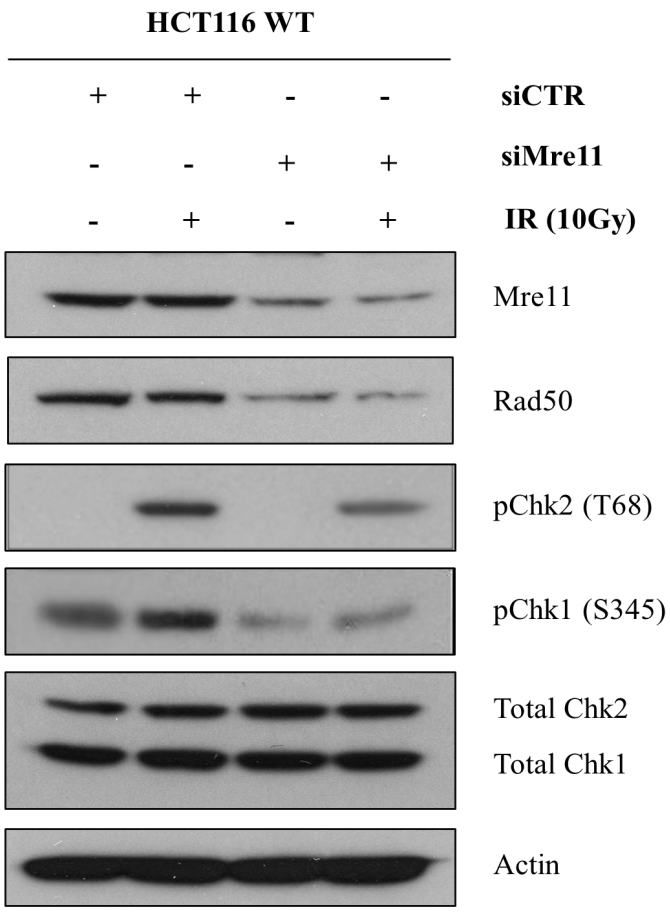


Figure 3.2: siRNA mediated depletion of Mre11 reduces Chk2 and Chk1 activation. Western blot analysis was performed on whole cell extracts from HCT116 WT cells 48hrs post-transfection with 200nM smart pool siRNA against Mre11 or non-targeting siRNA pool and 1hr post-irradiation with 10Gy. Actin provides the loading control.

In addition to this, the requirement for MRN complex and, in particular, for Mre11 nuclease activity in DDR signalling activation was also investigated using an Mre11 inhibitor, Mirin. Mirin is a new molecule produced to bind specifically Mre11, inhibiting its interaction with ATM and its exonuclease activity (Dupre et al., 2008; Garner et al., 2009). HCT116 WT cells were pretreated with two different concentrations of Mirin for 40 mins, exposed to 10Gy of radiation and Western blots were performed one hour later. As expected the level of Mre11 and Nbs1 remained unaltered by Mirin treatment (Figure 3.3). It has been previously shown that Mirin does not inhibit MRN complex formation or its recruitment to DBSs (Dupre et al., 2008; Lee et al., 2013). The inhibition of Mre11 with 100μM of Mirin was found to reduce the phosphorylation of Nbs1 on S343 and of Chk2 on T68, which confirms that Mre11 is necessary for the full activation of ATM. Chk1 activation via S345 phosphorylation was found to be reduced in a similar manner, indicating that Mre11 nuclease function is crucial for DSBs resection to ssDNA, which is required for the activation of ATR/Chk1 pathway.

Thus, the data so far indicate that the Mre11 expression is necessary for the stability of the entire MRN complex and that the presence of fully functional MRN complex is required for both full ATM/Chk2 and ATR/Chk1 activation.

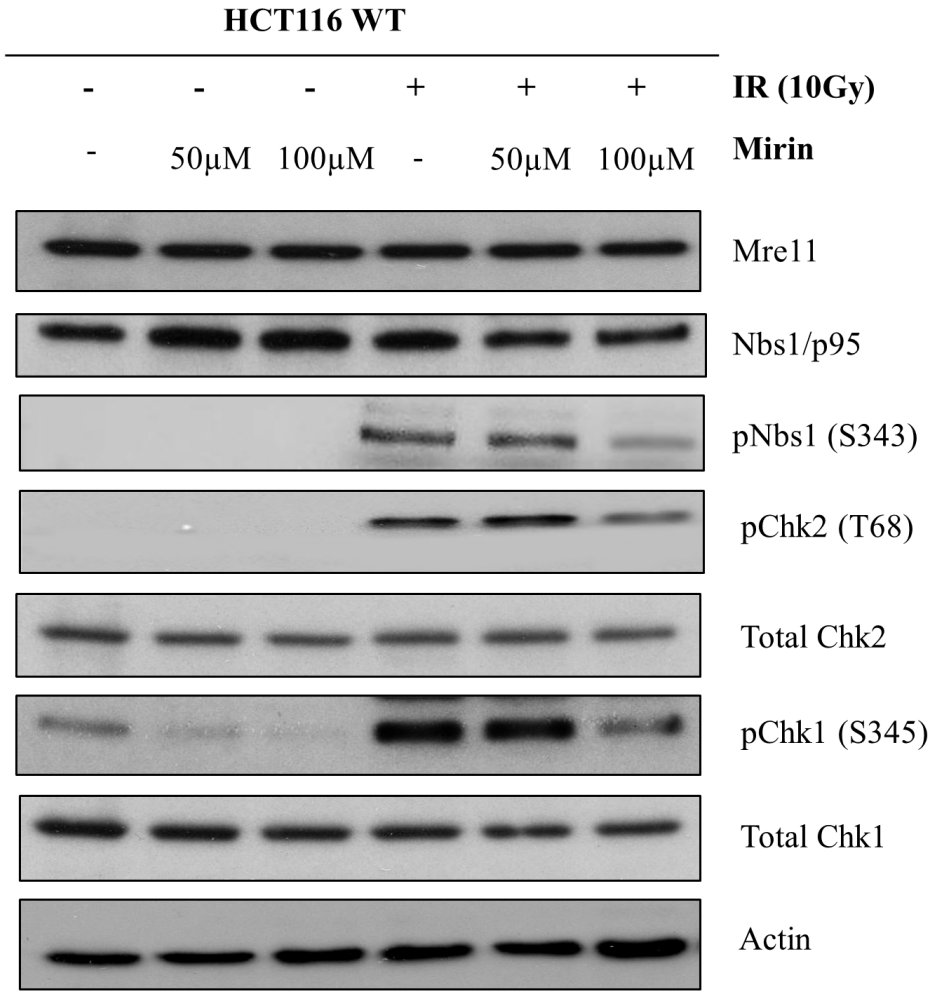


Figure 3.3: The inhibition of Mre11 activity by Mirin ablates Chk2 and Chk1 activation. Western blot analysis was performed on whole cell extracts from HCT116 WT cells, generated one hour after exposure to 10Gy irradiation and pre-treatment with 50μM and 100μM Mirin for 40 minutes. Actin provides the loading control.

3.2.3 Loss of PTEN causes defective HR due to reduced strand resection

As mentioned previously, DSBs are highly cytotoxic lesions that are mainly repaired in cells by two repair mechanisms, HR and NHEJ. It is also well known that the MRN complex is directly involved in early steps of DSBs repair. The effects of reduced MRN expression and activity in HCT116 cells as a result of PTEN loss or Mirin treatment on these two different repair mechanisms was further evaluated.

An elegantly designed fluorescent reporter system obtained from Gorbunova laboratory (Seluanov A., 2010) allowed the sensitive and qualitative measurement of these DNA repair processes. Two different constructs contain an engineered GFP gene, which is normally not expressed because it contains a disrupting exon or a mutation. In the case of the NHEJ reporter cassette, the GFP gene is disrupted by the insertion of the Pem1 intron, which contains an adenoviral exon cassette flanked by recognition sites for the I-SceI endonuclease (Figure 3.4A). These non-palindromic restriction sites are in an inverted orientation so as to generate two incompatible DNA ends upon cleavage, which mimics a DSB. Once the construct is digested with the I-SceI and the disrupting adenoviral exon removed, only repair with NHEJ can restore the GFP gene. The HR reporter cassette is based on the same Pem1 system. In this case, GFP-Pem1 has a 22bp deletion next to three restriction sites I-SceI/HindIII/I-SceI. The deletion ensures that repair with NHEJ cannot reconstitute an intact GFP ORF (Figure 3.4B). As before, the two I-SceI sites are in an inverted orientation so as to generate two incompatible ends. This GFP-Pem1 is followed by a second promoter-less/ATG-less first exon and intron of GFP-Pem1. After digestion with I-SceI, only HR via an intermolecular and intramolecular gene conversion between the two copies of GFP-Pem1 can restore a functional GFP ORF (Figure 3.4B) (Seluanov A., 2010). Thus, each construct is made with unique features to allow the successful repair of the I-SceI-induced breaks by one specific repair mechanism, HR or NHEJ.

The constructs were used in an extrachromosomal assay, where they were first digested *in vitro* by I-SceI and then transfected into HCT116 WT and PTEN knockout cells together with the DsRed plasmid expressing RFP, which controls for transfection efficiency. HCT116 WT cells were either pretransfected with a siRNA smart pool against Mre11 48 hours prior or pretreated with 15μ Mirin 40 mins prior to transfection with the linearised constructs. Cells well cultured in regular media or with Mirin for 24 hours post-transfection, following which they were harvested and the number of GFP and RFP positive cells was counted by flow cytometry (Figure 3.4C). The number of GFP positive cells was divided by the number of RFP positive cells in order to correct for transfection efficiency. The number obtained provides a quantitative measure of NHEJ or HR efficiency and is plotted in Figure 3.4D. The data suggest that the deletion of PTEN in HCT116 significantly affects their ability to repair DSBs with HR, which shows a 50% reduction compared to WT cells. On the contrary, NHEJ repair of DSBs shows a 30% increase, which possibly compensates for the inability to use HR. A similar decrease in HR was also observed for WT cells treated with Mirin or siRNA against Mre11. These data suggest that HR, but not NHEJ, is sensitive to the loss of the MRN complex.

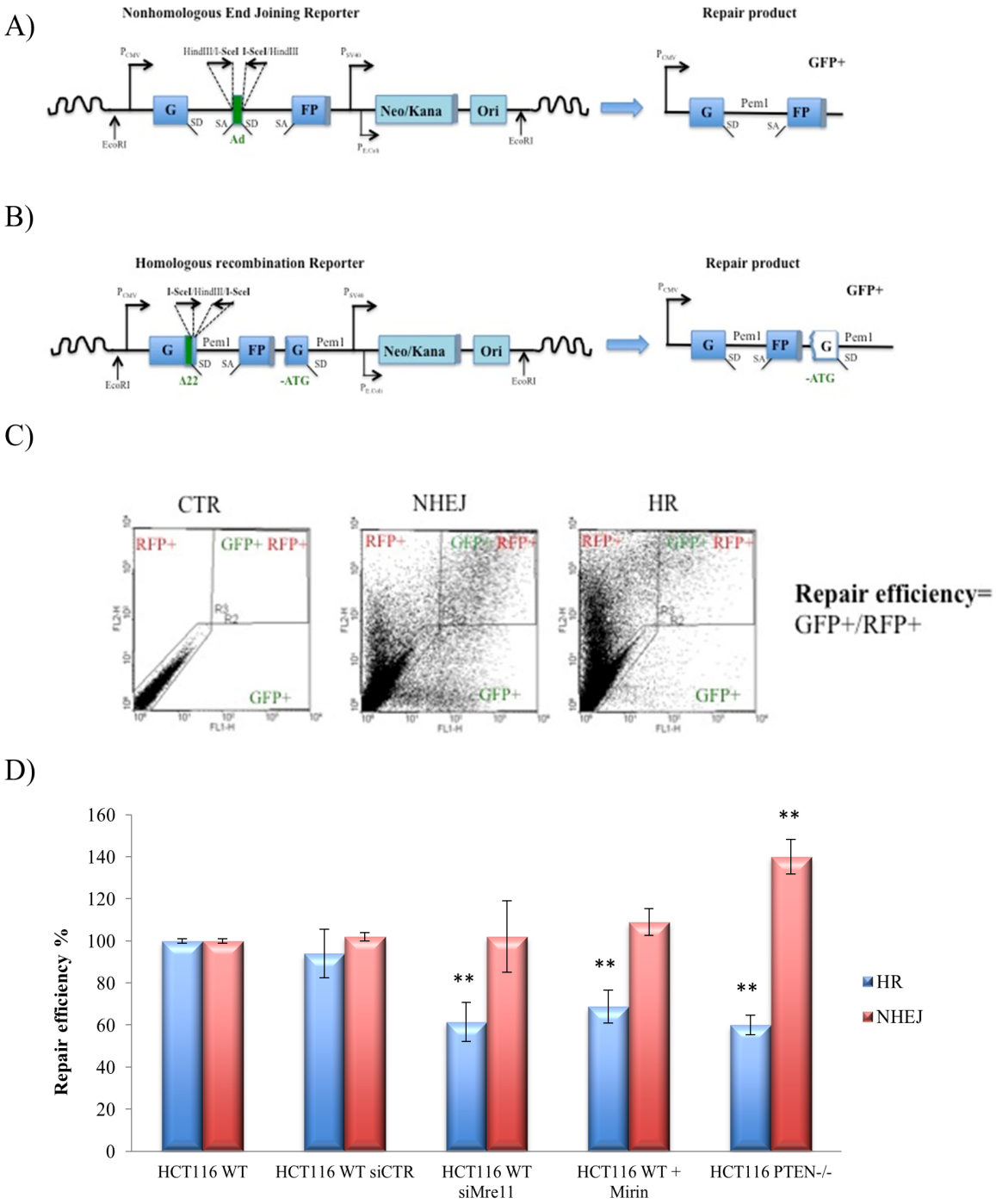
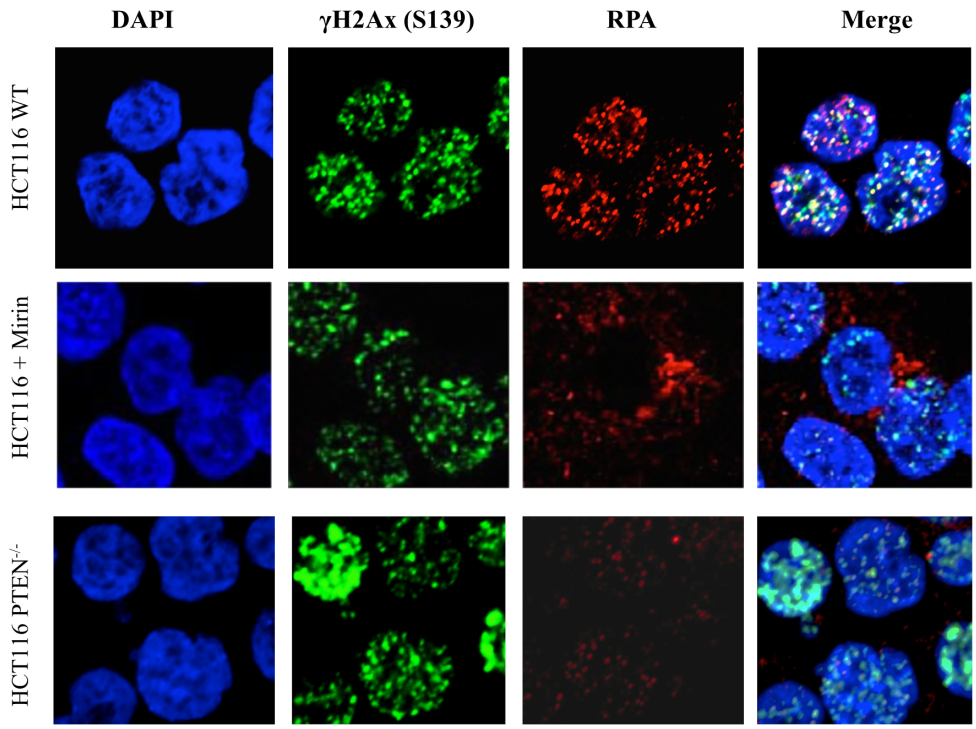


Figure 3.4: Efficient HR repair is sensitive to the loss of MRN complex. (A) (B) Schematics of the reporter constructs used to quantify NHEJ and HR repair [Figure adapted from Seluanov A., 2010]. (C) Representative FACS profiles with cells harvested 24 hours post-transfection with the reporter constructs presented in panel A. Cells transfected with empty vector provided the negative control. (D) Frequency of NHEJ and HR was analyzed with two independent NHEJ and HR constructs in HCT116 WT and PTEN$^{-/-}$ cell lines. The WT cell line was either pre-transfected with control siRNA or siRNA pool against MRE11 or pre-treated with 15μM Mirin for 40 mins. The ratio of GFP+/DsRed+ cells was used to measure repair efficiency and is presented as a percentage of the untreated WT control. The data represent three independent biological replicates. ** denote $p<0.01$ as calculated by Student's t-test.

Mre11 plays a crucial role in HR repair. Its nuclease activity in conjunction with additional factors, such as Ctip, opens DNA to catalyse the reaction 5´➔3´ single strand resection. This action is required for the formation of 3´ ssDNA for RPA loading and then for strand invasion, a step that characterises HR (Limbo et al., 2007; Sartori et al., 2007; Williams et al., 2008).

To investigate if the failure in HR repair in HCT116 PTEN knockout cells is due to a defect in this pivotal step of HR, the recruitment of RPA to DNA damage sites was analysed. Before performing the immuno-staining, cells were treated with a detergent-containing buffer to remove the nucleoplasmic and cytoplasmic proteins, leaving behind the chromatin bound and matrix-associated proteins. The number of RPA foci per nuclei was counted. As shown in figure 3.5A-B, immunofluorescence analysis showed that irradiation induced a marked persistence in concentration of RPA at γH2Ax foci in the majority of HCT116 WT cells. However, a dramatic reduction in chromatin-associated RPA foci was observed in PTEN null cells compared to WT. In addition to this, persistence of γH2Ax foci was also observed, especially at longer time points (data not shown), verifying a defect in the repair process. The pre-treatment of WT cells with Mre11 inhibitor Mirin was also found to inhibit RPA foci formation without affecting γH2Ax foci, resembling the phenotype observed in PTEN null cells.

Collectively, these data suggest that PTEN null cells, due to the low amount of MRN complex, fail to resect DSBs into ssDNA required for HR repair, thus impairing the recruitment of RPA and activation downstream of ATR/Chk1 pathway.



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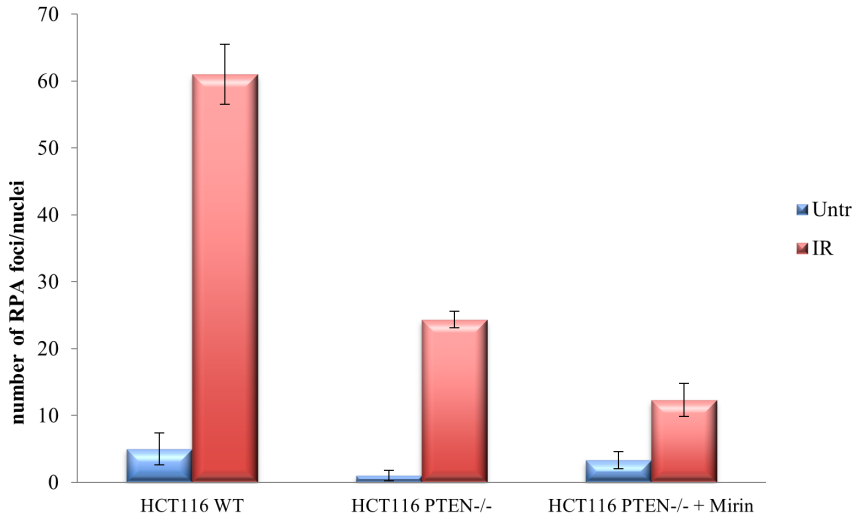

Figure 3.5: Loss of PTEN leads to reduced strand resection at DSBs. (A) Immunofluorescence analysis of phosphorylated H2Ax and RPA foci in HCT116 WT with or without pre-treatment with Mirin, and in PTEN null cells 4 hours after exposure to 10Gy IR. (B) Quantification of the number of RPA foci per nuclei. The data represent three independent biological replicates.

3.2.4 Loss of PTEN ablates effective G2/M checkpoint activation

The data so far suggest that reduced expression of MRN complex in cells without PTEN correlates with the compromised activation of the two checkpoint kinases. This event is apparently mediated by two different factors: failure to fully activate ATM due to low levels of MRN component proteins, and failure to activate ATR/Chk1 due to compromised strand resection. To ask whether failure to activate Chk1/Chk2 is linked to defective downstream signalling and G2 arrest, several analyses were performed.

To analyse the dynamics and timing of the G2/M checkpoint activation, both HCT116 WT and PTEN$^{-/-}$ were exposed to 10Gy of IR and the samples were analysed by flow cytometry at different time points. The distribution of cells along the cell cycle was visualized by PI incorporation. PI is a fluorescent dye that stains the DNA, thus allowing the visualisation of the cell cycle profile as two different peaks, the first one constituted by cells in G1 phase (2N DNA content) and the second one by cells in G2 phase (4N DNA content). In between these two peaks lie the cells in S phase. HCT116 WT displayed a proficient G2/M checkpoint, as seen from the progressive accumulation of cells in the G2 phase, which peaked at 9 hours (marked by an asterisk). This is followed by the release of the cells from G2 arrest by 24 hours (Figure 3.6). PTEN null cells also displayed a G2 accumulation following irradiation, however the G2 peak at 9 hours appeared smaller and the G1 peak bigger. At 9 hours, 74% of WT cells had arrested in G2 compared to 55% of PTEN$^{-/-}$ cells. This result indicates that the G2/M checkpoint is not fully activated in PTEN null cells, thus allowing the damaged cells to progress into mitosis and then renter the cell cycle.

