The regulation of p53 transcriptional activity by hnRNPUL-1 and the DNA damage response induced by a novel chemotherapeutic agent, ALX

By

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Summary

The tumour suppressor, p53, is a vital DNA damage response protein and its means of transcriptional regulation are vast. hnRNPUL-1 is a multifunctional protein and previous studies have implicated it in the modulation of p53 transcriptional activity, although this has been rather poorly understood. Results presented here demonstrate that hnRNPUL-1 represses p53 transcriptional activity and negatively regulates p21 levels. This is consistent with the depletion of hnRNPUL-1 leading to an increase in cells arrested in G1/S. Together these results confirm that hnRNPUL-1 acts as a p53 co-repressor with specific cellular targets.

Mutations in p53 and other DNA damage response proteins not only often contribute to the onset of tumourigenesis but can also be the cause of drug resistance during treatment. The development of a novel chemotherapeutic agent, Alchemix (ALX), was based on the requirement for effective treatment in the face of resistance mechanisms. Little has been known about the mechanism of action of ALX up to now. Findings here demonstrate that ALX treatment primarily induces an ATR-dependent DNA damage response that occurs specifically in cycling cells and culminates in cell death via mitotic catastrophe. Results also show that the response elicited by ALX requires TOP2α and TOP2β, as well as its alkylating ability, but does not involve ATM, p53, or components of the MMR and FA pathway. Therefore, ALX has the potential to treat tumours that have developed resistance to conventional chemotherapeutic drugs.

To my parents

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Abbreviations

53BP1	p53 binding protein 1
3meA	N^3-methyl adenine
7meG	N^7-methyl guanine
9-1-1	RAD9-RAD1-HUS1
ALKBH2/3	alpha-ketoglutarate-dependent dioxygenase alkB homologues 2/3
Alt-NHEJ	alternative NHEJ
ALX	Alchemix
AP	apurinic/apyrimidinic site
APS	ammonium persulphate
ASPP	apoptosis-stimulating proteins of p53
A-T	ataxia-telangiectasia
ATLD	ataxia-telangiectasia-like disorder
ATM	ataxia-telangiectasia mutated
ATR	ATM and Rad3-related
ATRIP	ATR interacting protein
Bak	BCL2 antagonist killer
Bax	BCL2-associated X
Bcl2	B-cell lymphoma 2
BBS	BRD7 binding site
BER	base excision repair
BRCA	breast cancer susceptibility gene

BRD7	bromodomain-containing 7
BSA	bovine serum albumin
CBP	CREB binding protein
cDNA	complementary DNA
CDK	cyclin-dependent kinase
Chk1/2	checkpoint protein 1/2
CIN	chromosomal instability
CKI	cyclin-dependent kinase inhibitor
CLL	chronic lymphocytic leukaemia
COG4639	clusters of orthologous groups of proteins 4639
CPT	camptothecin
CtIP	C-terminal binding protein interacting protein
DAPI	4', 6-diamidino-2-phenylindole
DBD	DNA binding domain
DDR	DNA damage response
DISC	death-inducing signalling complex
DMSO	dimethyl Sulphoxide
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DSB	double-strand break
DSBR	double-strand break repair
dsDNA	double-stranded DNA

ECL	enhanced chemiluminescence
EXO-1	exonuclease-1
FA	Fanconi anaemia
FACS	fluorescence-activated cell sorting
FCS	foetal calf serum
GADD45	growth arrest and DNA damage-inducible 45
GG-NER	global genome NER
γH2AX	phosphorylated histone H2AX
H3	histone variant H3
H3 S10-P	phosphorylated histone H3
HJ	holliday junction
hnRNPUL-1/2	heterogeneous nuclear ribonucleoprotein U-like-1/2
HR	homologous recombination
ICL	interstrand crosslink
IR	ionising radiation
kDa	kilodalton
LB	Luria broth
MDC1	mediator of DNA damage checkpoint protein 1
MDM2	murine double minute 2
MGMT	O^6-methylguanine-DNA methyltransferase
MMEJ	microhomology mediated end-joining
MMR	mismatch repair
mRNA	messenger RNA

MSI	microsatellite instability
NBS	nijmegen breakage syndrome
NBSLD	nijmegen breakage syndrome-like disorder
NER	nucleotide excision repair
NHEJ	non-homologous end-joining
O^6Cl-ethylG	O^6–chloroethyl guanine
O^6meG	O^6–methyl guanine
PARP	poly(ADP-ribose) polymerase
PBS	phosphate buffered solution
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PI	propidium iodide
PIKK	phosphatidylinositol 3-kinase-like protein kinase
pRB	retinoblastoma protein
PTM	post-translational modification
PUMA	p53 upregulated modulator of apoptosis
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RFC	RAD17-replication factor C
RGG	arginine/glycine-rich region
RNA	ribonucleic acid
RPA	replication protein A
rpm	revolutions per minute
SAF	scaffold attachment factor