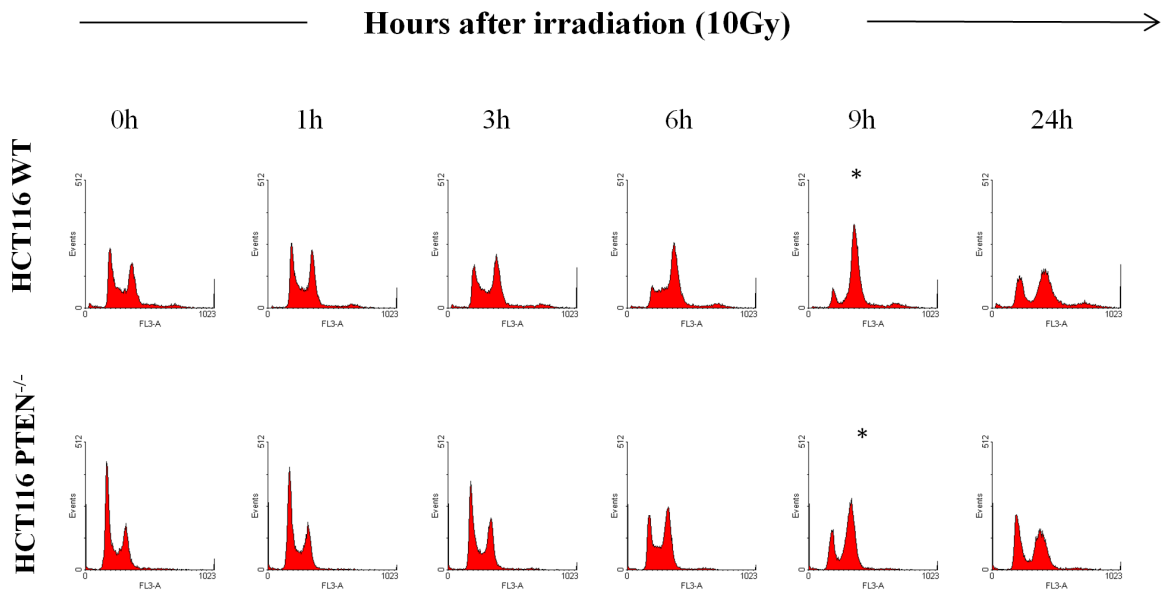

Figure 3.6: Loss of PTEN impairs the cell cycle checkpoint response to IR. FACS analysis measuring the incorporation of PI into HCT116 WT and PTEN null cells to analyse the cell cycle progression at different time points after exposure to 10Gy IR. The asterisk indicates the maximal accumulation of cells in G2 phase.

In order to more accurately assess the checkpoint proficiency, cells were subjected to a nocodazole trapping experiment. Cells with or without prior exposure to different doses of IR were incubated with nocodazole, and the accumulation of mitotic cells was quantified after 9 hours by evaluating the phosphorylation of histone H3 (pH3) by FACS (Figure 3.7A). Nocodazole is able to trap cells in mitosis by disrupting microtubules, thereby enabling the measurement of the rate at which cells enter mitosis from G2. This measurement can directly document the efficacy of the G2/M checkpoint activation in response to damage.

As shown in the figure 3.7A, HCT116 WT cells increasingly accumulated in G2 phase in an IR-dose dependent manner (4N DNA content) and less cells progressed to mitosis, as measured by reduced pH3 staining. However, PTEN null cells showed a comparatively higher number of pH3 positive mitotic cells. An IR-dose dependent increase in the difference between the two cell lines was observed (Figure 3.7B). Thus, the higher is the dose of IR, the greater is the difference in ability to activate the G2/M checkpoint between the two cell lines. Western blot analysis of lysates from HCT116 WT cells revealed that Chk1 activation as measured by S345 phosphorylation is IR dose-dependent (Figure 3.7C). This leads to a dose-dependent inhibition of the G2/M progression activator CDC25C, as measured by the inhibitory S216 phosphorylation. However, this trend is lost in HCT116 lacking PTEN.

Put together these results suggest that PTEN null cells are unable to activate the G2/M checkpoint as efficiently as the control parental cell line. This defect becomes increasingly apparent following incremental damage due to higher doses of IR.

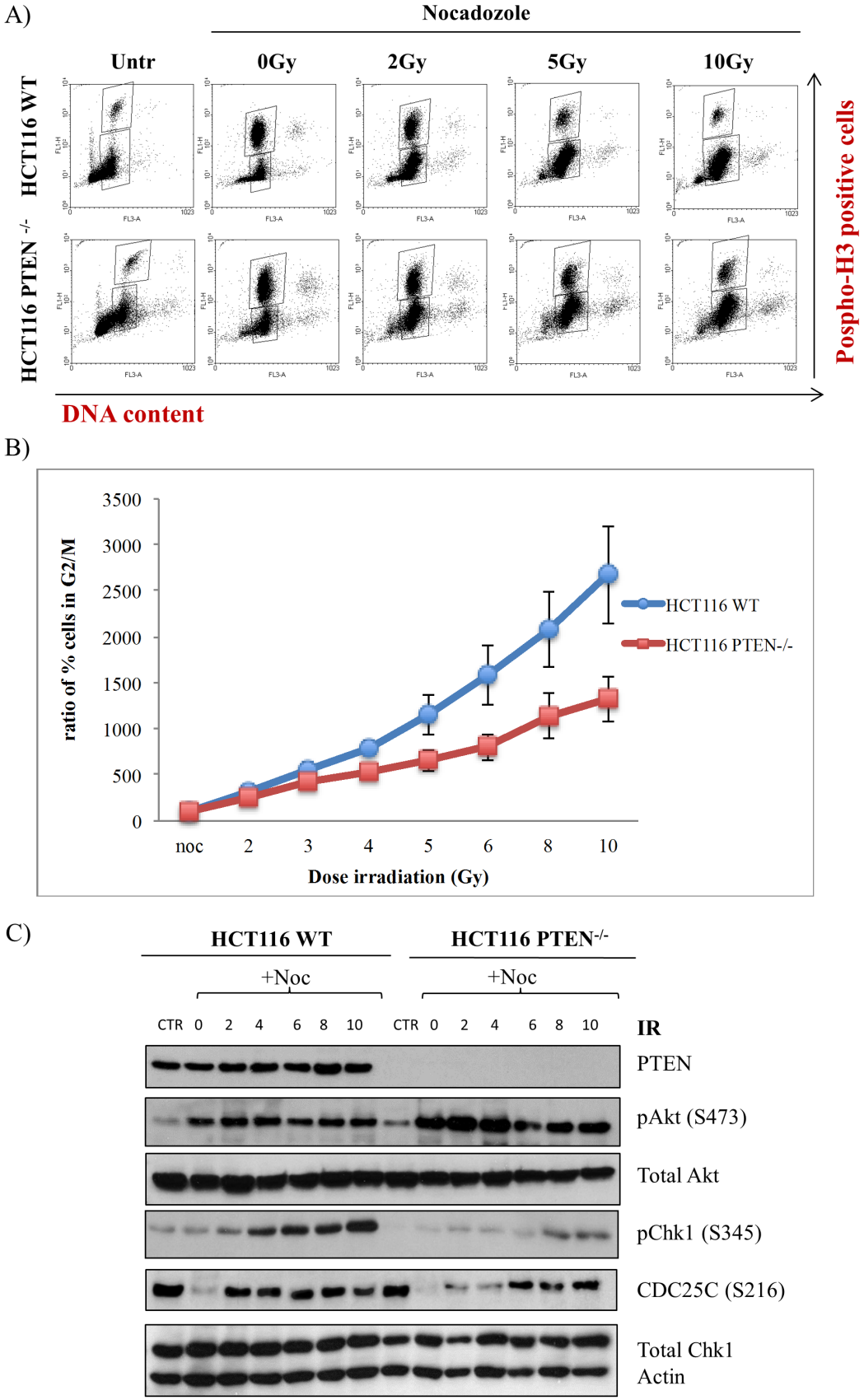

Figure 3.7: PTEN null cells show an impaired G2/M checkpoint. (A) FACS analysis measuring phopho-histone H3 levels and PI incorporation in replicate cultures of HCT116 WT and PTEN$^{-/-}$ cells with or without prior exposure to different doses of IR and incubation with Nocodazole for 9 hours. (B) FACS data presented as the ratio of cells in G2 (4N cells) over cells in M phase (positive for phosphorylated H3). The data are normalised to the Nocodazole (noc) sample not exposed to IR and average ± S.E.M. of n=3 are plotted. (C) Western blot analysis performed on lysates from cells in panel A. Actin provides the loading control.

3.2.5 PTEN loss or inhibition of Mre11 radiosensitises HCT116 cells

In order to test whether an inefficient activation of the G2/M checkpoint and HR impairment in PTEN null cells affects their ability to proliferate after exposure to ionising radiation, a clonogenic survival assay was performed. As Mirin was used to inhibit Mre11 in the clonogenic assay, it was necessary to establish the minimum dose that hits the target prior to the performing experiment. An initial clonogenic assay with varying doses of Mirin was performed in order to establish this optimal dose (Figure 3.8A). In addition to this, CellTiter-Glo® Luminescent Cell Viability Assay kit was also used to determine the number of viable cells in the plate after treatment with the titration of Mirin (Figure 3.8B). The assay measures the relative luminescence emitted by each sample, that is proportional to the amount of ATP present in the plate and used by the luciferase present in the reagent CellTiter-Glo®. Thus the amount of luminescence is a measure of the metabolic activity of cells and reflects the number of viable cells. From the results of both these cell viability measures, 15 μM was chosen as the highest least-toxic concentration of Mirin.

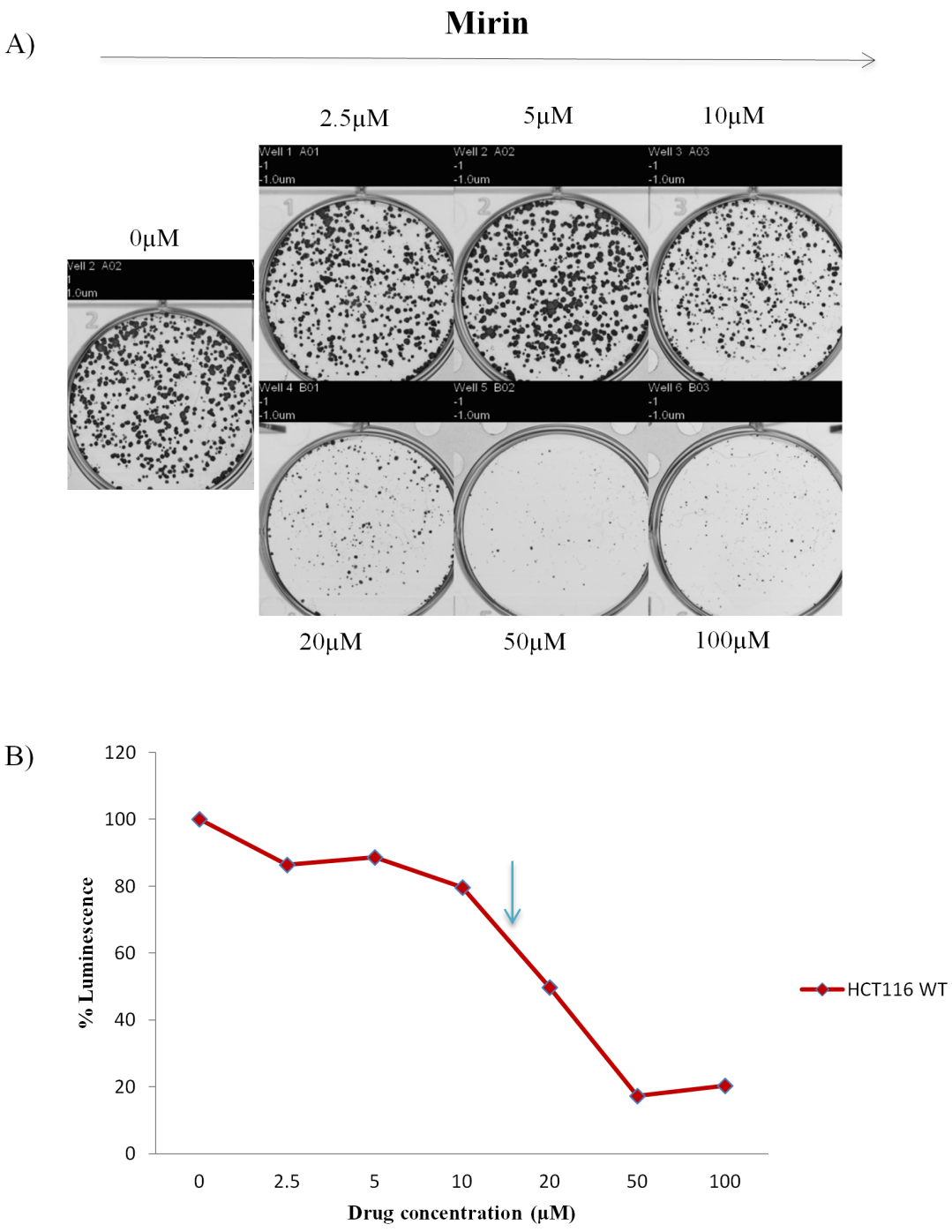

Figure 3.8: Dose tolerance curve for Mirin in HCT116 WT cells. (A) Clonogenic assay with HCT116 WT cells treated with a titration of Mirin for 24 hours and allowed to recover for 12 days. (B) CellTiter-Glo® Luminescent Cell Viability Assay performed on HCT116 WT cells treated with a titration of Mirin for 24 hours. The % Luminescence reflects the cell viability and the blue arrow points to dose used for further experiments.

The clonogenic assay showed that increasing doses of IR progressively impair the survival of HCT116 WT cells (Figure 3.9). The highest dose of IR reduced the % survival by a third in the WT cells. However, the PTEN null cells showed much greater sensitivity to IR than the WT counterparts, showing only 13% survival following treatment with 5Gy IR. Moreover, the treatment of WT cells with Mirin also sensitises them to IR, giving an intermediate response when compared to PTEN null cells. PTEN null cells were not treated with Mirin, however it may interesting to evaluate if additional Mre11 inhibition further reduces cell survival.

These results suggest that the impairment of HR repair due to the lack of a functional Mre11 causes the presence of high levels of unrepaired damage that then dramatically impairs cell survival.

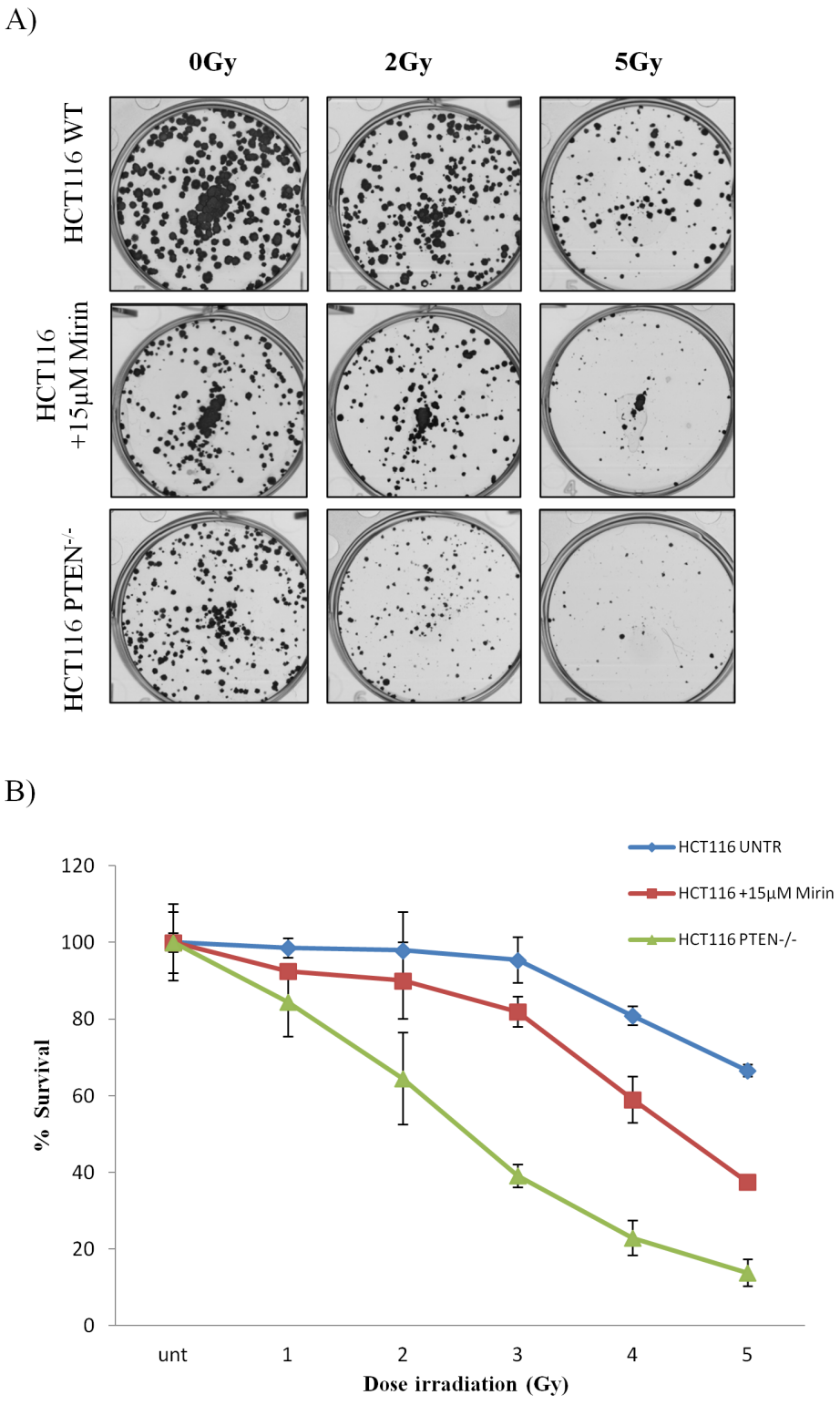

Figure 3.9: PTEN loss or Mirin treatment radiosensitises HCT116 cells. (A) Clonogenic assay performed on HCT116 WT cells with or without 40 min pre-treatment with 15 μ M Mirin or HCT116 PTEN$^{-/-}$ cells exposed to different doses of IR (0 to 5Gy). The cells were then cultured with or without Mirin for 24 hours and allowed to recover for 12 days. The colonies formed were visualized by Crystal violet staining. (B) Survival curves plotted with the number of colonies determined by the GelCount™. % Survival was calculated relative to the cells untreated with IR. The data represent three independent biological replicates.

3.2.6 Cells without PTEN are prone to genomic instability

Despite the overall reduction in cell survival compared to the wild-type cells, a percentage of PTEN null cells do survive and form colonies following irradiation in Figure 3.9. In order to assess the long-term effects of irradiation on the cell cycle in the checkpoint and HR deficient HCT116 PTEN$^{-/-}$ cells, FACS analysis similar to figure 3.6, at extended times after IR.

The data obtained revealed that a few days (120 hours) after irradiation, the WT cells recover perfectly from the damage and reacquire a normal cell cycle profile. On the contrary, PTEN null cells are still severely affected showing cell death as observed from the sub-G1 peak (Figure 3.10A). Moreover, a substantial proportion of the PTEN$^{-/-}$ cells also present with >4N DNA content as indicated by the arrow in figure 3.10A and quantified in figure 3.10B. 8% of the surviving PTEN$^{-/-}$ population was detected to be polyploid, whereas just 1% of the wild-type cells demonstrated this phenotype.

DAPI staining of HCT116 PTEN$^{-/-}$ nuclei confirmed the presence of nuclear abnormalities consistent with polyploidy (Figure 3.10C). In addition to this, these cells also showed the presence of micronucleation 72 hours post-irradiation. These data indicate that the loss of PTEN leads to genomic instability following exposure to IR.

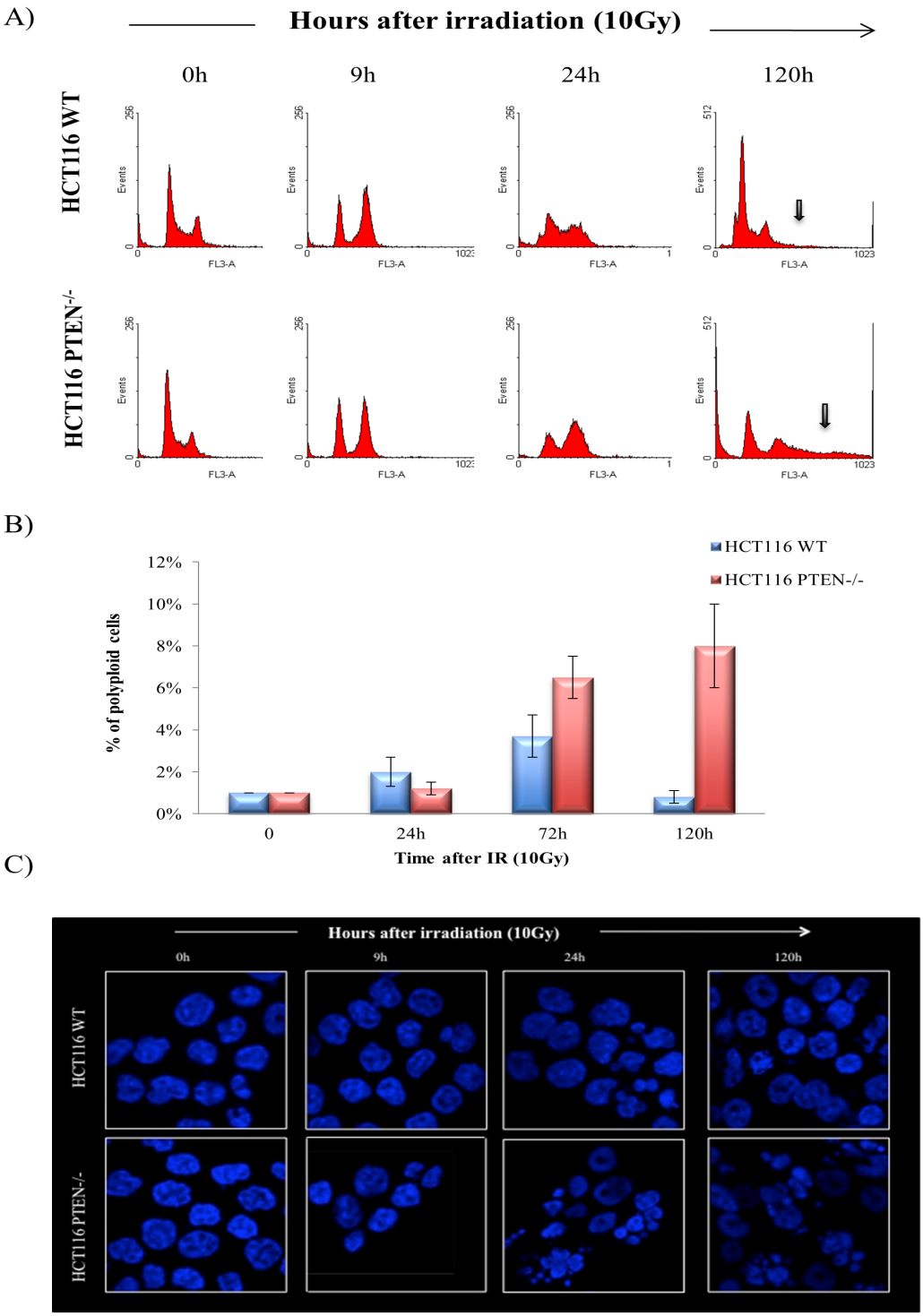

Figure 3.10: PTEN$^{-/-}$ cells present elevated genomic instability following IR exposure. (A) FACS analysis measuring the incorporation of PI into HCT116 WT and PTEN null cells at different time points following exposure to 10Gy IR. (B) Quantification of polyploid cells in HCT116 WT and PTEN null cells from the FACS analysis. The data represent three independent biological replicates. (C) DAPI staining of HCT116 WT and PTEN null cells at different time points after exposure to 10Gy IR.

3.3 Discussion

Cells are constantly exposed to various sources of stress that cause DNA damage and if not dealt with, this can threaten genomic integrity. Therefore, cells have evolved powerful mechanisms to contend with damaged DNA. The activation of cell cycle checkpoints is crucial for preventing the accumulation and inheritance of damage to daughter cells (Tyson et al., 1995). Furthermore, checkpoint-mediated arrest is necessary for allowing the cells to resolve the damage with the appropriate DNA repair pathway (van den Bosch et al., 2002). Loss of function or mutations in any of DDR components that govern these cellular mechanisms, result in abnormal genomes with chromosomal rearrangements and aneuploidy, thus predisposing cells to cancer (Jackson and Bartek, 2009).

Akt is often found activated in several human cancers driving neoplastic transformation (Calvo et al., 2009). Several publications have shown DDR signalling to be a target of Akt signalling, however the mechanism behind this is still unknown. However, other studies suggested that also PTEN is correlated with DNA repair. PTEN deletion has been shown to affect DNA repair causing elevated levels of unrepaired DSBs (Pappas et al., 2007; Shen et al., 2007b).

Using a PTEN null background, we present evidence suggesting that PTEN deletion affects the expression of the components of the MRN complex in two human colon carcinoma cell lines. However, more experiments are required to ascertain whether activated Akt or PTEN deletion leads to this reduction and the mechanism by which it is achieved. This is required in order to fathom the full implications of the findings in this chapter and the following chapter will address this issue further.

Mre11 is among the first proteins to be recruited to DSB sites. Helped by the other two components Rad50 and Nbs1, it binds the broken ends of the DNA mediating the recruitment of ATM machinery to activate the DDR signalling (Uziel et al., 2003). It has been shown that MRN-ATM interaction, mediated mainly by Nbs1, is pivotal for its full activation after damage (You et al., 2005). Therefore, the reduction of MRN complex affects the phosphorylation and activation of Chk2, the main target of ATM. In agreement with this, the reduction in the components of MRN complex in $PTEN^{-/-}$ cells was also found to reduce the downstream DDR signalling via the ATM/Chk2 and ATR/Chk1 pathways in response to irradiation. This reduction in signalling can be replicated by the

inhibition of MRE11 with Mirin in wild-type cells, and is therefore likely to be a direct result of reduced MRE11 expression in PTEN$^{-/-}$ cells.

A reduction in MRE11, due to loss of PTEN or treatment with Mirin, was found to cause reduced HR efficiency in HCT116 cells. HR is able to mediate a faithful repair of DNA damage preventing the accumulation of any illegitimate repair, which may contribute to cell transformation (Johnson and Jasin, 2001). DNA strand resection is an essential step in HR repair, and is dependent on the nuclease activity of MRE11. Thus, a substantial reduction in RPA foci is observed in wild-type cells treated with Mirin or PTEN$^{-/-}$ cells. RPA32 is a ssDNA binding protein and RPA foci are widely used as markers for ssDNA. However, MRN complex is not necessary for ATM or ATR or DNA-PK-mediated phosphorylation of γH2Ax. This explains why no substantial reduction in γH2Ax foci was observed.

The recruitment of ATRIP-ATR is strictly dependent on ssDNA-RPA via PCNA like 9-1-1 checkpoint clamp (Delacroix et al., 2007). Activated ATR recruits a second mediator, Claspin, which binds Chk1 bringing it in proximity of ATR for its phosphorylation and activation. All these events are regulated by the formation of single-stranded DNA. Therefore, the reduced strand resection would severely impair Chk1 activation. Chk1 and Chk2 are checkpoint kinases. These proteins are essential for the regulation of many nuclear and cytoplasmic substrates downstream ATM/ATR (Lukas et al., 2003). The extent of their activation is directly proportional to the severity of the damage. High level of damage requires high amount of Chk1 active to induce a longer cell cycle arrest by inhibition of CDC25C phosphatase.

PTEN$^{-/-}$ cells were found to display a defective G2/M checkpoint and reduced clonogenic survival in response to IR exposure. This was consistent with wild-type cells treated with Mirin, suggesting a crucial role of MRE11 in the observed phenotypes. The lack of a strong activation of ATM/ATR by the MRN complex is likely to have impaired the Chk1-dependent activation of G2/M checkpoint. This would allow a big part of the population to progress to mitosis, despite containing unrepaired damage. Moreover, in the cells that do arrest, deficiency in HR would lead inefficient and error-prone repair. Together, this contributes to make PTEN null cells extremely sensitive to radiation.

Damaged DNA compromises the fidelity of Mitosis, promoting aneuploidy. Thus, a significant percentage population surviving following IR insult was found to display

genomic instability and micronucleation. These data indicate that cells with a PTEN deletion or hyperactive Akt are more likely to attain unstable genomes due to reduced repair and checkpoint efficiency. Thus, despite a reduction in cell viability following irradiation, the surviving population is more mutagenic and possibly more prone to tumourigenesis. Therefore, loss of PTEN or Akt hyperactivation could be acquired by tumour cells to gain more genomic instability, necessary to promote tumour progression and invasion.

Chapter 4: Activated Akt impairs the stability of MRN complex through S6K activity

4.1 Introduction

The Mre11-Rad50-Nbs1 is an evolutionary conserved complex, and orthologues of Mre11 and Rad50 can be found in all taxonomic kingdoms (van den Bosch et al., 2003). Mre11 is uniformly spread in the nuclei of resting undamaged cells. Following treatment with agents that induce DNA DSBs such as IR, Mre11 is dynamically redistributed (Nelms et al., 1998). Mre11 associates with the DNA at these breakage points forming discrete nuclear foci (Maser et al., 1997). Mre11 foci appear in a dose- and time-dependent way in cells treated with DSB-inducing agents. However, this is not the case in cells treated with DNA damaging agent such as UV that induce other type of DNA lesions (Limoli et al., 2000; Maser et al., 1997). Moreover, it has been observed that MRN complex associates to chromatin even in unperturbed conditions (Costanzo et al., 2001; Mirzoeva and Petrini, 2003). These and other evidences showing the localization of MRN complex at replication forks have suggested that MRN complex has also a preventive role against the accumulation of DBSs during replication (Mirzoeva and Petrini, 2003; Olson et al., 2007; Robison et al., 2004; Tittel-Elmer et al., 2009).

MRN complex *in vivo* is constituted by a core complex $Mre11_2Rad50_2$, further stabilized by Nbs1 binding giving an overall stoichiometry of $Mre11_2Rad50_2Nbs1_2$ (Trujillo et al., 1998). The intra-molecular interactions between the three components are essential for the physiological roles of the complex itself. The interaction of Rad50-Mre11 stimulates both Mre11 exo- and endo-nuclease activity, which is further stimulated by Nbs1 interaction (Paull and Gellert, 1998, 1999). The knockdown of one component of MRN decreases the levels of the other two members. Thus, mutations in Mre11 cause reduction levels of Rad50 and Nbs1 resulting in a strongly destabilized complex (Paull and Gellert, 1999; Stewart et al., 1999; Zhong et al., 2005). The addition of wild type Mre11 in these cells restores the normal level of Rad50 and Nbs1 (Stewart et al., 1999; Uziel et al., 2003).

Emerging data also suggest an important role for post-translational modifications in the regulation of the MRN complex. Upon its recruitment by the MRN complex to the DNA damage sites, ATM along with ATR phosphorylates all three components of the MRN complex (Matsuoka et al., 2007; Mu et al., 2007). This suggests that MRN complex activity is regulated by a feedback loop mechanism that involves phosphorylation. Mre11 in particular contains eight putative phosphorylation sites for PIKK kinases. Its phosphorylation is crucial for regulating the entire dynamic of DDR, whereby the phosphorylation facilitates its dissociation from the chromatin and is necessary for its

inactivation after completion of repair. This allows the downregulation of the DNA damage signalling and the recovery from checkpoint arrest. However, the inhibition of ATM and ATR with caffeine or wormannin only partially inhibits Mre11 phosphorylation, suggesting that other kinases might be involved in Mre11 phosphorylation (Di Virgilio et al., 2009).

Moreover, Mre11 ATM-mediated phosphorylation is mediated by Nbs1-ATM interaction. Thus Nbs1 deficient cells lose Mre11 phosphorylation, which also impairs Mre11 foci formation (Dong et al., 1999). Several ATM-dependent phosphorylation sites have also been mapped on Nbs1 by mass spec analysis, and these are found to be evolutionary conserved in vertebrates (Tauchi et al., 2001; Tauchi et al., 2002). These sites seem to be important for the regulation of different cellular processes such as intra-S checkpoint activation and p53-independent apoptosis (Iijima et al., 2008a; Iijima et al., 2008b). Nbs1 can be also acetylated by PCAT and p300 histone acetyl transferases (HATs), whereas SIRT1 regulates its deacetylation when it is bound to Mre11-Rad50. Interestingly, hyperacetylation of Nbs1 impairs DNA damage-induced activation mediated by ATM phosphorylation, suggesting that phosphorylation and acetylation work antagonistically in regulating Nbs1 activity (Yuan et al., 2007).

However, other post-translational modifications, such as methylation, have been shown to impact Mre11 activity. At least nine methylated arginines were identified in the C-terminus of Mre11 protein, which are important for the function of this region (Boisvert et al., 2005b). PRMT-mediated methylation of Mre11 occurs before Mre11 associates with Rad50 and Nbs1 to form the complex. However, the impairment of Mre11 methylation doesn't affect the formation of the complex, but it affects the binding to DNA and its nuclease activity (Boisvert et al., 2005b; Dery et al., 2008). Thus, Mre11 methylation seems to be crucial for its recruitment to the damage sites, regulating DSB processing and intra-S checkpoint activation.

As mentioned previously, Akt signalling has previously been shown to influence DNA repair and checkpoint response. In particular, Akt-mediated phosphorylation can affect the expression or subcellular localisation of its targets. In some cases of human cancer, activated Akt phosphorylates Brca1 inducing its cytoplasmic retention inhibiting HR repair (Plo et al., 2008; Tonic et al., 2010). Akt also mediates Chk1 phosphorylation at S280, inducing its translocation to the cytosol and then inhibiting its checkpoint function (King et al., 2004; Puc et al., 2005; Shtivelman et al., 2002). This phosphorylation also affects its

interaction with Claspin and its full ATR-mediated activation (Pedram et al., 2009). Therefore, it has been observed in many cancers that high level of active Akt correlates with resistance to treatment as chemotherapy and/or radiotherapy (Bellacosa et al., 2005).

However, Akt exerts its full oncogenic potential through the activation of other protein kinases such as mTORC1 and S6K. Therefore, the inhibition of mTOR by rapamycin has been shown to induce cell cycle arrest and/or apoptosis in tumours with activated Akt (Alam et al., 2004; Altomare et al., 2004; deGraffenried et al., 2004). mTOR, often called the master regulator of cell growth, is also found to regulate the abundance of several proteins involved in maintenance of chromosomal integrity and DDR activation (Li et al., 2006). The knockdown of mTOR or its inhibition by rapamycin induces ATM activation by upregulating the steady-state level of unidentified proteins in the DDR pathway, which are responsible for ATM activation (Bandhakavi et al., 2010). However, this can be achieved via indirect regulation through other direct targets and more studies are necessary to clarify the link between mTOR and DDR signalling.

mTOR controls growth, proliferation and translation efficiency through two downstream signalling molecules, S6K1 and S6K2. Recently evidences have linked S6K to tomourigenesis. It has been shown that activated Akt induces the tumour formation in mouse pancreas through S6K1 protein activation (Alliouachene et al., 2008). Other studies have shown how mTOR-S6K pathway controls p53-mediated DNA damage response in presence of genotoxic stresses. S6K1 adapts the damage response to the growth condition and energy levels of the cells, regulating cell death by MDM2 inhibition (Lai et al., 2010). Therefore, mTOR-S6K pathway can be another pathway involved in tumourigenesis, through the regulation of DDR.

This chapter investigates the potential mechanisms by which PTEN loss or activated Akt signalling ablates MRN complex expression and activity.

4.2 Results

4.2.1 S6K1 but not Akt impairs MRN complex stability

The data so far indicate that in HCT116 cells the deletion of PTEN causes a reduction in MRN complex which compromises both the G2/M checkpoint and the DNA repair response, associated with a higher basal level of active Akt. To investigate whether activated Akt or the lack of PTEN is most directly responsible, HCT116 WT cells were transiently transfected with a constitutively active form of Akt (GAG-Akt), and its effect on levels of MRN complex components was analyzed by Western blot at different time points post-transfection. GAG is a viral sequence, which anchors Akt to the plasma membrane where it is fully activated by PDK1 and mTORC2. High levels of active GAG-Akt (reflected by its phosphorylation on S473) were expressed in WT cells for 72 hours, identified as the upper band above the endogenous Akt (Figure 4.1). Kinase activity was determined by the phosphorylation of p70-S6K, and showed a substantial increase following expression of GAG-Akt. The expression of active GAG-Akt in WT cells correlated with the gradual reduction of both Mre11 and Rad50 levels, dropping to lowest levels at 72 hours. These data are consistent with the idea that activated Akt mediates the downregulation of MRN complex in HCT116 PTEN$^{-/-}$ cells.

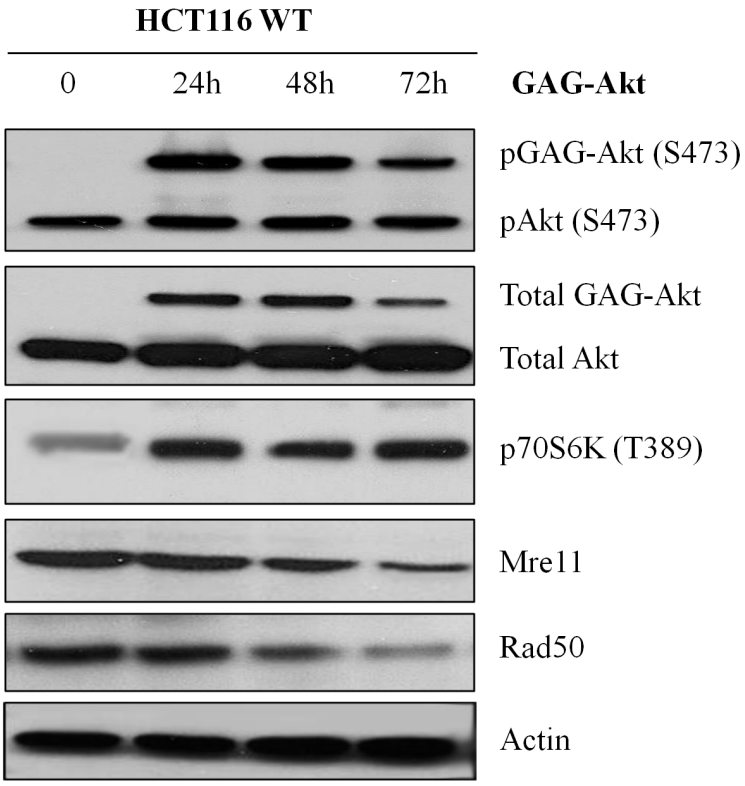

Figure 4.1: Expression of hyperactive Akt in HCT116 WT cells reduces expression of MRN complex. Western blot analysis of HCT116 WT cells transiently transfected with GAG-Akt and lysed at the indicated time-points post-transfection. Actin provides the loading control.

Constitutive activation of Akt induces the activation of other protein kinases downstream in the signalling pathway. To further investigate if the downregulation of MRN complex is directly mediated by Akt or one of its downstream effectors, PTEN null cells were treated with several inhibitors targeting various kinases at different levels in the Akt/mTORC1/S6K pathway.

Cells were treated with inhibitors for 72 hours, following which the levels of MRN complex and DDR pathway activation upon IR exposure were analysed by Western blotting. A pleckstrin homology (PH) domain inhibitor at the concentration of 1μM was used to inhibit the activation of both Akt1 and Akt2, which resulted in a strong reduction of its phosphorylation on S473 (Figure 4.2). Surprisingly, the inhibition of Akt did not affect the activation of mTORC1/S6K pathway. 500nM Everolimus, a Rapamycin analogue, was used to selectively inhibit mTORC1 activity. This was found to efficiently inhibit mTORC1-mediated activation of S6K, as observed from the lack of its activating phosphorylation and from the decreased phosphorylation of its substrate, the ribosomal S6 protein. Furthermore, PF4708671 at 20μM was used to inhibit S6K1 activity, confirmed by the strongly reduced phosphorylation of S6 protein. The inhibition of mTORC1 not only restored Mre11 and Rad50 levels, but also Chk1 and Chk2 activation after exposure to IR. Interestingly, inhibition of S6K1 also had the same effect on the DDR pathway, despite not affecting mTORC1 activity. This suggests that downregulation of DDR following depletion of PTEN depends on the Akt-mediated activation of its effector S6K1.

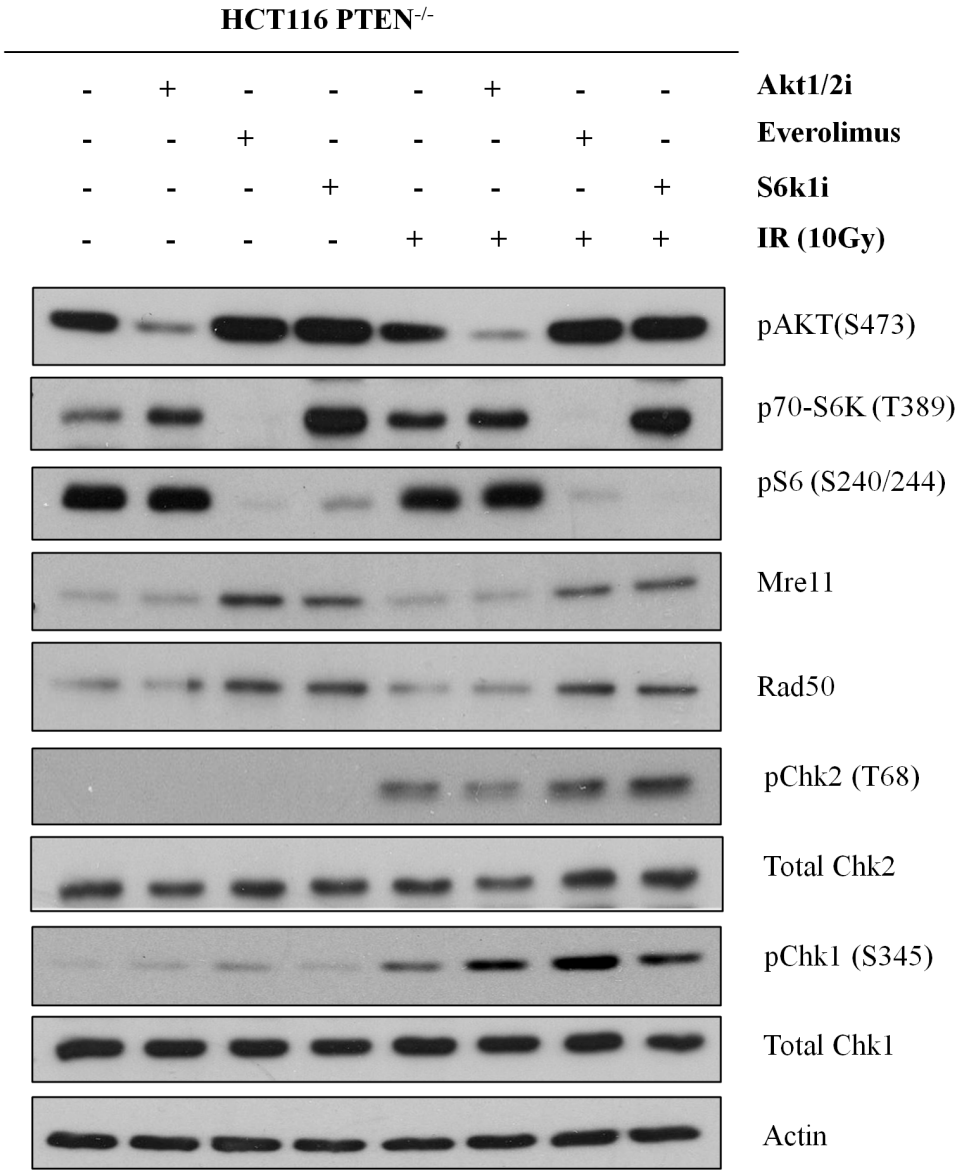

Figure 4.2: The inhibition of S6K1 activity in PTEN$^{-/-}$ cells restores MRN complex levels and DDR signalling following IR. Western blot analysis of HCT116 PTEN$^{-/-}$ cells treated for 72 hours with different inhibitors targeting various kinases within the Akt/mTOR/S6K pathway. Cells with or without IR exposure were analyzed. Actin provides the loading control.

Since S6K1 inhibition restores the expression of MRN complex in PTEN null cells, a likely rescue of its strand resection activity was investigated. The inhibition of S6K1 was found to rescue RPA foci formation following the exposure to IR (Figure 4.3A-B). These data indicate that in absence of S6K1 activity, not only is DDR signalling restored as judged by Chk1/2 phosphorylation, but also the processing of DSBs into ssDNA is rescued. I conclude that S6K1 contributes to the downregulation of MRN complex stability and function, and decreased activity of the ATM/Chk2 and ATR/Chk1 pathways, in PTEN$^{-/-}$ cells.