siRNA	small interfering RNA
SPRY	spore lysis A/ryanodine receptor
SSA	single-strand annealing
ssDNA	single-stranded DNA
SDSA	synthesis-dependent strand annealing
SMC1	structural maintenance of chromosomes-1
SSB	single-strand break
SSBR	single-strand break repair
TAD	transactivation domain
TC-NER	transcription-coupled NER
TOP1/2/3	topoisomerase I/II/III
TOPBP1	topoisomerase-binding protein-1
TOPcc	topoisomerase cleavage complex
TLS	translesion synthesis
UV	ultraviolet light
WT	wildtype
XP	xeroderma pigmentosum
XRCC	X-ray repair cross-complementary protein

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CHAPTER 1

INTRODUCTION

CHAPTER 1 INTRODUCTION

1.1 HALLMARKS OF CANCER

The development of cancer is a multi-step process characterised by distinct events that drive the progression of normal cells into malignant ones. These hallmarks include: an evasion of apoptosis, sustained angiogenesis, tissue invasion and metastasis, self-sufficiency in proliferation, insensitivity to growth-inhibitory factors and a limitless replication potential (Hanahan and Weinberg, 2000). Recently, further traits that contribute to the process of tumourigenesis have emerged. These include the evasion of immune destruction and the ability to modify cellular metabolism (Hanahan and Weinberg, 2011). These hallmarks are essentially alterations within the cell that enable tumour cells to survive and proliferate. The ability of cancer cells to acquire these capabilities depends on the genomic instability of that cell. Chromosomal instability (CIN) is the most common form of genomic instability present in cancer cells and this can lead to mass chromosomal lesions (Negrini *et al.*, 2010). Microsatellite instability (MSI) has also been described as a form of genomic instability and this is characterised by the disruption of oligonucleotides present in repetitive DNA sequences known as microsatellites (Thibodeau *et al.*, 1993). Both types of genomic instability can generate mutations that lead to the acquisition of the hallmarks listed above.

The integrity of the genome is constantly under threat of damage from both exogenous and endogenous agents that induce DNA lesions. These are recognised by components of the DDR pathway, which trigger the activation of cellular responses that can, in turn, lead to the maintenance of genomic integrity through cell cycle arrest and repair of the DNA lesion or apoptosis if the damage to the DNA is too great. Very often, genomic instability is

attributable to mutations in genes that would otherwise recognise and detect these lesions; these are known as the "caretakers" of the genome (Kinzler and Vogelstein, 1997). Known caretaker genes involved in the DDR whose mutations lead to genomic instability include *BRCA1, BRCA2, ATM* and genes involved in repair pathways.

1.2 THE DNA DAMAGE RESPONSE

Key regulators of the DDR include members of the phosphatidylinositol 3-kinase-like protein kinase (PIKK family) – ATM (ataxia-telangiectasia mutated), ATR (ATM- and Rad3-related) and DNA-PK (DNA-dependent protein kinase). Other members of the PIKK family include SMG1 (suppressor with morphological effect on genitalia family), mTOR (mammalian target of rapamycin) and TRAPP (transformation/transcription domain-associated protein). Although ATM, ATR and DNA-PK are the main PIKKs that regulate the DDR, there is evidence that SMG1 is implicated in the response to genotoxic stress (Brumbaugh *et al.*, 2004).

Of the PIKKs that are involved in the DDR, ATM and DNA-PK are primarily activated in response to double strand breaks (DSBs), which are the most deleterious of DNA lesions since failure to properly detect and repair them leads to chromosomal instability and consequently to tumourigenesis (Jeggo and Lobrich, 2007). In contrast, ATR responds mainly to RPA-coated single-stranded DNA (ssDNA) regions that are generated at stalled replication forks and DSBs (Cimprich and Cortez, 2008). This is of particular importance during DNA replication when replication forks are under threat from both exogenous and endogenous agents. If not dealt with correctly, a stalled replication fork is detrimental to the cell since it can develop into unstable structures that can, in turn, lead to genomic instability (Paulsen and

Cimprich, 2007). DNA-PKcs (DNA-dependent kinase catalytic subunit) plays a major role in the DSB repair pathway of non-homologous end-joining (NHEJ) where it is recruited to sites of damage and coordinates the signalling cascade that results in the repair process. In this way, DNA-PK differs from ATM and ATR in that it is involved primarily in DNA repair mechanisms themselves rather than the signalling of the break and subsequent responses such as the induction of cell cycle checkpoints.