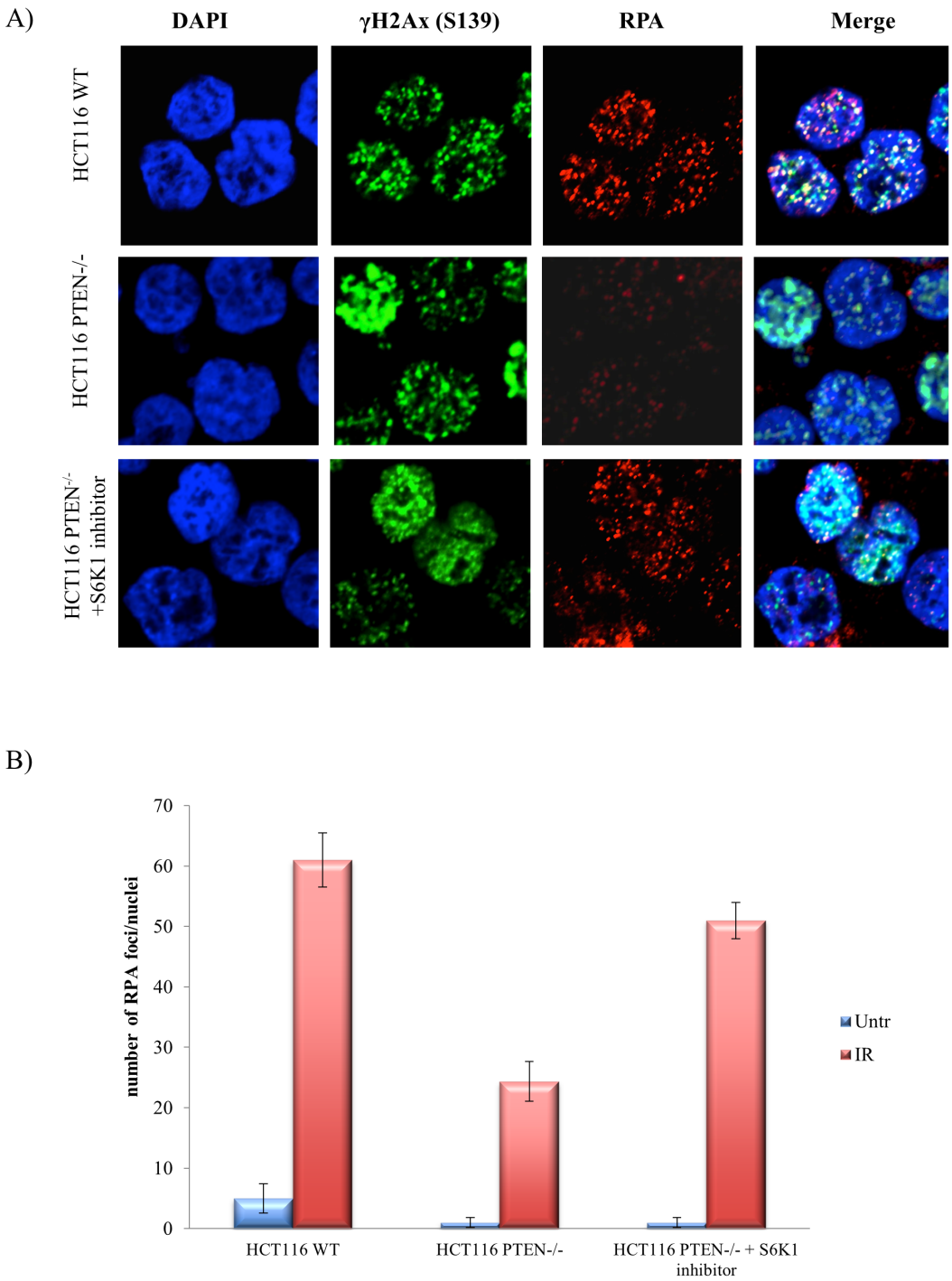

Figure 4.3: S6K1 inhibition restores the resection of DSBs into ssDNA in PTEN$^{-/-}$ HCT116 cells. (A) Immunofluorescence analysis of phosphorylated H2Ax and RPA foci in HCT116 WT and PTEN null cells minus or plus 72 hours pre-treatment with S6K1 inhibitor, 4 hours after exposure to IR. (B) Quantification of the number of RPA foci per nuclei. The data represent three independent biological replicates.

4.2.2 S6K1 affects MRN protein stability

Several analyses were performed in order to clarify the mechanism by which S6K1 altered MRN levels in HCT116 PTEN$^{-/-}$ cells. Firstly, a quantitative PCR analysis was used to analyse any significant alterations in the level of Mre11, Rad50 and Nbs1 transcripts between the WT and PTEN$^{-/-}$ cell lines. The expression levels were normalized to Actin mRNA levels. The clear absence of PTEN transcripts in the null cells validates the assay and the gene deletion. Surprisingly, the levels of Mre11, Rad50 and Nbs1 consensus transcripts were found to be comparatively similar in both WT and PTEN$^{-/-}$ cells (Figure 4.4). These data indicate that the reduction in protein levels of MRN complex components observed in PTEN$^{-/-}$ cells is not due to alteration in transcription or mRNA stability.

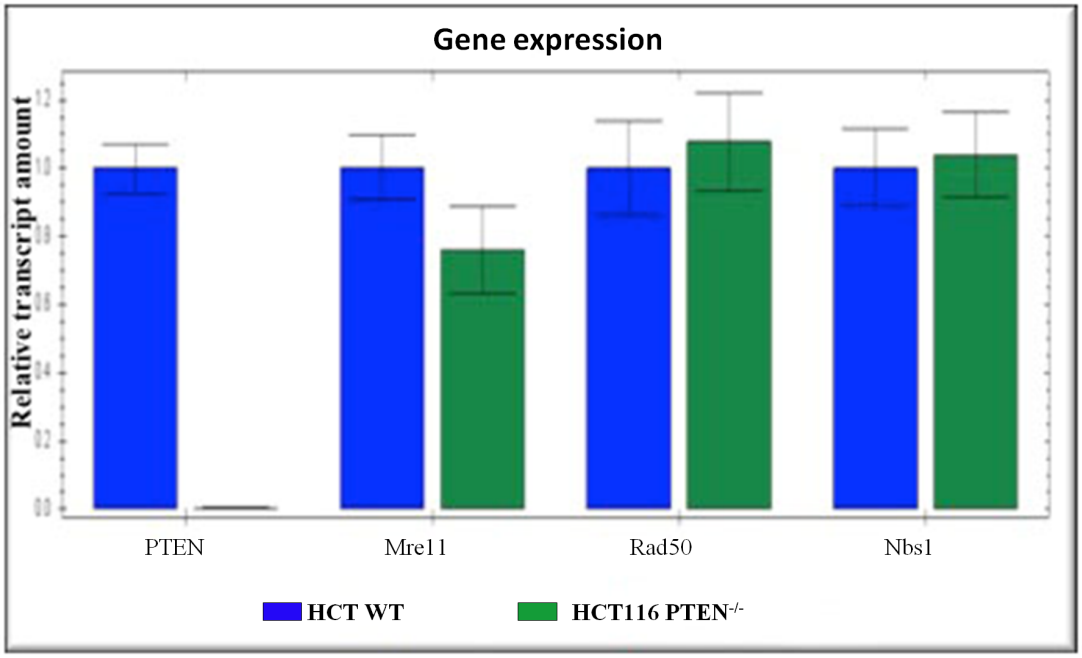

Figure 4.4: Transcript levels for components of MRN complex are unaltered upon PTEN deletion. RT-qPCR analysis of Mre11, Rad50 and Nbs1 mRNAs in HCT116 WT and PTEN$^{-/-}$ cells. Data was normalized to Actin mRNA and is presented relative to the WT signal. The error bars represent the standard deviation of three independent biological replicates.

To examine whether the reduction of MRN complex is due to reduced protein stability, the HCT116 cells were cultured in presence of Cycloheximide for 24 hours, and stability of the complex was analyzed by Western blot at 7 and 24 hours post-treatment. Cycloheximide is an inhibitor of protein synthesis, which inhibits the polypeptide elongation step during translation. The levels of p53 were drastically reduced in both cell lines following Cycloheximide treatment, expected for this highly unstable protein. 24hr treatment did not alter the levels of MRN components in WT cells, whereas MRN complex levels were drastically reduced following 7 hours of treatment of $PTEN^{-/-}$ cells (Figure 4.5A). As quantified in Figure 4.5B, 80% reduction in MRE11 signal was observed in $PTEN^{-/-}$ cells. This result suggests that active S6K1 reduces MRN complex in PTEN null cells by severely impairing its protein stability.

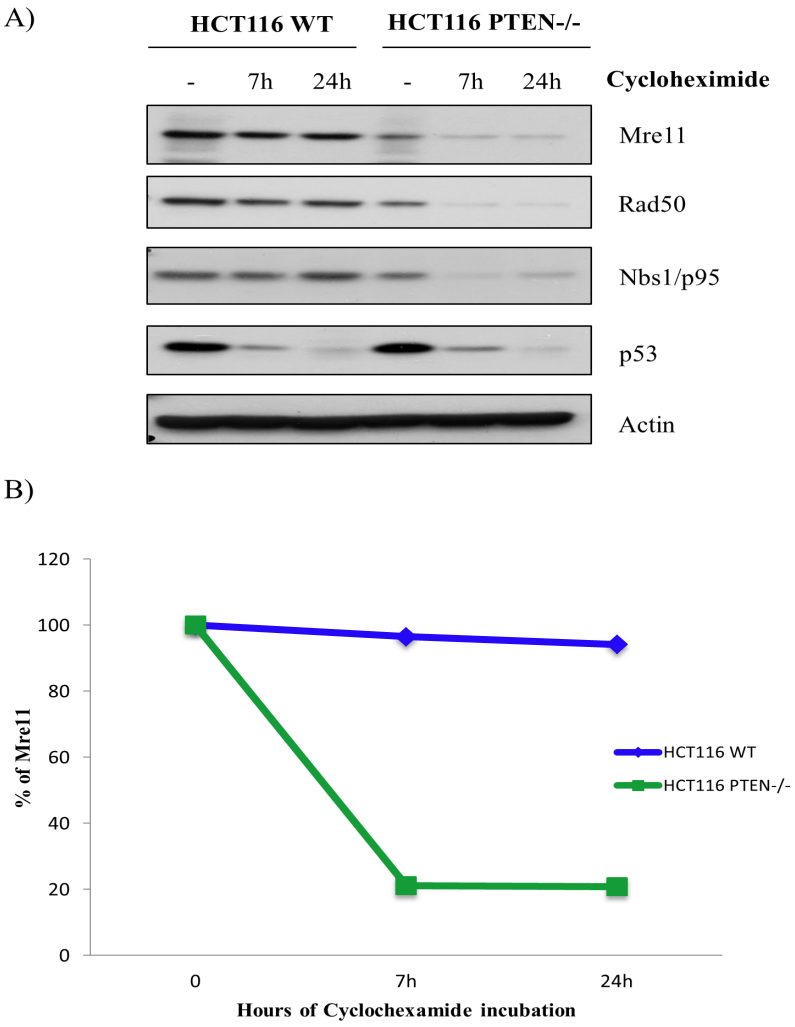

Figure 4.5: MRN complex is unstable in PTEN null cells. (A) Western blot analysis of HCT116 WT and PTEN$^{-/-}$ cells following treatment with Cycloheximide for 7 and 24 hours. p53 provides the positive control and Actin provides the loading control. (B) Quantification of the Mre11 Western blot analysis using ImageJ.

To further test the stability of the MRN complex, PTEN WT and null cells were transfected with a vector to ectopically express Mre11A. The expression of ectopic Mre11-Myc-flag was identified by Western blot using an anti-Myc antibody. As seen in figure 4.6A, the ectopic Mre11-Myc was already well expressed at 24 hours post-transfection in WT cells, reaching its maximal peak at 32 hours. Conversely, PTEN null cells showed a significantly lower expression of ectopic Mre11-Myc, and by 48 hours post-transfection it returned to basal levels. Moreover, the transient expression of Mre11 transiently increases Rad50 levels in both cell types, confirming that the ectopically expressed protein is functional. This further validates the idea that Mre11 protein regulates the levels of the other two components of MRN complex.

However, the low level of Mre11A-Myc in PTEN-null cells could be due to reduced expression of the ectopic gene or could be caused by the rapid degradation of the protein itself. In order to address this, HCT116 cells expressing the maximal level of Mre11A-Myc (32 hours time point) were treated with Cycloheximide for up to 24 hours. Similar to the endogenous protein, the treatment was found to cause rapid reduction in the ectopically expressed protein (Figure 4.6B). The quantification of the Western blot clearly shows that Mre11A-Myc is strongly unstable in $PTEN^{-/-}$ cells compared to the parental cell line (Figure 4.6C). Thus, Mre11 protein stability is severely impaired in PTEN null cells as a result of high level of active S6K1.

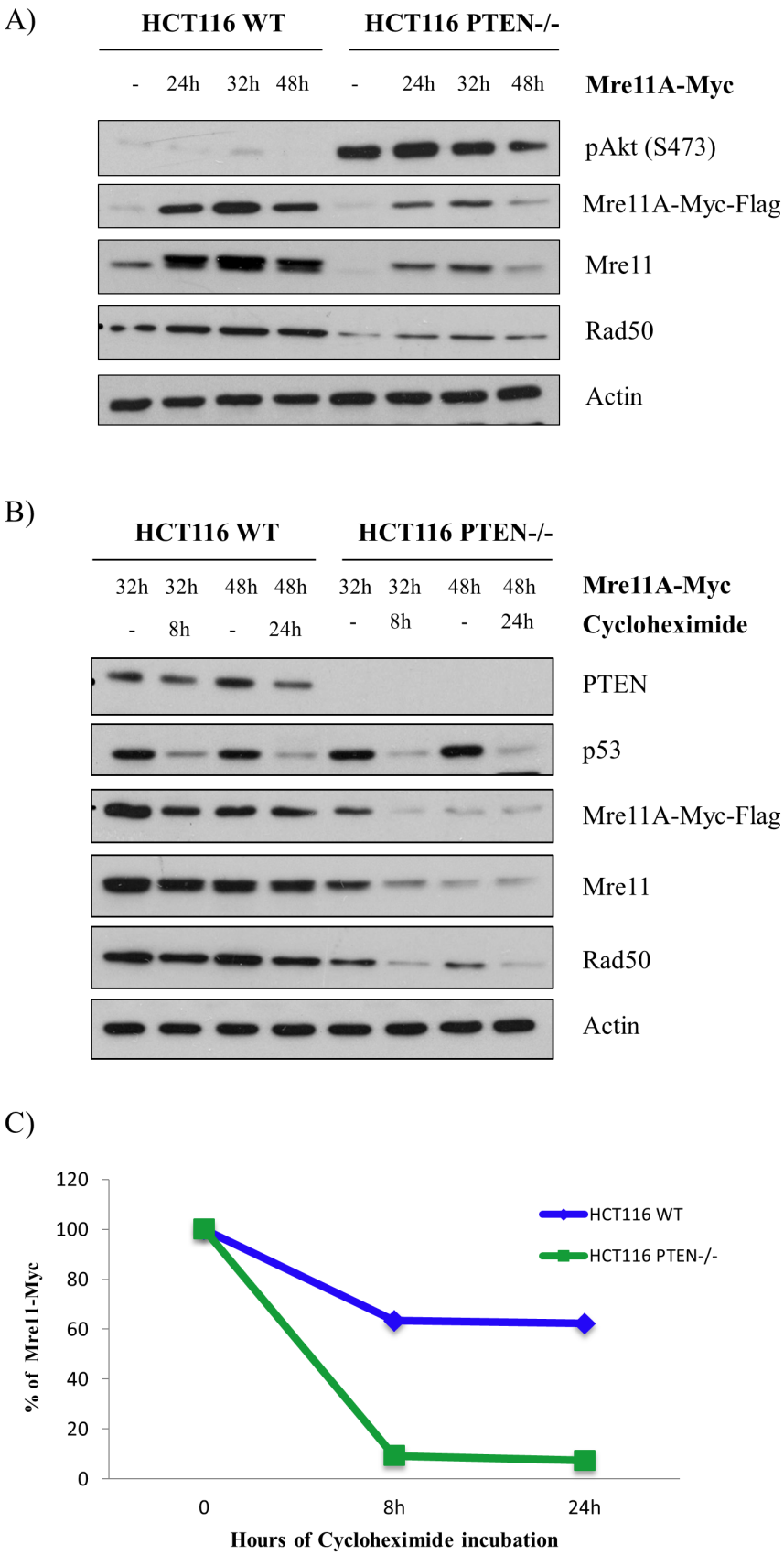

Figure 4.6: Ectopically expressed Mre11A is unstable in PTEN null cells. (A) Western blot analysis of HCT116 and PTEN-null cells harvested at different times following transfection with ectopic Mre11A-Myc-Flag. (B) Western blot of HCT116 WT and PTEN null cells expressing ectopic Mre11A with or without Cycloheximide treatment for 8 and 24hours. (C) Quantification of the Mre11A-Myc-Flag Western blot signal by Image J.

4.2.3 Cause of MRN complex instability

Considering that p70-S6K is a kinase, in order to clarify the possible mechanism by which active S6K1 may regulate MRN complex stability, a bioinformatics analysis was performed to investigate if Mre11 could be a direct target. The preferred S6K1 phosphorylation motif RXRXXS/T (where X is any amino acid) was found in the sequence of Mre11 isoform1 (Figure 4.7A). The analysis identified a Threonine in position 597 as a possible target of S6K1 phosphorylation, suggesting the possibility of another regulatory site for Mre11 DNA-binding and stability. Interestingly, this potential residue is located within the glycine-arginine rich (GAR) motif of Mre11 (Figure 4.7B). Several studies have shown that the methylation of 9 arginines between 565-600 in this region is crucial for Mre11-mediated DSB repair and checkpoint activation (Boisvert et al., 2005a; Dery et al., 2008; Yu et al., 2012). The resulting positive charge of this domain controls Mre11 DNA binding and then its nuclease activity. Therefore, the phosphorylation of this site could alter the net positive charge of this domain, thus compromising the ability of Mre11 to bind DNA and hence its stability and function.

A)

Protein ID	Protein name	Residue No.	S6 consensus RXRXXT/S
Mre11 homolog 1 human	Full meiotic recombination 11 homolog 1	T597	GQNSASRGGSQRGRADTGL

B)

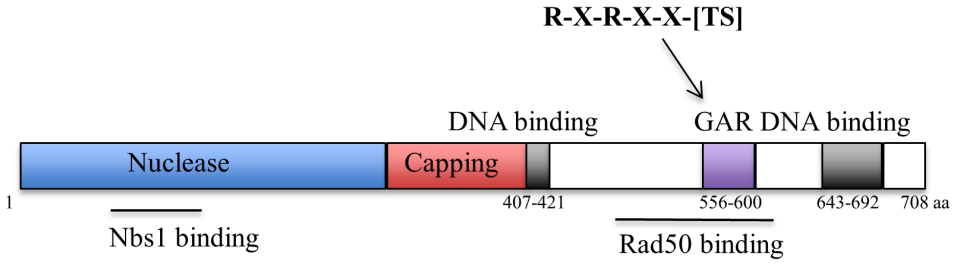

Figure 4.7: Mre11 isoform 1 has a possible phosphorylation site for S6K1. (A) Sequence analysis of Mre11A reveals the presence of S6K1 consensus sequence. The analysis identified a site on T597. (B) Schematic of domains arrangement in human Mre11 [Figure adapted from Park et al., 2011].

An *in vitro* kinase assay was performed to confirm if Mre11 is a direct substrate for S6K1 activity. Briefly, various amounts of recombinant S6K1 were incubated with 480ng of Mre11A recombinant protein for 30 min at 30°C. Following this, the proteins were resolved on an SDS-PAGE gel and ^{32}P incorporation into Mre11 was visualised by autoradiography (Figure 4.8A). The levels of recombinant Mre11A and S6K1 were visualised by Coomassie staining. The Mre11 band from each lane was cut out carefully avoiding the S6K1 band. The incorporation of ^{32}P for each sample was then quantified by the scintillation counting, and the values were plotted in the graph shown in Figure 4.8B. As seen in figure 4.8A, increasing concentration of the kinase resulted in increasing incorporation of the ^{32}P into the kinase itself due to auto-phosphorylation. This verifies that kinase is functional. However, no substantial increase was observed for ^{32}P incorporation into Mre11A protein by autoradiography. Scintillation counting did reveal incremental ^{32}P incorporation into Mre11A, however this could be due to cross contamination from the S6K1 band.

Therefore, increased Akt activity leads to a reduced Mre11 protein stability in $PTEN^{-/-}$ cells. Mre11 contains a S6K1 target consensus site, however, it is still unclear if S6K1 directly or indirectly regulates its stability.

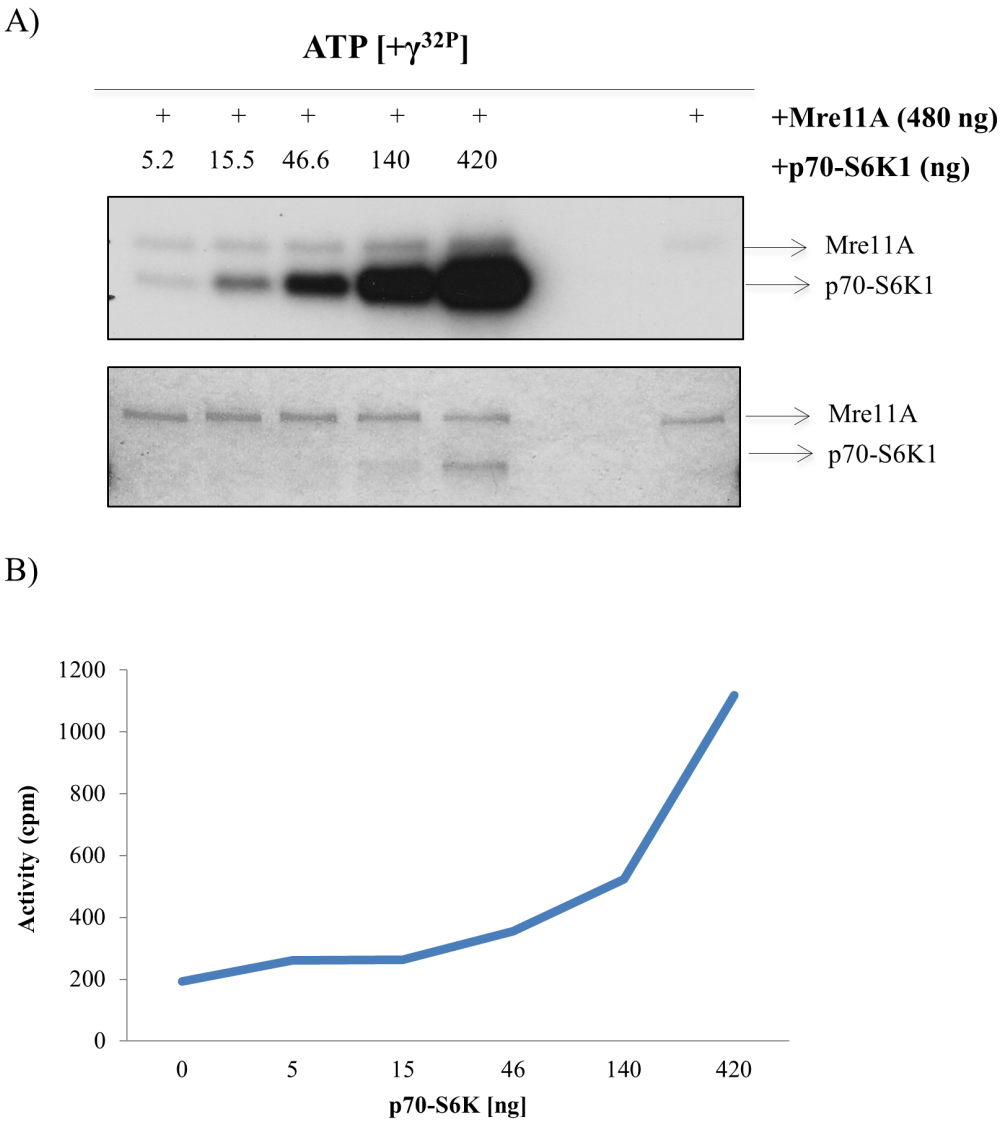

Figure 4.8: S6 Kinase assay for Mre11A. (A) SDS-PAGE performed on recombinant Myc-Mre11A incubated with Υ^{32}P-ATP and varying amounts of recombinant S6K1. The lower panel shows coomassie staining. (B) Scintillation counts of Mre11 bands cut from the SDS-PAGE gel quantifying ^{32}P incorporation into Mre11.

4.3 Discussion

PTEN is a well-known tumour suppressor that controls cell growth and apoptosis by inhibiting PI3K/Akt signalling. Several lines of evidence have shown that both PTEN and Akt are involved in regulating DNA repair and DNA damage response, however the mechanism for this still remains unclear (Kandel et al., 2002; King et al., 2004; Pappas et al., 2007; Plo et al., 2008; Puc et al., 2005; Puc and Parsons, 2005; Shen et al., 2007b). The previous chapter showed that a reduction in the components of MRN complex correlates with the reduced ability of $PTEN^{-/-}$ cells to achieve checkpoint arrest and efficiently perform DNA repair. Therefore, it seemed necessary to clarify if the reduction in MRN complex was most directly caused by PTEN deletion or Akt activation. The transient expression of constitutively active form of Akt in WT cells confirmed that oncogenic Akt specifically ablates MRN complex levels, suggesting that the effects of PTEN deletion are exerted via Akt.

The activation of Akt induces the indirect activation of other major kinases downstream in the PI3K pathway, such as mTORC1 and S6K. Akt regulates important cellular functions such as proliferation, growth, survival and metabolism through these kinases. The use of chemical inhibitors to target the PI3K pathway in $PTEN^{-/-}$ cells revealed that the inhibition of S6K1 was able to restore MRN complex levels, and subsequently the activation of DDR pathway in response to radiation. The inhibition of mTORC1 by Everolimus was also found to reduce S6K1 activity and thus rescues the expression of MRN complex and DDR response. However, the direct inhibition of Akt was surprisingly unable to rescue MRN expression. It has been previously demonstrated that Pdk1 is able to compensate for the lack of Akt activity, directly phosphorylating and activating the p70-S6K (Pullen et al., 1998). In agreement with this, the levels of active p70-S6K were not reduced upon inhibition of Akt1/2 in $PTEN^{-/-}$ cells, consistent with the idea that S6K activity is responsible for reduction in MRN expression. This new finding suggests that the deregulation of Akt activation could affect DDR response through elevated S6K1 activity. Therefore, inhibition of S6K1 could provide a potential new therapeutic avenue for tumours with PTEN loss or activated Akt.

Not much is known about the role of S6K1 in tumourigenesis. It was just recently that Lai and colleagues showed that in presence of genotoxic stress, S6K1 modulates DDR response according to cellular energy conditions (Lai et al.). The regulation of MRN complex could be the mechanism through which S6K1 fine-tunes DDR according to

cellular environment. This hypothesis motivated us to investigate the details of the mechanism by which S6K1 may regulate MRN complex stability.

The observed reduction in MRN complex level could be caused by a reduced transcription, translation or protein stability. In ATLD cells, the observed reduction in MRN complex is caused by a mutation in Mre11 gene, which alters the stability of its transcript, and consequently of the other two components Rad50 and Nbs1 (Bartkova et al., 2008). However, cycloheximide treatment and ectopic expression experiments revealed that the reduced levels of MRN complex in PTEN null cells are due to impaired Mre11 protein stability.

It is well known that protein phosphorylation can mark proteins for ubiquitylation and proteasomal degradation (Swaney et al., 2013). For example, the phosphorylation of Cyclin D1 induces its nuclear export and ubiquitination–mediated degradation (Alt et al., 2000). Mre11 contains several serine and threonine residues within its carboxyl-terminus, which are phosphorylated by the PIKK family members in presence of DNA damage, thus regulating its association with chromatin and its function (Di Virgilio et al., 2009). Several studies have shown that inhibition of PIKK proteins doesn't abolish Mre11 phosphorylation, indicating that other proteins are involved in phosphorylating Mre11 (Di Virgilio et al., 2009). Bioinformatic analysis revealed the presence of a phosphorylation consensus site for S6 kinase in the carboxyl-terminus of Mre11. However, an *in vitro* kinase assay with recombinant S6K1 failed to show significant phosphorylation of Mre11. Despite this, due to doubts regarding substrate purity one cannot rule out Mre11 as a S6K1 substrate. Thus, further definitive analysis is required to investigate this possibility. In addition to this, mass spectrometric analyses are in progress to investigate other possible sites and/or post-translational modifications of that may impact Mre11 stability in the $PTEN^{-/-}$ HCT116 cells.

Although, phosphorylation has been demonstrated to cause ubiquitination-mediated proteolysis of certain proteins, MG132 mediated inhibition of the proteasome does not rescue the levels of MRN complex in $PTEN^{-/-}$ cells (data not shown). This suggests that degradation of MRN components is not proteasome-mediated, but must occur through alternative pathways. Thus, further work is required to define the mechanism by which S6K affects MRN complex stability.

Chapter 5: Activated Akt enhances RAS-induced senescence by inhibition of MRN complex

5.1 Introduction

As previously mentioned, the abrogation of DDR pathway by the alteration of its components results in genomic instability, which significantly contributes in driving tumourigenesis. The activation of an oncogene has been established to be a powerful trigger of DDR signalling. The expression of the oncogenic HRAS leads to an initial burst of proliferation causing high level of DNA replication stress, after which cells slow down and eventually undergo senescence (Di Micco et al., 2007). DNA replication stress is caused by several factors: an increased number of simultaneously active origins of replication, DNA re-replication events, accumulation of single stranded DNA, impaired progression of replication forks and subsequent formation of DSBs (Bartkova et al., 2006; Di Micco et al., 2006). These events, together with aberrant activation of cell cycle regulators and associated transduction pathways, lead to the activation of DDR, formation of senescence-associated DNA damage foci (SDFs) and accumulation of DNA damage. DDR activation is indeed crucial for the establishment and maintenance of senescence. Early stage preneoplastic lesions show high levels of DDR activation, which coincides with the expression of senescence markers. For example, skin papillomas harbouring RAS mutations or precancerous colon adenomas demonstrate a positive correlation between DDR activation and markers of senescence (Bartkova et al., 2006; Di Micco et al., 2006). However, inactivation of DDR bypasses senescence, thus significantly contributing to cancer progression. In agreement with this, it has been observed that DDR activation peaks at early dysplastic stages of tumourigenesis in lung, bladder and colon, but its activation is lost during the progression to advanced stages of the cancer (Bartkova et al., 2005; Gorgoulis et al., 2005; Nuciforo et al., 2007; Tort et al., 2006).

Therefore, senescence poses a fundamental barrier to cell (Braig et al., 2005; Chen et al., 2005b; Collado et al., 2005; Michaloglou et al., 2005). The expression of an oncogene is called a driver mutation because it provides the bases for tumour initiation and progression. However, additional mutations are required in order to switch this driver mutation from a senescence trigger to a driver of cellular transformation. For example, 90% of invasive pancreatic ductal adenocarcinoma (PDAC) present activating KRAS mutation, in conjunction with inactivation of various tumour suppressor genes such as BRCA1, TIP53, SMAD4 and CDKN2A (Hruban et al., 2000).

The most important effectors of RAS are PI3K and its downstream effector Akt. These function as mediators of RAS-induced cell survival and proliferation (Castellano and

Downward, 2010). Activating mutations of PI3K, deletion of PTEN or Akt overexpression are observed in several cancers. Moreover, an increasing level of Akt activity was also found to correlate with melanoma progression (Dai et al., 2005). Interestingly, the combinations of activating RAS mutations and PI3K/PTEN/Akt signalling mutations are commonly found within the same tumours (Repasky et al., 2004). Endometrial cancer, thyroid cancer and acute lymphoblastic leukemia have all been shown to harbor the simultaneous mutation of RAS and genes encoding proteins for the PI3K signalling (Yeang et al., 2008). Published data suggest that 25% of human colon cancers contain mutations in both KRAS and PI3K-associated genes (Parsons et al., 2005). Moreover, 60% of human PDAC show PTEN loss, due to deletions, mutations or epigenetic silencing (Biankin et al., 2012).

Since PI3K/Akt is an effector of RAS, the molecular advantage of these co-occuring mutations within the same pathway is still not fully understood. Several studies have suggested that inactivation of PTEN or activation of Akt promotes escape from senescence and confers a selective advantage for tumour growth (Dankort et al., 2009; Kennedy et al., 2011; Vredeveld et al., 2012). In primary human fibroblasts, Akt overexpression leads to a reduction in percentage of cells with SAHF and suppression of p16 expression. In addition to this, RASG12D/Pten$^{fl/+}$ mice show a reduction in p21 and β-gal expression, along with increased proliferation in pancreatic intraepithelial neoplasia (PanIN) (Kennedy et al., 2011). Acute shRNA-mediated depletion of PTEN was shown to reduce β-gal expression and rescue proliferation defect in BRAF expressing human melanocytes. PTEN depletion was also found to drive tumour formation from BRAF expressing nevi (Vredeveld et al., 2012). Thus in these models, an activation of PI3K signalling leads to senescence suppression and confers a growth advantage.

Considering my findings regarding the involvement of Akt in suppressing DNA damage sensing and processing, it is necessary to establish if Akt induces or bypasses senescence and if this is achieved via the inhibition of DDR.

5.2 Results

5.2.1 Activated Akt suppresses DDR in RAS-expressing cells

In order to investigate the effects of activated Akt on oncogene-induced senescence (OIS), a specific cell type was used as a model. IMR90's are primary human diploid fibroblasts (HDFs) isolated from foetal lung. This cell line has been utilized for nearly 30 years in the study of replicative lifespan and senescence (Nichols et al., 1977). The expression of constitutively active mutant H-RAS allele (HRASG12V) has been shown to induce senescence in HDFs (Serrano et al., 1997). Early passage IMR90 cells were infected with either control retroviruses or retroviruses encoding HRASG12V or activated myristoylated Akt (Myr-Akt) or both (hereon in referred to as Control, RAS, Akt or RAS/Akt cells). After infection, cells were subjected to antibiotic selection, and harvested after 12 days when a majority of the RAS cells demonstrate a flat enlarged and vacuolated morphology that is typical of senescent cells.

As expected, infection with HRASG12V retrovirus causes high RAS expression and infection with Myr-Akt retrovirus causes elevated total Akt as detected by Western blotting (Figure5.1). Myristoylation causes Akt to localize to the plasma membrane where it is activated by PDK1 and mTORC2. Thus, Myr-Akt expression also leads to an increase in the phosphorylation of Akt on S473, confirming that it is fully active. Elevated Akt activity leads to increased phosphorylation of its direct target GSK3α/β and the activation of the mTORC1-S6K pathway, as observed by an increase in S6 protein phosphorylation. RAS expression was found to cause activation of DDR signalling, as observed from the phosphorylation of Chk2 on T68 and of Chk1 on S345. This is in agreement with several studies published by d'adda di Fagagna and colleagues showing that activating mutations of RAS induce hyperproliferation-mediated replication stress, which leads to the formation of DBSs causing the activation of DNA damage response (Evan and d'Adda di Fagagna, 2009; Suram et al., 2012). As observed in HCT116 cells, the expression of active Akt in IMR90's was found to cause a reduction in all three components of the MRN complex. Moreover, in the presence of DNA damage caused by RAS, active Akt also inhibited the activation of Chk2 and Chk1.

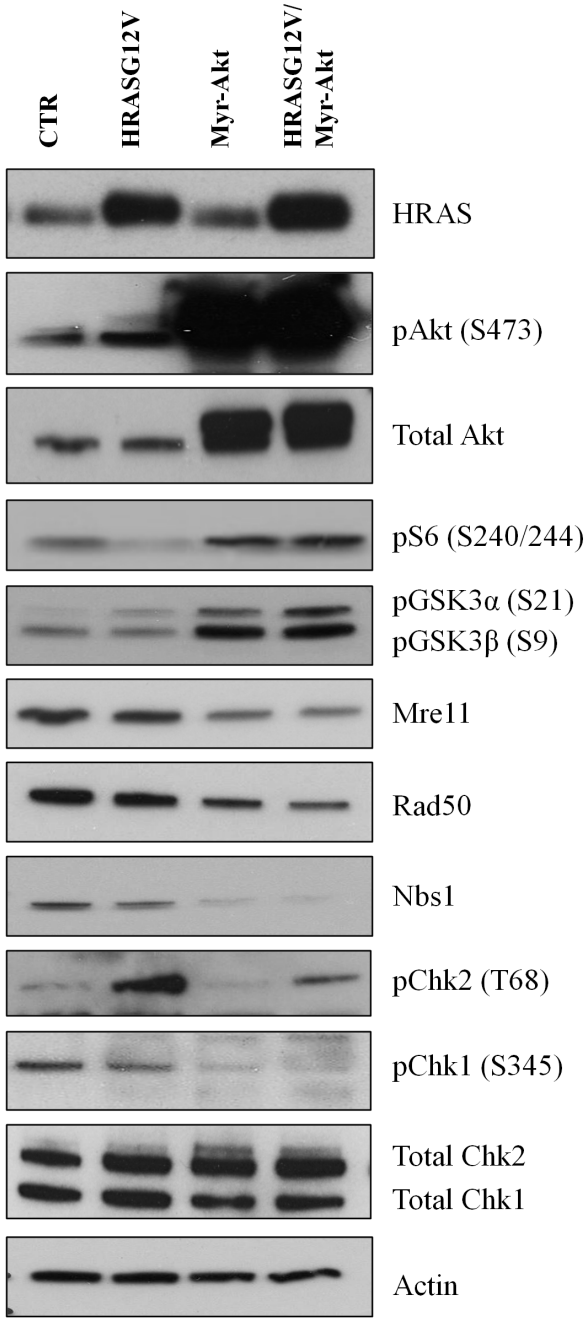

Figure 5.1: Activated Akt suppresses DDR in RAS-expressing IMR90's via a reduction in MRN complex. Western blot analysis on IMR90 cells twelve days post-infection with retroviruses expressing CTR, HRASG12V, Myr-Akt or HRASG12V/Myr-Akt. Actin provides a loading control.

Since Akt expression in RAS cells demonstrates a dramatic inhibition of DDR signalling, it is highly likely that the genomic integrity of these cells would be compromised. Alkaline comet assay was used to assess the integrity of the DNA in these cells (Figure 5.2A), where intact DNA forms the head of the comet and damaged DNA forms the tail due to its increased electrophoretic mobility. Thus, total DNA damage can be measured and expressed as a percentage of DNA migrating in the tail of the comet (Figure 5.2B). Cells expressing Myr-Akt similarly to CTR cells present low amount of damage DNA. The majority of RAS comets present a small tail, suggesting the presence of small amount of broken DNA compared to the CTR cells. However, the presence of much higher levels of DNA breaks was evident from the long tail of RAS/Akt comets compared to RAS cells. This difference was found to be statistically significant ($p<0.001$) and suggests that the reduced expression of MRN complex leads to inefficient repair in these cells.

Interestingly, this assay also revealed a marked difference in nuclei size and shape among the three different cell types (Figure 5.2A). The control cells presented relatively small nuclei and with fairly round morphology. RAS cells showed an increase in nuclei size, whereas the RAS/Akt cells showed a large proportion of comets with irregular and larger nuclei. Therefore, in presence of DNA damage caused by the expression of oncogenic RAS, activated Akt inhibits DDR activation and repair via the reduction in MRN expression, thus causing an accumulation of unrepaired DNA.

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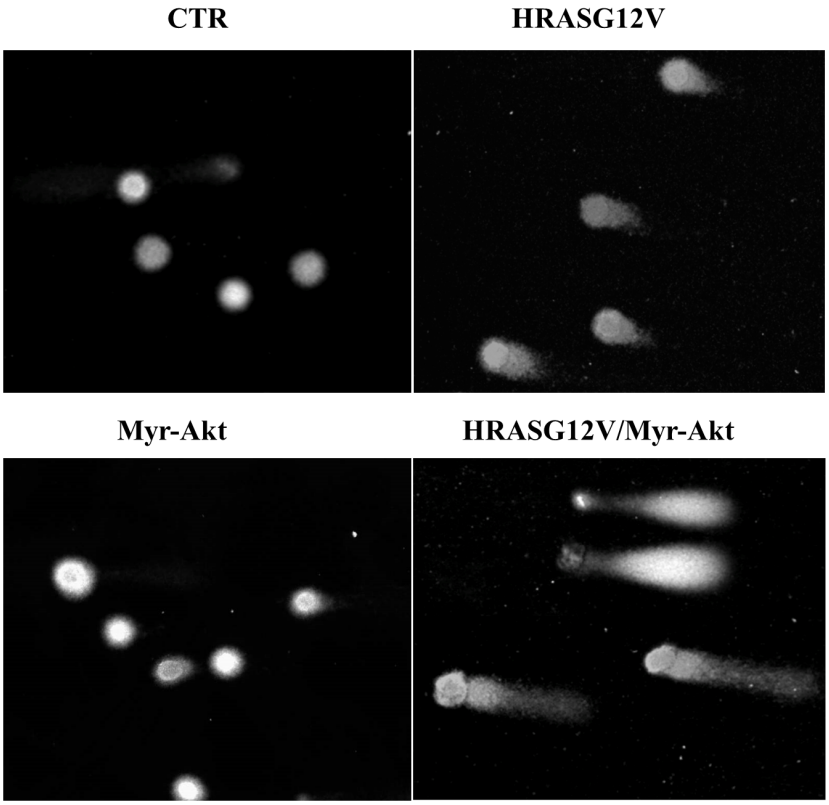

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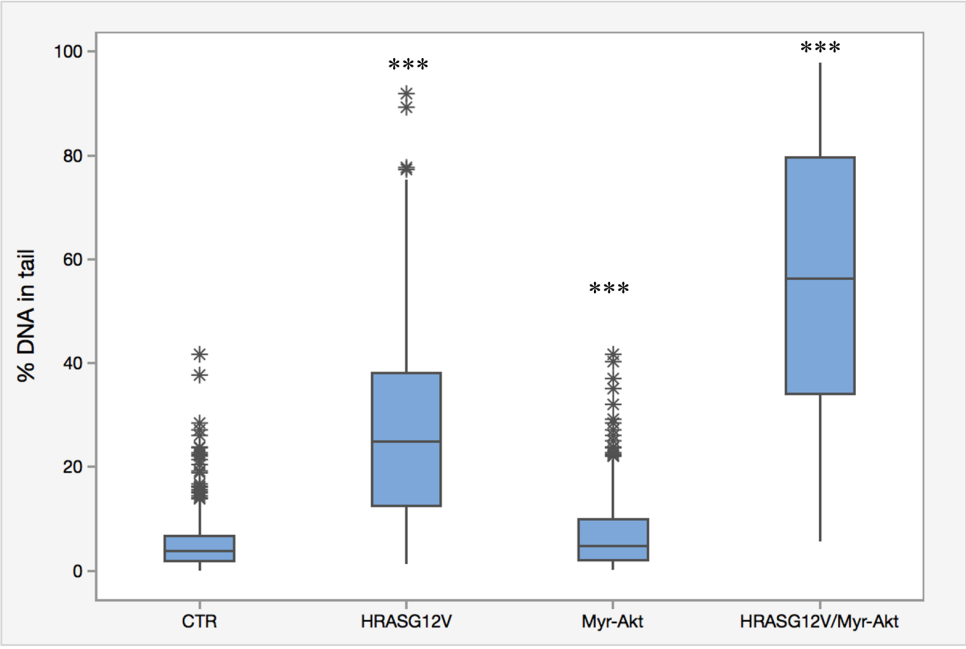

Figure 5.2: Activated Akt leads to high levels of DNA breaks in RAS-expressing IMR90's. (A) Immunofluorescence images obtained following an alkaline comet assay with IMR90 cells 12 days post-infection with CTR, HRAS G12V, Myr-Akt or HRAS G12V and Myr-Akt retroviruses. (B) Total DNA damage (sum of single and double DNA breaks) was measured as percentage of DNA migrated in the tail of the comet over the total signal from the head and tail. *** denotes $p<0.001$ as calculated by Student's t-test. Data was quantified by using ImageJ OpenComet, and it was derived from three independent biological replicates.

5.2.2 Activated Akt reinforces RAS-induced senescence

Previous work from our lab showed that activated Akt *in vitro* suppresses some features of RAS-induced senescence. Oncogenic Akt suppresses the upregulation of p16 and the formation of SAHF, but it didn't suppress the RAS-induced proliferation arrest as measured by cyclin A expression (Kennedy et al., 2011). So far, my results have suggested that overactivation of Akt signalling inhibits DDR and DNA repair via suppression of MRN complex. Since DDR plays a crucial role in triggering RAS-mediated proliferation arrest, we set out to investigate the impact of activated Akt on the establishment of OIS.

To do this, the expression of several markers of cell proliferation and senescence were analyzed. RAS cells showed a flattened and bigger cell morphology, which is characteristic of senescence induced by the expression of this oncogene (Figure 5.3A). Akt cells appeared larger and flatter compared to control cells, but smaller compared to the RAS cells. This is consistent with the idea that the expression of active Akt can induce some feature of senescence, but it is much less potent in this regard than activated RAS (Kennedy et al., 2011). Interestingly, the combined expression of activated RAS and Akt in IMR90 cells caused a peculiar phenotype. These cells were much bigger than the RAS cells with various protrusions emerging from the cellular body. This morphological difference suggests that the expression of active Akt in RAS-expressing cells may have an important qualitative impact of RAS-induced senescence.

High levels of cyclin A and phosphorylated pRb, along with low levels of cell cycle inhibitors p21WAF1 and p16INK4a as seen by Western blot in control IMR90 cells indicate normal cell cycle progression (Figure 5.3B). Contrary to this, the expression of oncogenic RAS was found to induce a robust expression of p16INK4a and p21WAF1. An increase in ATM-dependent S15 phosphorylation of p53 was also detected. In addition to this, cyclin A and phosphorylated pRb were reduced, suggesting that the cells had undergone cell cycle arrest and were senescent. Akt cells depicted a similar reduction of proliferation markers, however they were found to have reduced induction of p21 and p16 compared to RAS cells. This supports the idea that Akt is a less potent inducer of senescence when compared to RAS, as reported in Kennedy's paper (Kennedy et al., 2011). On the other hand, extremely low levels cycln A and phosphorylated pRb, along with higher levels of p16 and p21 were detected in RAS/Akt cells. This finding suggests that the activation of Akt in the presence of oncogenic RAS leads to a stronger senescent phenotype. It must be noted that no increase in S15 phosphorylation of p53 was observed in cell line expressing Akt, possibly due to the lower ATM activity in the absence of Mre11.

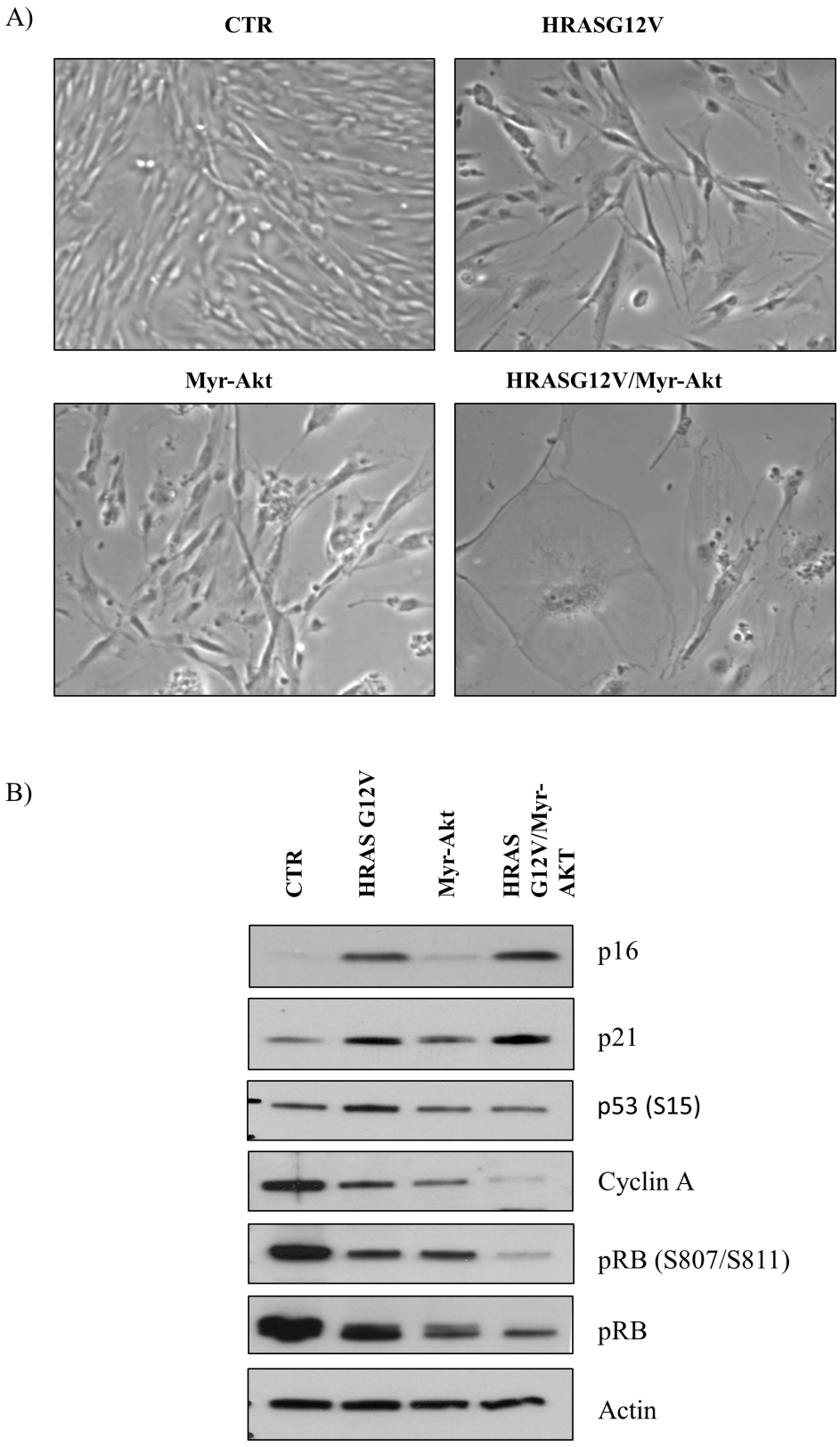

Figure 5.3: RAS/Akt cells show a stronger senescent phenotype. (A) Light microscopy images of IMR90 fibroblasts 12 days post-transduction with CTR, HRASG12V, Myr-Akt or both HRASG12V and Myr-Akt retroviruses. (B) Western blot analysis of lysates from cells shown in (A). Actin provides a loading control.

In order to establish if the senescence program in RAS cells is altered by Akt expression, a further marker crucial for defining the senescence status was analyzed. High level of β-galactosidase activity is normally used as diagnostic tool to distinguish senescent cells from other cellular states. Its activity can be visualized as perinuclear blue precipitate in cells incubated with a reaction mix containing the substrate X-gal. The number of β-galactosidase positive cells at 6 and 13 days post-infection were quantified and expressed as a percentage of the total number of cells. As expected, control cells were found to have few cells (<5%) positive for β-galactosidase activity 13 days post-infection (Figure 5.4). However, Akt expression lead to nearly 40% of the cell population to be positive for the staining 12 days post-infection, suggesting that Akt by itself is weak inducer of senescence.

RAS cells were found to enrich with positive cells at 6 days post-transfection, and by day 13 the majority of them (70%) were found to demonstrate a robust staining for β-galactosidase activity. However, the co-expression of activated Akt lead to an earlier and increased enrichment of β-galactosidase positive cells. The RAS/Akt cells demonstrated a lower cell density compared to the RAS or Akt only cells, however 40% of cells at day 6 and 90% of cells at day 12 were found to stain positive. This provides further evidence to suggest that co-expression of Akt compounds oncogenic RAS-induced senescence.

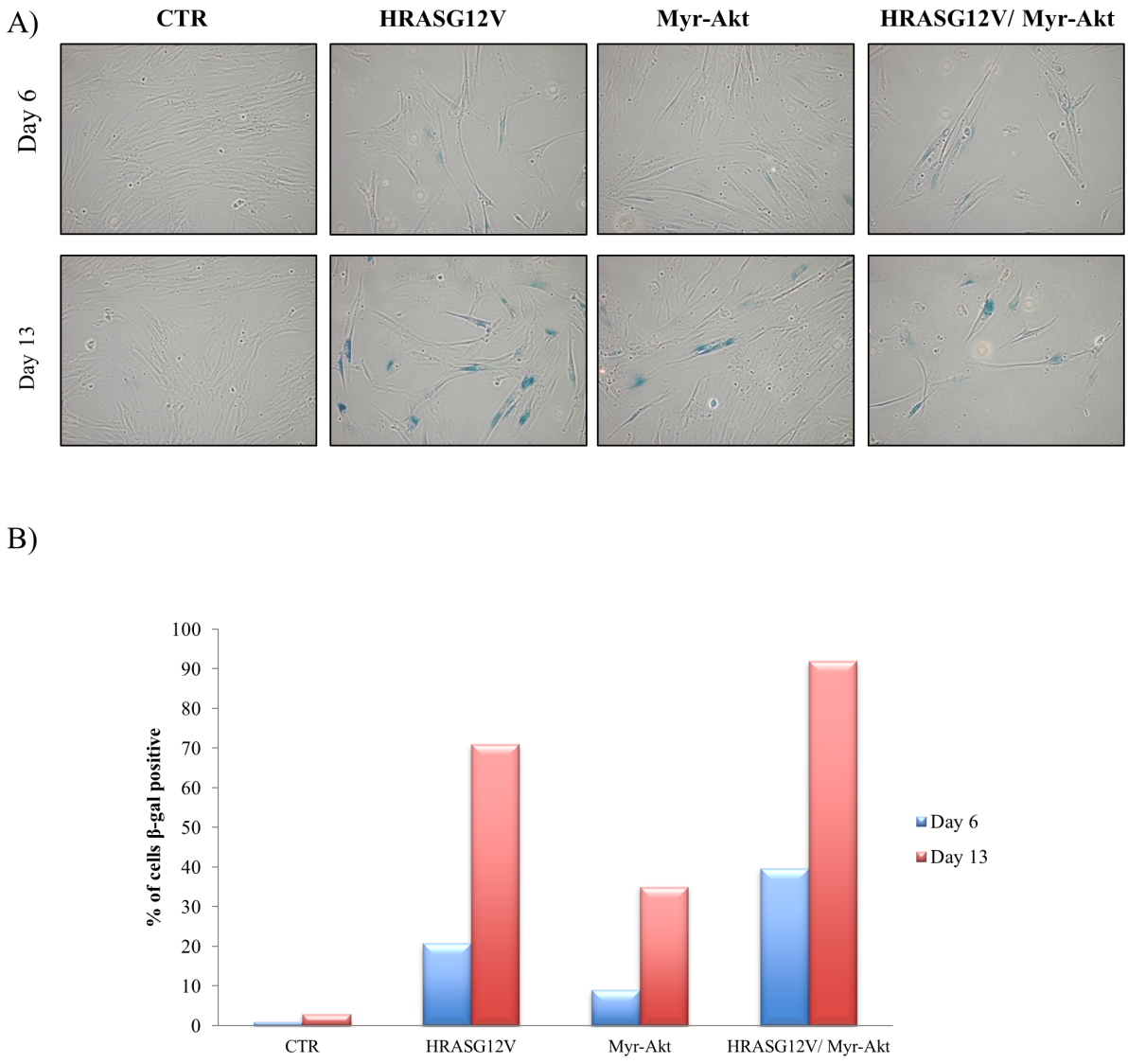

Figure 5.4: Majority of activated RAS/Akt cells are β-galactosidase positive. (A) Bright field of microscopy images of CTR, HRAS G12V, Myr-Akt or HRAS G12V/Myr-Akt cells stained for β-galactosidase activity. (B) Quantification of SA β-galactosidase positive cells plotted as a percentage of total cells. The quantification was performed on the average of 100 cells per sample.

The effects of these retrovirally expressed proteins on cell proliferation were investigated using a cell proliferation assay. The cells were pulsed for 24 hours with BrdU 12 days post infection, following which BrdU and PI incorporation was measured by flow cytometry (Figure 5.5A). The percentage of cells in each phase of the cell cycle was then calculated for each condition (Figure 5.5B). Control cells showed a cell profile typical of actively proliferating cells, with nearly 70% of cells in S phase. Consistent with the previous data, RAS cells demonstrated a severe reduction in cell proliferation, with just 20% of the cell population in S phase. This is caused by the majority of the cells arresting in G1 and G2 phases of the cell cycle. Activated Akt had a similar impact on cells, however they were found to be slightly more proliferative (30% S phase cells) with a higher G1 accumulation, consistent with the result obtained in Chapter 4 that activated Akt partly inhibits G2/M checkpoint activation. The simultaneous expression of both oncogenic RAS and Akt had a catastrophic effect on the proliferative potential of IMR90 cells with only 5% in S-phase, inducing a stronger G1 cell cycle arrest. Despite the inhibition of Chk1 and Chk2 activation, a small part of RAS/Akt cells still arrest in G2. This could be sustained by the higher level of p21, which is known to be one of the effector of the cell cycle arrest in both G1 and G2/M phases (Cayrol et al., 1998; Wu et al., 1996). Interestingly, the FACS analysis showed the presence of a sub-G1 peak in Akt cells, which is severely amplified (5 fold) by the simultaneous presence of activated RAS. This generally indicates the presence of cell debris and is in agreement with the lower cellular density observed in the β-galactosidase staining of these cells (Figure 5.4A). This could suggest that the inhibition of DDR by Akt produces such high levels of damaged DNA in some cells, that it is incompatible with cell viability.

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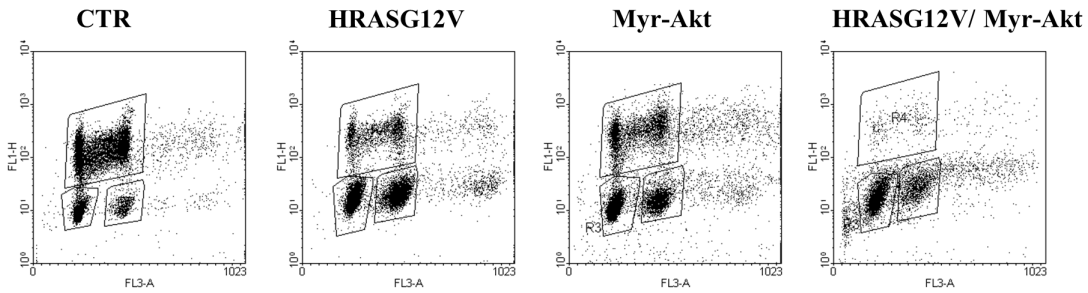

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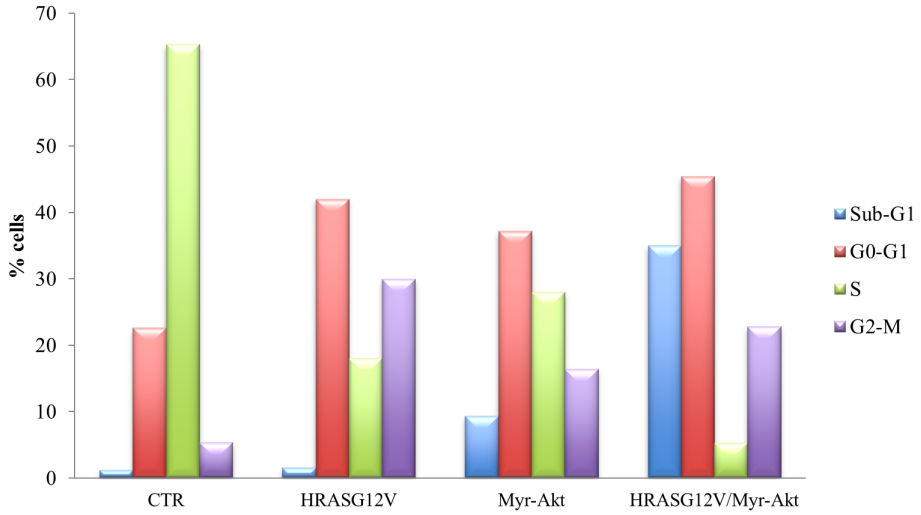

Figure 5.5: Simultaneous expression of RAS/Akt in IMR90 cells induces a drastic cell cycle arrest and cell death. (A) FACS analysis measuring the incorporation of BrdU after 24 hours of incubation, and Propidium Iodide (PI) into CTR, HRASG12V, Myr-Akt or HRASG12V/Myr-Akt cells 12 days post infection. (B) Quantification of BrdU and PI incorporation from the FACS analysis .

Considering the presence of the substantial sub-G1 peak, a cell death assay was used to determine whether RAS/Akt co-expression could kill cells due to unrepaired DNA lesions. Cells were incubated with Sytox green, a dye that stains nucleic acid in dead cells, and imaging by an IncuCyte imager was used to quantify the number of stained cells 10 to 12 days post-infection. This value was then divided by the confluence factor to correct for cell density, and the resulting values were plotted in figure 5.6. Control cells showed minimal cell death, and cells expressing activated Akt or activated RAS do not show a substantial increase in cell death either. However, when both these activated protein are expressed simultaneously a substantially larger proportion cells incorporate Sytox green indicating a dramatic elevation in cell death. However, this cell death is unlikely to be caused by p53-mediated apoptosis, since RAS/Akt cells did not show an increase in p53 levels (Figure 5.3B).

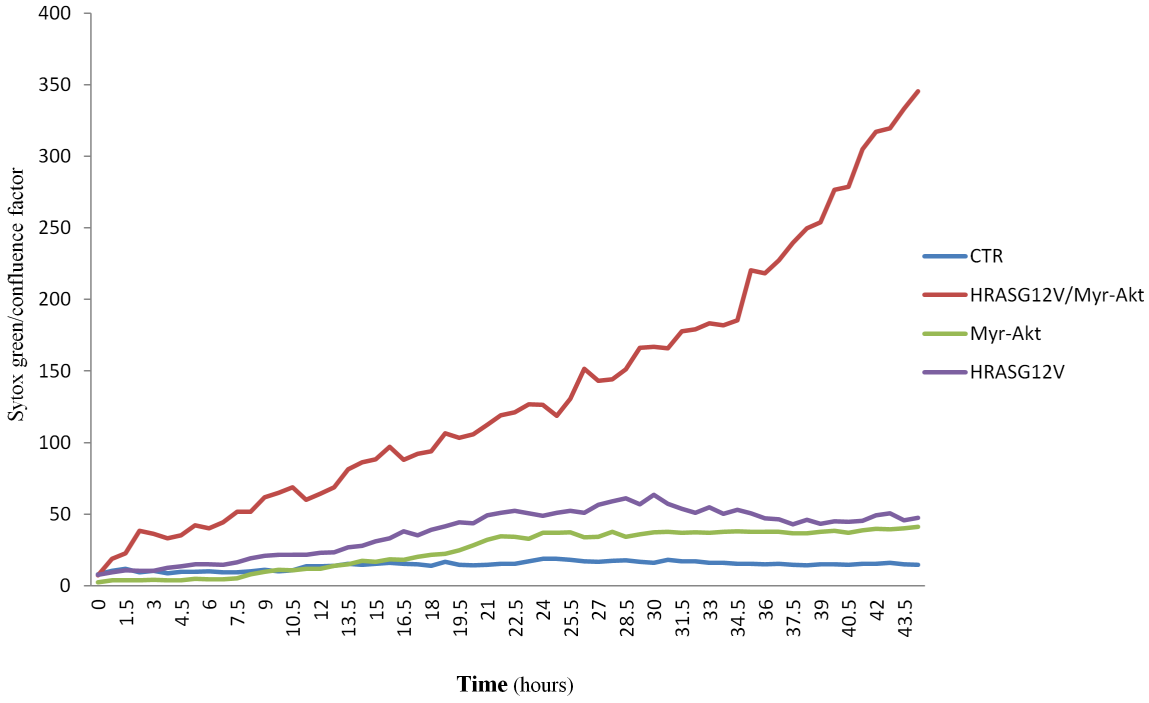

Figure 5.6: Simultaneous expression activated Akt and RAS induces cell death. Time lapse quantification of Sytox Green uptake by IMR90 cells 10-days post-infection with CTR, HRASG12V, Myr-Akt or HRASG12V/Myr-Akt retroviruses using an IncuCyte imager. Data were normalised to the confluence factor.

In order to assess the proliferative potential of the RAS/Akt cells, a clonogenic survival assay was performed with the surviving cells 14-days post infection. As expected, in the absence of senescence, the proliferation proficient control cells formed a large number of colonies as observed from the crystal violet staining (Figure 5.7A). Akt cells formed 70% of colonies compared to control cells, confirming that these cells are actively proliferative (Figure 5.7B). RAS cells instead formed 60% less colonies relative to control cells, however this confirms that a component of this population is still proliferative. On the other hand, the surviving senescent RAS/Akt cells appeared to form very few colonies. Importantly, the RAS/Akt cells did not lose viability, but persisted as viable cells with a classic senescent morphology in culture. Altogether, these data suggest that the combination of Akt and RAS in IMR90 cells results in a more robust senescence program compared to activated RAS alone.

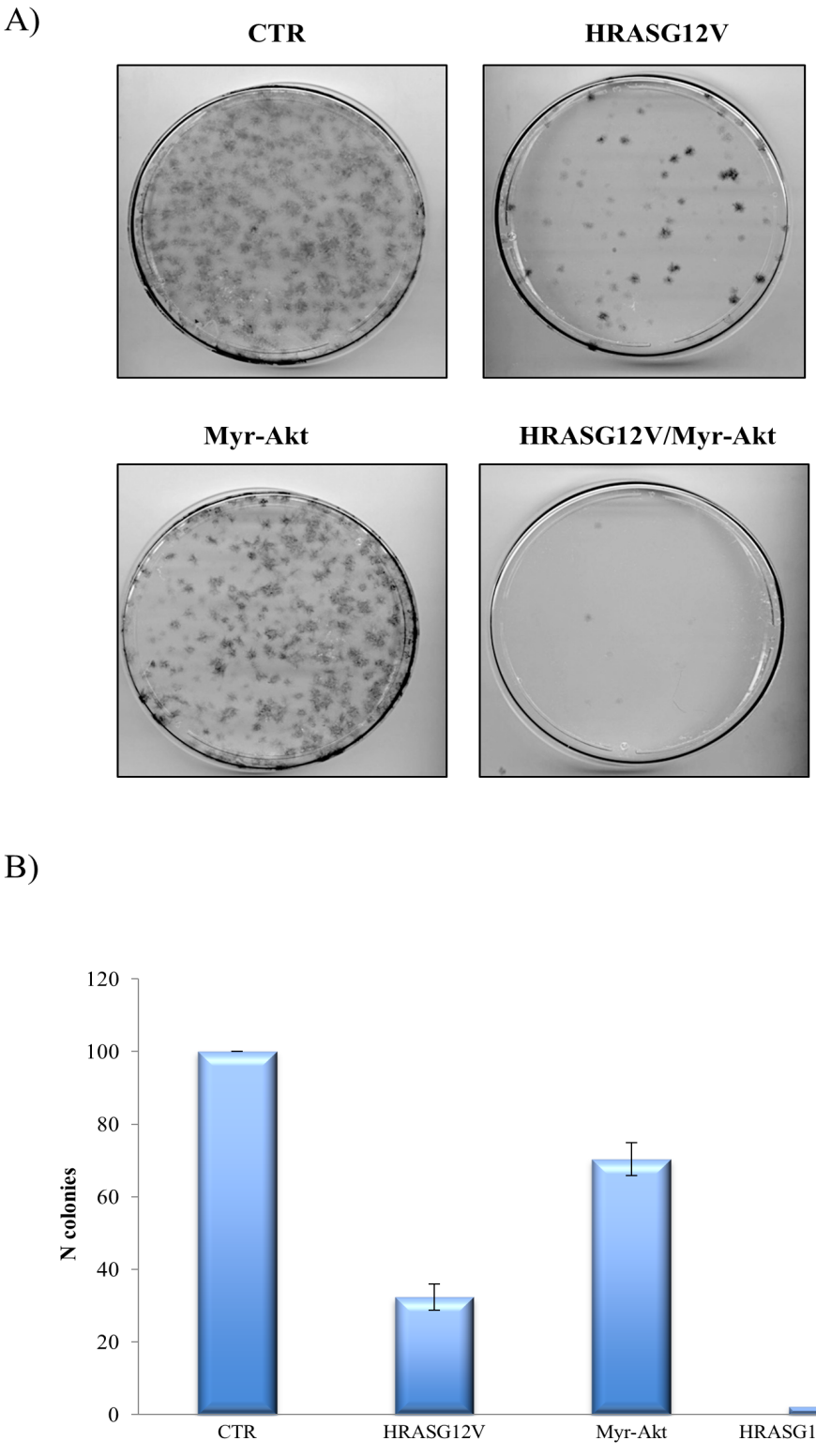

Figure 5.7: RAS/Akt cells have a markedly reduced proliferative potential. (A) Clonogenic survival analysis of CTR, HRASG12V, Myr-Akt and HRASG12V/Myr-Akt senescent cells 2 weeks post-infection. Cells were reseeded in equal numbers and were then cultured for 2 weeks. The colonies formed were visualized by Crystal violet staining. (B) Quantification of the number of colonies relative to the CTR cells. The data represent three independent biological replicates.

5.2.3 Mre11 inhibition in ER-RAS-induced cells reproduces RAS/Akt phenotype

The data so far have suggested that the expression of activated Akt in presence of a genotoxic stress of RAS activation suppresses DDR activation by reduction of MRN complex, causing accumulation of damaged DNA. This correlates with a strong cellular senescence in response. To confirm this phenotype in RAS/Akt cells is achieved via the reduction in MRN complex expression and activity, RAS expressing cells were treated with Mre11 inhibitor Mirin.

For these studies, a model system dependent on the chemical induction of a form of activated RAS fused to ER-α (Estrogen Receptor Alpha) was used Littlewood (Littlewood et al., 1995). This fusion protein ER-RAS is formed by the in-frame fusion of the mouse ER Hormone Binding Domain (HBD) and the full-length HRASG12V encoded by the retrovirus pLNC-RAS:ER. The ER part has a mutation at the codon 525 (G525R) that makes it insensitive to 17β-estradiol, but responsive to its synthetic ligand 4-hydroxytamoxifen (4OHT) (Reuter and Khavari, 2006). In absence of 4OHT, ER-RAS is kept inactive by HSP90 in the cytoplasm, whereas 4OHT biding to the receptor ER releases it from this repression.

ER-RAS cells were treated with 100nM of 4OHT for 8 days to allow for the induction of senescence by activated RAS, in the absence or presence of 20μM of Mirin. The dose of Mirin was chosen in preliminary experiments as the minimum dose that hits the target. The inhibition of MRN activity by Mirin treatment was monitored by immunofluorescence analysis of DNA damage markers such as S139 phosphorylated γH2Ax, and T68 phosphorylated Chk2. Control cells with or without Mirin treatment did not show any visible signs of DDR activation (Figure 5.8). Contrastingly, RAS-induced cells presented strong activation of DDR response, as seen from the presence of several γH2Ax foci of different sizes dispersed in the nucleus. Chk2 phosphorylation was observed in smaller and less sparse foci, which in some places were focused in large spots. It has been reported that the formation of these big foci in OIS is the result of unrepaired DSBs, which are usually localized within telomeric regions and are crucial for the maintenance of senescent phenotype (Suram et al., 2012). The co-treatment with Mirin leaded to a substantial reduction in γH2Ax and phospho-Chk2 foci. However, a prevalence of cells with larger and comparatively fainter phospho-Chk2 foci was noticed. The inhibition of DDR by reduction in Mre11 activity may lead to an increased amount of permanently damaged

DNA in ER-RAS-induced Mirin treated cells, potentially causing the formation of these bigger foci. The presence of these larger foci is likely to also contribute to a qualitative difference in the senescence phenotype of these cells.

In the control proliferating cells, the DAPI signal was found to be relatively uniform across the nucleus. However, ER-RAS-induced cells treated with Mirin had much bigger nuclei compared to control cells, which contained large foci of intense DAPI staining demarcating the presence of SAHF. Interestingly, in ER-RAS-induced Mirin treated cells the DAPI staining revealed a different nuclear conformation with a prevalence of large DAPI unstained areas that are apparently depleted of DNA content.

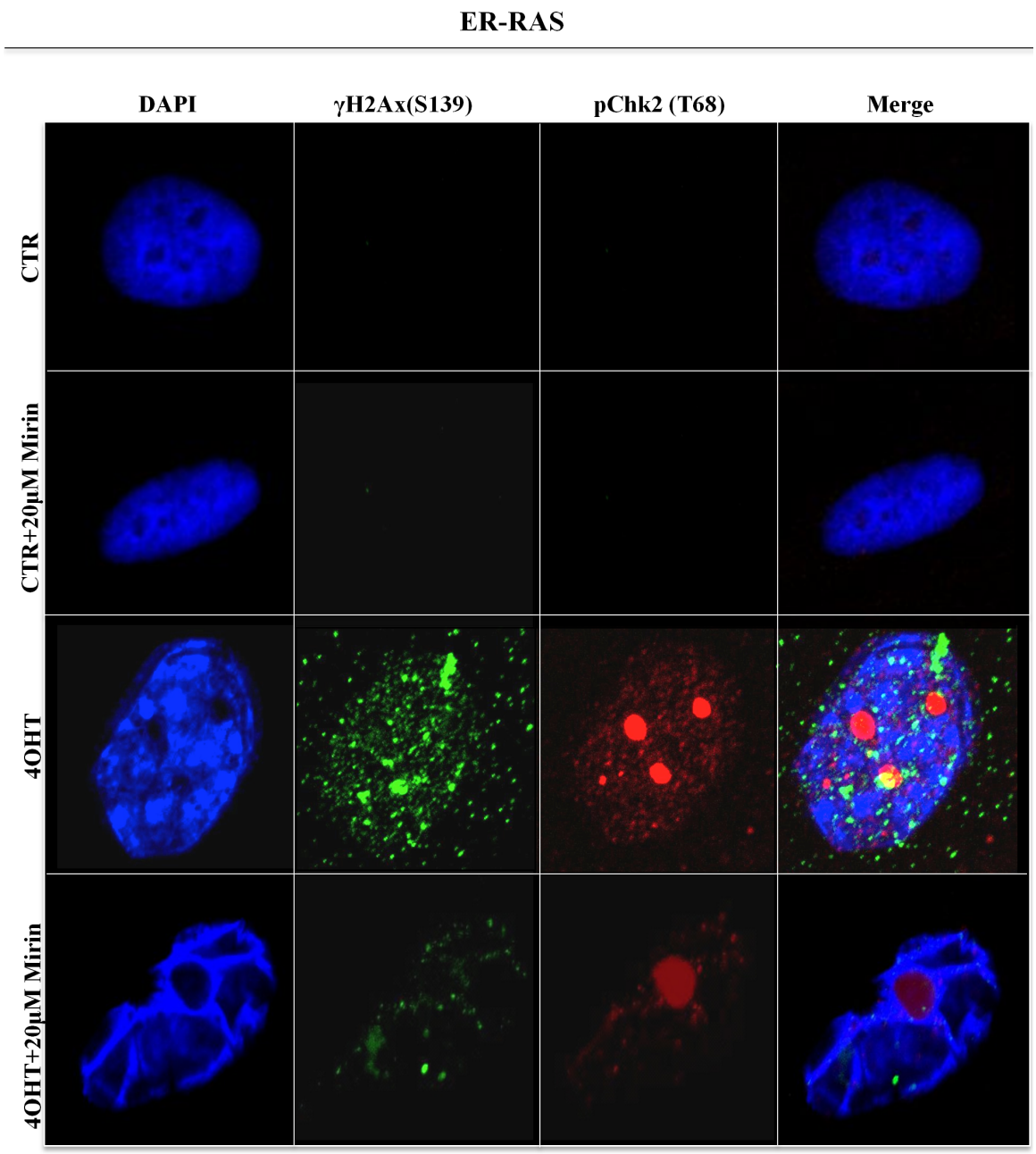

Figure 5.8: Mirin treatment of ER-RAS-induced cells leads to a reduction in DDR activation. Immunofluorescence analysis of phosphorylated γH2Ax and phosphorylated Chk2 in ER-RAS cells with or without induction with 100nM 4OHT in the absence or presence of 20μM Mirin for 8 days. DAPI was used for nuclear staining.

ER-RAS-induced cells were found to present the typical large flattened morphology of senescent cells (Figure 5.9A). The treatment with 20μM Mirin drastically reduced the number of cells, and the surviving cells appeared even longer and flattened. Control cells retained normal spindly morphology of fibroblast, both in the absence or presence of Mirin.

Western blot analysis demonstrated that the use of 4OHT in ER-RAS cells efficiently induced the activation of RAS (Figure 5.9B). As expected, this was associated with high levels of p16 and p21, and low levels of cyclin A along with activated pRb, indicating that these cells were senescent. Control cells with or without Mirin treatment presented high levels of proliferation markers and undetectable level of p16 and p21, indicating a normal cell proliferation status. ER-RAS-induced Mirin treated cells showed a strong reduction of proliferation markers. As previously seen for the RAS/Akt IMR90 cells (Figure 5.3B), Mirin treatment lead to higher levels of p16 and p21 when compared to ER-RAS-induced only. The increase in p21 is unlikely to be due to DDR signalling. It is still unclear how p21 levels are unregulated despite the inhibition of DDR by Mirin, but a stress-dependent induction model is a distinct possibility. It has been shown that various stress signals by TGF-β1 activation increase p21 levels by p38α and JNK1 mediated phosphorylation (Kim et al., 2002).

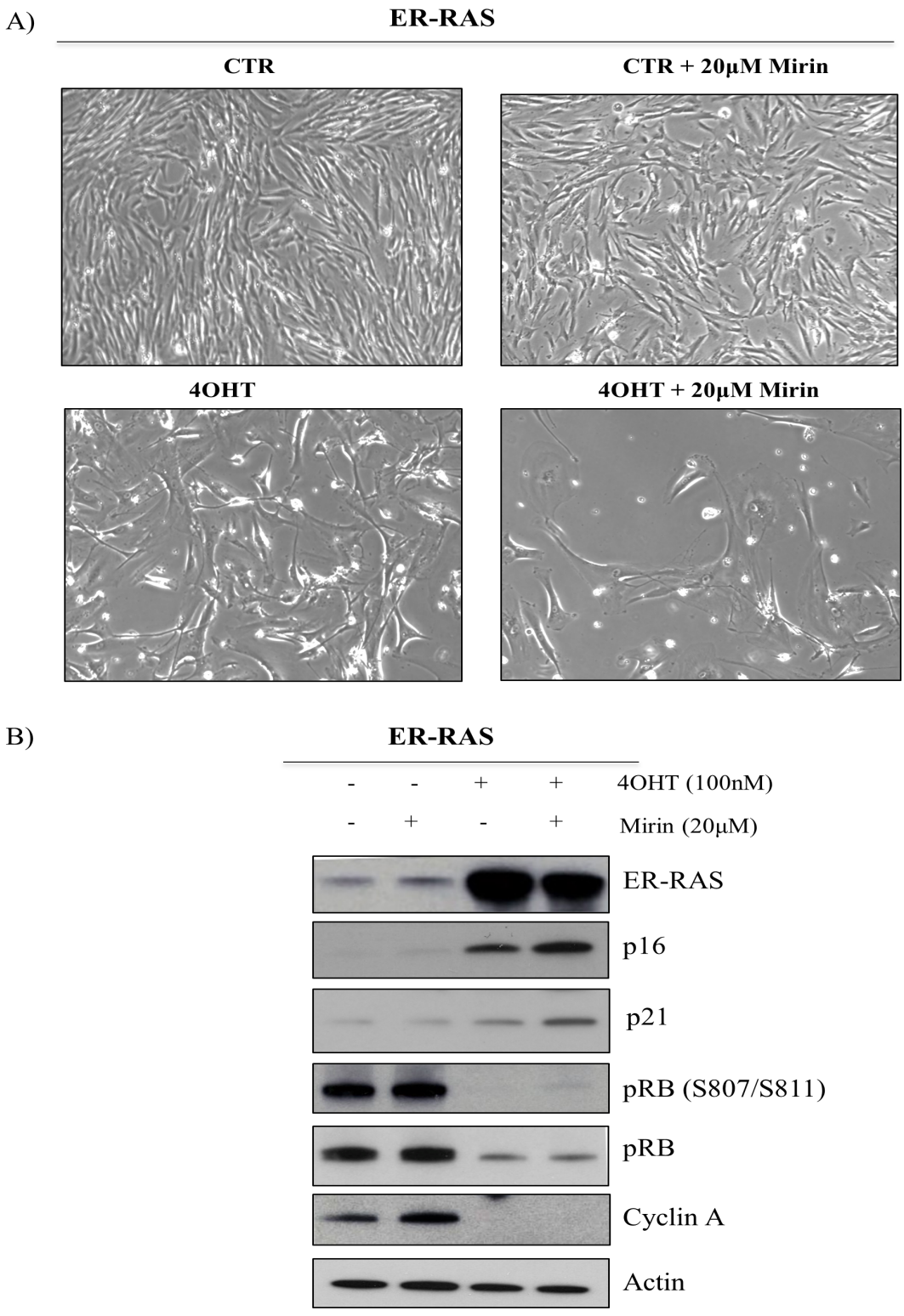

Figure 5.9: 4OHT treatment of ER-RAS cells in the absence or presence of Mirin induces senescence. (A) Bright field images of ER-RAS cells 8 days post-treatment with or without 100nM 4OHT in the absence or presence of 20μM Mirin. (B) Western blot analysis of lysates from cells shown in A. Actin provides a loading control.

Since the induction of ER-RAS caused a strong reduction in cell proliferation markers (Figure 5.9), it is likely to alter the cell cycle profile of these cells. Moreover, higher levels of p16 and p21 in Mirin treated cells could suggest a stronger cell cycle arrest compared to the untreated cells. Thus, to evaluate the cell cycle profiles, cells were incubated with BrdU for 24 hours and then BrdU/PI was analysed by flow cytometry (Figure 5.10A). The majority of control cells were found to have undergone DNA replication, demonstrating that these cells were actively proliferating (Figure 5.10B). Control cells treated with Mirin showed a similar trend to untreated cells, indicating that Mirin alone did not have any significant effect on cell proliferation. Contrary to this, the expression of activated ER-RAS for 8 days was found to drastically reduce the proliferating population to 25%, arresting most of the cells in G1 and fewer in G2 phases of the cell cycle. The inhibition of Mre11 by Mirin treatment in ER-RAS cells lead to an even greater number of cells to arrest in G1, with just 10% of the cell population detected to have undergone S phase. These data clearly demonstrate that Mirin contributes to a stronger cell cycle arrest caused by oncogenic RAS.

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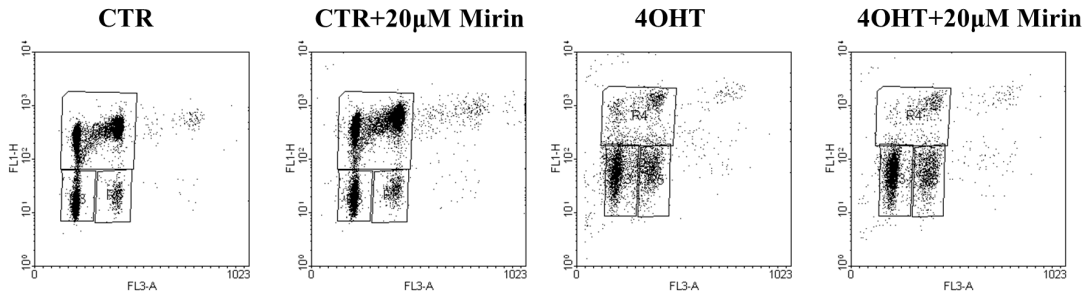

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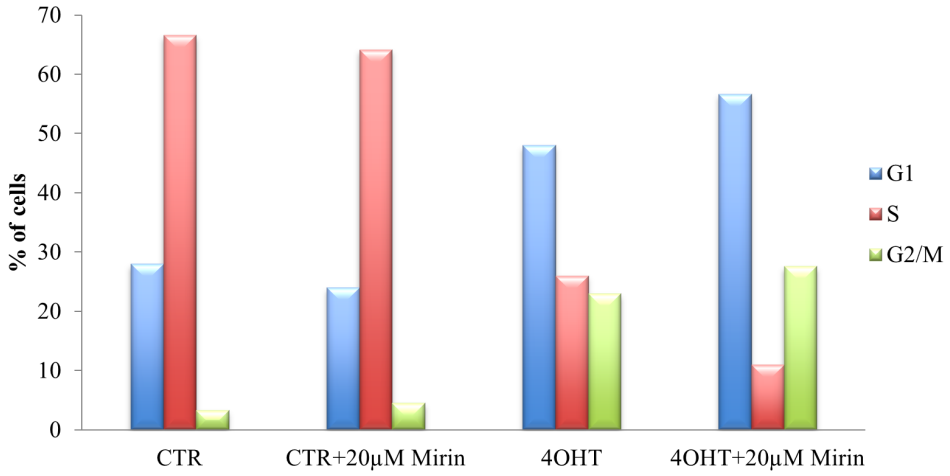

Figure 5.10: Mirin treatment enhances ER-RAS-induced cell cycle arrest. (A) FACS analysis measuring the incorporation of BrdU after 24 hours of incubation, and Propidium Iodide (PI) into ER-RAS cells with or without treatment with 100nM 4OHT in the absence or presence of 20µM Mirin for 8 days. (B) Quantification of BrdU and PI incorporation from the FACS analysis.

Considering that ER-RAS induction caused a strong cell cycle arrest, SA β-galactosidase activity was analyzed to establish the senescent status of these cells. Less than 10% of the control cells with or without Mirin treatment were found to be positive for lysosomal β-galactosidase activity (Figure 5.11). The ER-RAS-induced cells instead showed high number (70%) of blue cells positive for β-galactosidase activity, demonstrating that ER-RAS expression caused cellular senescence. Moreover, Mirin treatment of ER-RAS-induced cells was found to cause a 15% increase in β-galactosidase positive cells compared to untreated cells, and as shown previously they are also sparser (Figure 5.9A).

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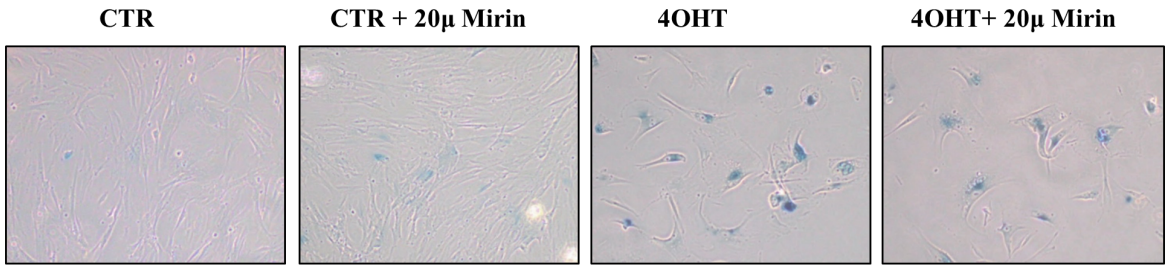

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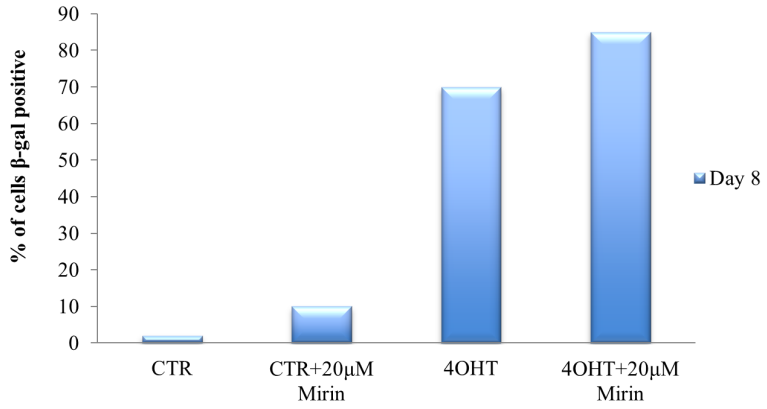

Figure 5.11: Mirin treatment reinforces cellular senescence in ER-RAS-induced cells. (A) Bright field of microscopy images of ER-RAS cells with or without induction with 100nM 4OHT in the absence or presence of 20µM Mirin for 8 days. (B) Quantification of β-galactosidase positive cells plotted as a percentage of total cells. The quantification was performed on the average of 100 cells per sample.

In addition to this, the presence of numerous floating dead cells was observed when ER-RAS-induced Mirin treated cells were inspected under the microscope. In order to confirm this observation, cells were incubated with Sytox green and the number of positive cells was measured by IncuCyte. The treatment of control cells with Mirin does not affect cell viability, however ER-RAS induced cells presented a subtle cell death phenomenon, which is significantly enhanced by Mirin treatment (Figure 5.12). This is in agreement with the observed reduction of cells ER-RAS/Mirin cells (Figure 5.9A), and previous observations in the RAS/Akt cells (Figure 5.6).

Collectively, these results suggest that inhibition of Mre11 activity by Mirin inhibits DDR activation and damage processing that results in high cell death in response to genotoxic stress and accumulation of DNA damage caused by oncogenic RAS. However, the majority of the surviving cellular population is strongly arrested in G1 phase of the cell cycle, resulting in a strong cellular senescence response. Thus, the robust induction of cell senescence in RAS/Akt cells is likely to be due to the reduction in MRN levels caused by Akt-induced S6K activity.

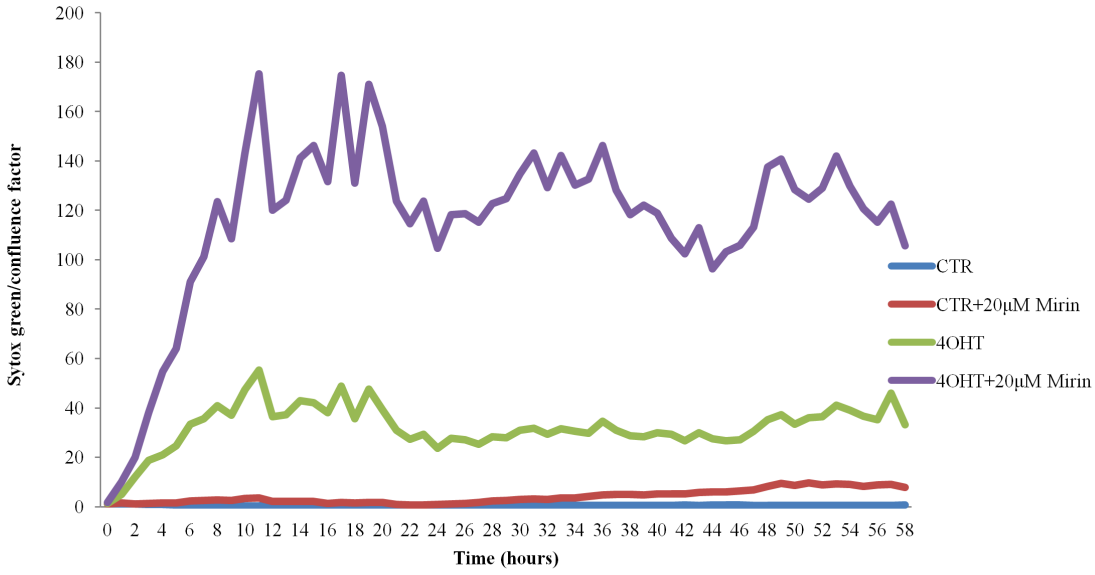

Figure 5.12: Mre11 inhibition induces cell death in presence of ER-RAS. Time-lapse quantification of Sytox Green uptake by ER-RAS cells 8-days post-treatment with or without 100nM 4OHT in the absence or presence of 20µM Mirin using an IncuCyte imager. Data were normalised to the confluence factor.

5.3 Discussion

The previous chapters have shown that the hyperactivation of the Akt pathway severely impairs the stability of the MRN complex in human colon carcinoma cell lines. The same phenomenon was found to occur in primary human IMR90 fibroblasts expressing activated Akt. It is well known that the expression of constitutively active oncogenes such as HRASG12V induces the DNA damage response by engaging both the ATM and ATR pathways (Bartkova et al., 2006; Di Micco et al., 2006). The expression of HRASG12V in IMR90 cells was found to induce the activation of both checkpoint kinases Chk1 and Chk2, however this was abrogated by co-expression of activated Akt.

As it was demonstrated in previous chapters, the full activation of these checkpoint kinases relies on optimal MRN complex activity, which directly regulates the recruitment and activation of ATM machinery, and causes the activation of ATR by resecting DSBs into ssDNA. Chk1 and Chk2 are essential for stopping damaged cells from completing DNA replication (intra-S checkpoint) and progressing into mitosis (G2/M checkpoint), thus preserving genome stability. Comet assay analysis revealed the presence of severely damaged DNA in cells co-expressing activated RAS and Akt compared to cells only expressing RAS, suggesting that reduced checkpoint activation and lack of MRN-complex mediated HR repair severely affects genomic integrity. However, a substantial part of RAS/Akt cells were also found to undergo cell death. This could be due to unbearable levels of damaged DNA accumulate in the cells due to reduced Mre11 function, thus leading to cell death. An increase in damage-mediated ROS formation could also be a secondary cause of cell death.

Despite the lack of checkpoint activation, the majority of RAS/Akt cells were found to arrest dramatically in G1 phase of the cell cycle and present a marked senescence phenotype. It has been previously shown that activated RAS can cause p21 activation via DNA damage-mediated increase in p53 activity (Di Micco et al., 2006; Fikaris et al., 2006). This is reflected in increased S15 phosphorylation of p53, which is known to be dependent on ATM activity (Saito et al., 2002). Also, Akt activity can contribute to upregulation of p21 by direct phosphorylation of p21 (Li et al., 2002). Furthermore, increased damage causes a rise in ROS levels that leads to elevated p16 activation (Takahashi et al., 2006). In the absence of Mre11 activity, the increased amount of unrepaired damage thus provides an essential trigger for p16-mediated senescence in response to oncogene activation. This likely causes the majority of the RAS/Akt cells to

arrest in G1, even in the absence of Chk1/2 activation. The additive effects of p21 activation by RAS and Akt, along with elevated p16 activation due to damage-induced ROS could be responsible for the even stronger proliferation arrest in RAS/Akt cells.

Activating RAS and PI3K pathway mutations often correlate with more aggressive tumours (Biankin et al., 2012; Dai et al., 2005). Previous studies have shown that activation of Akt or PTEN deletion facilitates to bypass RAS or RAF-induced senescence. Contrary to these studies, the data in this chapter propose that oncogenic Akt and RAS cooperate to induce a qualitatively stronger senescence.

Vredeveld and colleagues found that the depletion of PTEN in human fibroblasts led to reduced BrdU incorporation, however this does not seem to cause a reduction BRAF-induced p16 expression (Vredeveld et al., 2012). Activating PI3K mutations were also found to confer a proliferative advantage. It must be noted that the BrdU incorporation data in this study was obtained 3 days post-infection, whereas the Western blot and β-gal incorporation analyses were performed 8-days post-infection. Thus it is unclear if these cells were fully engaged in the senescence program when the PI3K/PTEN loss-mediated increase in BrdU incorporation was measured. Thus, it cannot be concluded that PI3K/PTEN loss enables cells to fully evade the senescence program. The data in this chapter use BrdU incorporation in conjunction with PI staining and FACS analysis to quantitate cells in various stages of the cell cycle 13 days following RAS expression, and therefore is likely to reflect the effect of Akt expression on the long term establishment of senescence.

In agreement with this, Kennedy *et al* found that co-expression of RAS and Akt for 7 days did not lead to a rescue in the expression of cyclin A as measured by immunofluorescence. Moreover, Akt expression on its own was also found to reduce proliferation of IMR90 fibroblasts. A reduction in SAHF formation in RAS/Akt cells was also observed, however this might not confer a growth advantage since SAHFs have been reported to not correlate with growth arrest (Di Micco et al., 2011). Thus, previously published data have demonstrated that loss of PTEN or Akt activation can lead to partial reprieve from the senescence phenotype *in vitro*, however, a full escape from the senescence program is not evident.

Both the above studies use PTEN deletion *in vivo*, however PTEN loss may not be functionally equivalent to Akt activation. PTEN loss loads leads to genomic instability via

multiple mechanisms and may therefore provide a more potent mechanism by which cells can escape senescence (Yin and Shen, 2008). DDR and the checkpoint machinery provide a powerful barrier against tumourigenesis. Tumour cells in clinical specimens from different tissues present the constitutive activation of DNA damage signalling, as seen from the presence of γH2Ax foci and the activation of ATM/ATR machinery. Several studies have shown that any breach of this defence barrier due to mutations and/or loss of expression of DDR components facilitate early cancer progression (Bartkova et al., 2005; Gorgoulis et al., 2005). Therefore, it seems reasonable that the inhibition of DDR by Akt-mediated suppression of MRN complex may provide a growth advantage to RAS expressing cells.

However, it seems that senescence provides a secondary barrier that must be overcome. Genomic instability following PTEN loss or Akt activation is likely to provide the mechanism by which these cell eventually evade senescence. For example, cells with genomic instability would have an increased probability of loss of function mutations within the p16 or p53 pathways, therefore not only enabling the escape from senescence but also providing these cells with a substantial growth advantage.

Chapter 6: Final Discussion

During the past decade, an increasing number of studies have highlighted the significance of the DNA damage response pathway in the evolution and treatment of cancer. Genomic instability is the common feature of cancer cells, which fuels high mutation rates that lead to an accumulation of oncogenic and tumour suppressor mutations. The DDR is regulated by two kinase-signalling cascades, i.e. ATM/Chk2 and ATR/Chk1, which are primarily activated by DSBs and ssDNA respectively (Matsuoka et al., 2007). From the data presented, it is clear that the functions of the MRN complex are essential for coordinating the activation dynamic of these two pathways in HCT116 cells following exposure to irradiation. Full activation of ATM at DSBs completely relies on MRN complex and therefore a reduction in MRN complex levels results in reduced phosphorylation of its downstream target Chk2. Activated ATM is not only crucial for assembling and activating the DDR machinery, but also for the phosphorylation of many well-known repair factors, including Mre11 and Nbs1 (Di Virgilio et al., 2009; Horejsi et al., 2004; Lavin, 2007). Thus, a reciprocal activation loop exists between ATM and MRN complex, whereby the lack of one of the two factors severely impairs the activity of the other.

Moreover, activated MRN complex is also required for enzymatic processing of the damaged DNA (Paull and Gellert, 1998). Mre11 nuclease activity is indispensible for nucleolytic resection of DSBs in ssDNA, which is crucial for the initial steps of HR repair. Therefore, its reduced availability or the inhibition by the drug Mirin results in reduced HR repair efficiency, and consequently reduction of chromatin RPA-associated foci formation. RPA foci are essential for marking the sites of DNA damage to which HR factors are recruited. They also signal the activation of the cell cycle checkpoint through ATRIP/ATR recruitment and Chk1 activation. Thus, a reduction in RPA foci impairs activation of the Chk1 protein in presence of IR-induced damage. Thus it is clear that MRN complex is central to DDR response, and its suppression impairs genome stability.

Multiple studies have shown that the overactivation of Akt can promote genome instability by its inhibitory effect on the DDR pathway (Henry et al., 2001; Hirose et al., 2005; Kandel et al., 2002; King et al., 2004; Plo et al., 2008; Tonic et al., 2010), however no specific mechanism has been proposed. The data obtained here demonstrates that this may be achieved via reduction in the expression and activity of the MRN complex.

PTEN has a crucial role in the maintenance of various cellular processes. It targets a vast range of proteins, from membrane-bound receptor tyrosine kinases and cytoplasmic signalling molecules to transcriptional factors within the nucleus. However, the PI3K/Akt

pathway is considered the primary physiological target of PTEN, where it counteracts Akt activation by PI3K (Leslie and Downes, 2004; Sulis and Parsons, 2003). In normal growth conditions, Akt kinase promotes growth, proliferation and survival by regulating the activity of kinases such mTORC1 and S6K (Figure 6.1). Moreover, in response to damage it is activated in PIKK family members-dependent manner (Caporali et al., 2008; Toulany et al., 2008; Viniegra et al., 2005). In this case, it provides the pro-survival signal required to allow the reengagement of the cell cycle post repair. The observed inhibition of MRN expression by Akt is likely to help fine-tune the amplitude of the DNA damage response. This would act as a feedback mechanism to avoid hyperactive DDR signalling. Contrary to this, when Akt is hyperactivated due to oncogenic stimulus (activating PI3K mutations) or loss of tumour suppression (PTEN loss), the dramatic downregulation of the MRN activity makes this prosurvival mechanism to become detrimental due to accumulation of unrepaired DNA damage. In agreement with this, a study in breast cancer lines has shown that Akt hyperactivation suppressed RAD51 and BRCA1 foci formation, thus inhibiting homologous recombination. This correlates with supernumerary centrosomes and aneuploidy in hamster ovary cells (Plo et al., 2008). Thus it can be hypothesised that this interplay between active Akt signal and MRE11 activity provides a crucial two-way link between the PI3K pathway and DDR, i.e. growth control and growth arrest.

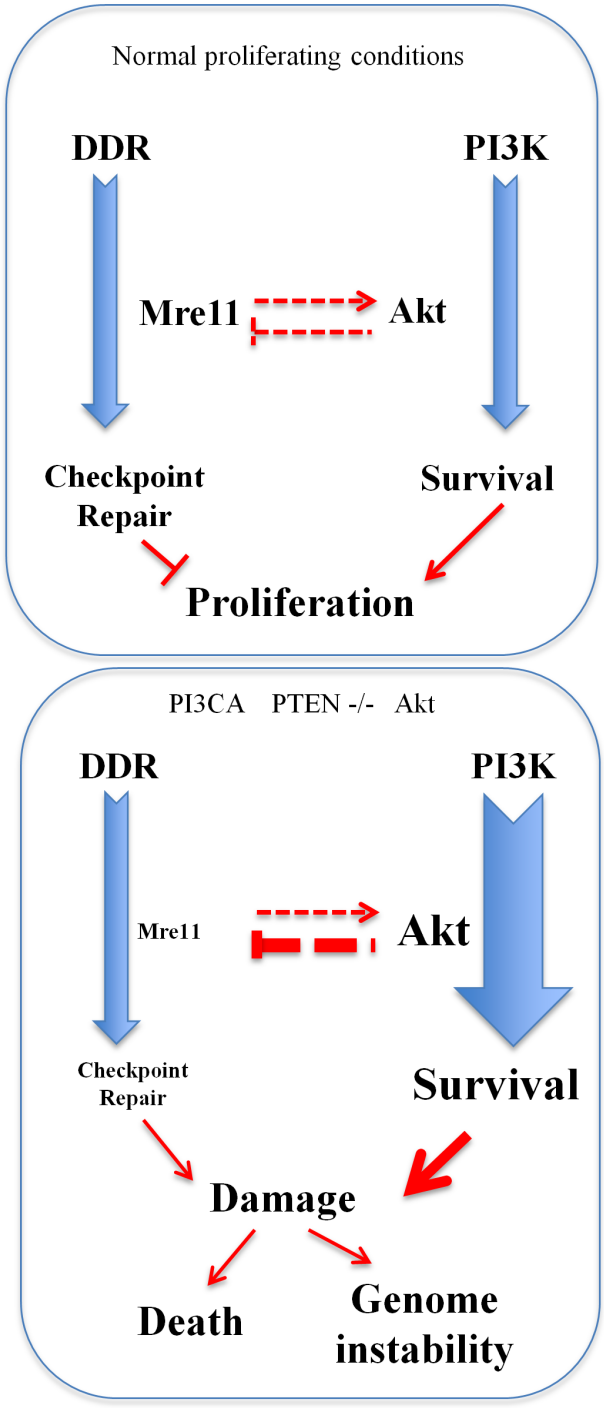

Figure 6.1: Model for the interplay between the DDR and PI3K pathways.

Activating RAS and PI3K pathway mutations often correlate with more aggressive tumours (Biankin et al., 2012; Dai et al., 2005). These findings suggest a new mechanism by which activated Akt pathway can contributes to tumourigenesis. Downregulation of DDR and DNA repair could be a mechanism to allow the cells to keep proliferating despite the presence of damaged DNA. However, it is still unclear how Akt or S6K activity regulates stability of the MRN components. Further work is required to investigate potential alterations in the post-translational modifications of MRE11 in response to Akt activation. This will provide mechanistic insights into how this finely tuned network functions.

Senescence poses a fundamental barrier against cellular transformation (Mathon and Lloyd, 2001). In the primary human fibroblast IMR90 cell line, the expression of activated oncogene HRASG12V induced formation of DSBs due to its hyper-proliferative effects (Di Micco et al., 2006). However, as expected in this non-transformed cells line, persistent DDR activation caused a p16 and p21-mediated cell cycle arrest. On the contrary and as has been shown by previous studies, activated Akt was found to be a weak inducer of senescence (Alimonti et al., 2010; Kennedy et al., 2011). However, these cells did not show an increase in DNA damage, which is consistent with the idea that Akt activation due to PTEN deletion induces a different form of senescence that is not associated to DDR and hyper-replication (Alimonti et al., 2010; Miyauchi et al., 2004). Moreover, as observed in the human colorectal carcinoma cell lines, IMR90 cells expressing activated Akt were also found to demonstrate a loss of MRN expression and activity, which impaired their ability to induce a DDR.

In contrast to previous studies (Kennedy et al., 2011; Vredeveld et al., 2012), the activation of Akt in RAS-expressing cells was found to reinforce the RAS-induced senescence. In presence of DNA damage due to expression of activated RAS, the reduction of MRN activity was associated with the accumulation of unrepaired DNA lesions. This, in-turn, was linked to stronger G1 arrest likely due to an increased p21 upregulation, which is unlikely to be p53-mediated. This arrest was further reinforced by a ROS-induced increase in p16. Contrary to this, activated Akt partly abrogated RAS-mediated G2 arrest, which relies on the activity of MRN complex. The use of Mirin to inhibit MRN complex activity in presence of RAS-induced damage supports the idea that the activated Akt reinforced RAS-induced senescence by inhibiting MRN complex-mediated repair. However, a rescue experiment will be required to verify if the reintroduction of Mre11 in RAS/Akt cells

reverts to the RAS-only senescence phenotype. This will be necessary to ascribe the suppression of MRN complex to Akt-mediated effects in RAS cells.

Senescence triggered by RAS or by RAS/Akt presented diverse severity of damaged DNA, suggesting that these senescent cells are not the same. In particular, the RAS/Akt cells showed a large amount of broken DNA, which could affect the genome integrity of these cells. In addition to this, the observation that the expression of several DDR genes is suppressed during cancer progression strengthens the idea that this genomic instability caused by activated Akt will eventually contribute to bypass senescence, thus stimulating tumour formation and progression (Bartkova et al., 2006; Di Micco et al., 2006).

Therefore, it can be said that all oncogenes are not equal in their ability to cause cellular transformation or senescence. *In vitro,* oncogenic RAS induces senescence due to accumulation of unresolved DNA damage (Figure 6.2); however, *in vivo* this creates the genetic environment required for benign precursor lesions. Comparatively, hyperactive Akt is a weak inducer of senescence on its own (Kennedy et al., 2011). But by affecting the DNA repair and checkpoint activation, Akt enhances RAS-induced damage, which still causes senescence. However, this equips the cells with higher mutation rates by inhibiting DDR and perhaps allows the eventual bypass of this senescence. Typical examples for this are pancreatic ductal adenocarcinomas (PDACs), which have the poorest prognosis of the solid tumours. The progression of PDAC from preneoplastic lesions (PanINs) is associated with accumulation of genetic alterations. The activating mutation of H-RAS in the pancreas of mice induces the development of PanINs. However, in 90% of cases the progression of these PanINs into PDAC is associated with additional loss of crucial tumour suppressors such as PTEN (Altomare et al., 2002; Asano et al., 2004; Biankin et al., 2012; Tuveson and Hingorani, 2005). Furthermore, activation or amplification of Akt2 and Akt1 is been reported in 60% of these pancreatic cancers (Altomare et al., 2002; Ruggeri et al., 1998; Schlieman et al., 2003). A large proportion of PDAC (8%) have also presented somatic mutations in ATM, suggesting the importance of DNA damage response mechanisms in driving this disease. Interestingly, abnormalities in homologous recombination mechanism have been associated with pancreatic cancer too (Biankin et al., 2012; Goggins et al., 1996; Klein, 2012; Roberts et al., 2012). In line with this, neoplastic tissue from pancreas of mice with HRASG12V and loss of PTEN were found to contain significant levels of aberrant mitosis (Kennedy et al., 2011).

The data obtained in this study suggest that activated Akt may contribute to the high genomic instability that drives RAS-mediated tumourigenesis upon PTEN loss. It is known that genome instability can cause the loss of tumour suppression, which can lead to bypass of senescence, thus driving uncontrolled proliferation. However, it is unclear if this can be achieved just via MRE11 inhibition. Therefore, it could be interesting to test if the treatment of mice with Mirin could accelerate the progression of RAS PanINs to PDAC.

In conclusion, this study could explain the molecular basis of the cooperation between oncogenic mutations of the RAS and PI3K pathways in human tumours.

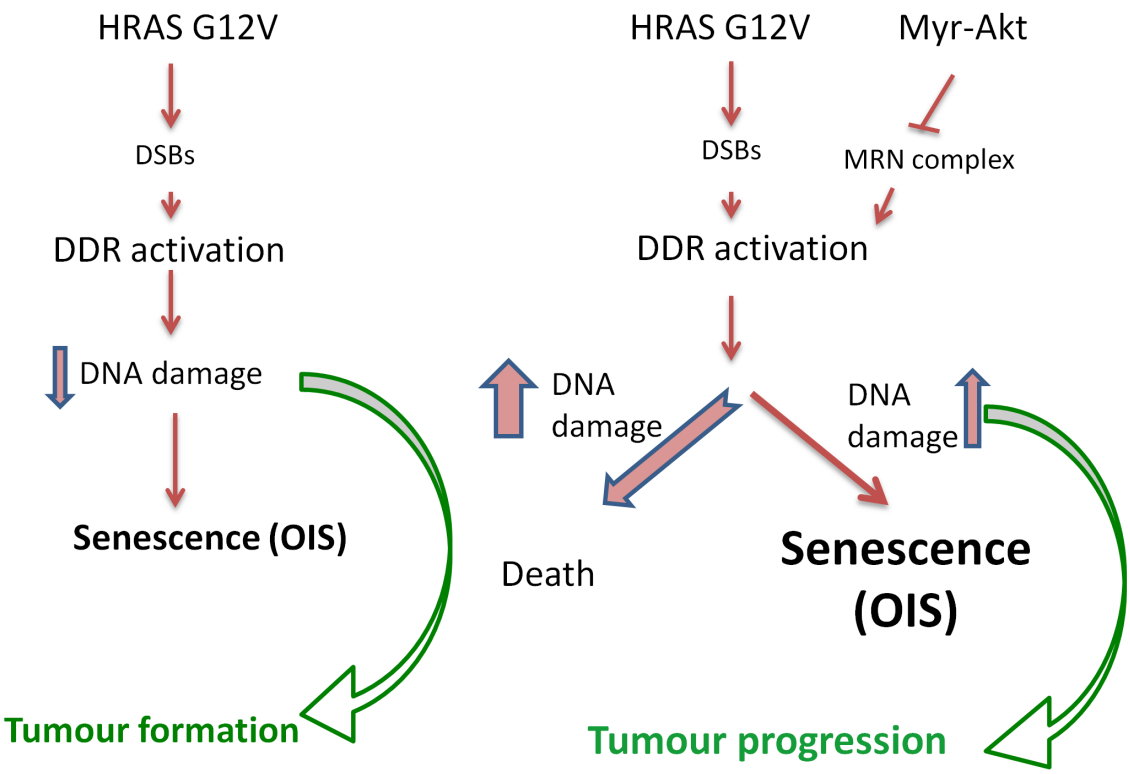

Figure 6.2: Model for the contribution of Akt to RAS-induced senescence.

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