1.2.1 ATM-mediated DNA damage response

The recruitment of ATM to sites of DSBs is dependent on the MRE11-RAD50-NBS1 (MRN) mediator complex, which senses DSBs and binds to DNA through two DNA binding sites located in MRE11 whilst a conserved region at the C-terminus of NBS1 is responsible for the binding of ATM (Falck *et al.*, 2005, van den Bosch *et al.*, 2003). In this way, the MRN complex facilitates the recruitment and activation of ATM at sites of DNA damage and is therefore a prerequisite for ATM-dependent signalling. In unstressed cells, ATM exists as an inactive dimer that allows the protein to remain stable but unable to phosphorylate its downstream targets (Bakkenist and Kastan, 2003). Evidence shows that the activation of ATM upon DNA damage requires its autophosphorylation on serine residues S1981, S367 and S1893 (Bakkenist and Kastan, 2003, Kozlov *et al.*, 2006). These phosphorylation events result in the dissociation of ATM dimers into catalytically-active monomers. Activated ATM is able to phosphorylate histone H2AX on serine residue 139 (referred to as γH2AX), which is an early event in the response to DSBs and initiates chromatin rearrangement in the immediate vicinity of the DSB to allow the accumulation of repair or damage-signalling components to sites of damage (Xu and Price, 2011). This establishes phosphorylation as

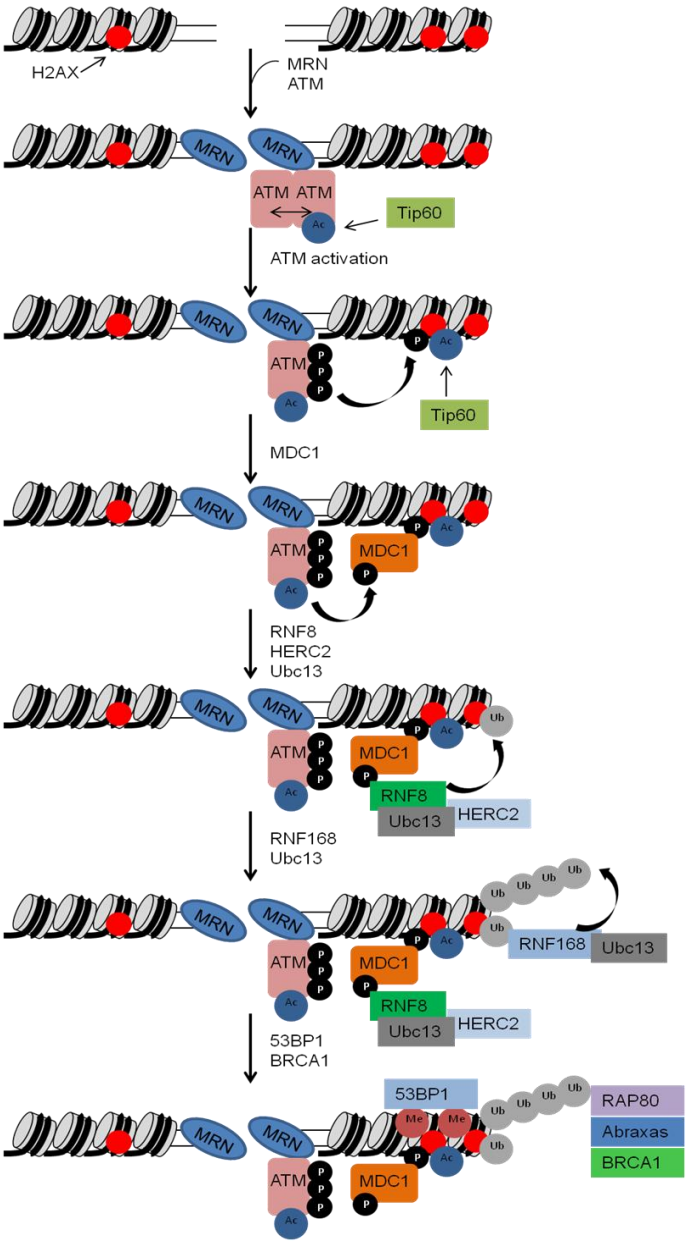

Figure 1.1 ATM-mediated DDR
DSBs that are generated from IR treatment or collapsed replication forks are sensed by the MRN complex, which binds to DNA and recruits ATM. This facilitates ATM kinase activation through its autophosphorylation and Tip-60-mediated acetylation. Activated ATM phosphorylates H2AX, which triggers MDC1 localisation. phosphorylation by ATM results in its association with RNF8, which then ubiquitinates H2A-type histones. RNF168 binds to these ubiquitylated histones and catalyses their poly-ubiquitylation, promoting a more open chromatin conformation. This consequently leads to the recruitment of further DDR proteins, 53BP1 and BRCA1 (adapted from Ciccia and Elledge, 2010).

critical to the activation of an ATM-mediated DDR. However, acetylation by the histone acetyltransferase, Tip60 has also proven to be indispensible since autophosphorylation of ATM at S1981 only occurs if ATM has been previously acetylated by Tip60 (Sun *et al.*, 2005). In addition, Tip60 is also required for the acetylation of histones that leads to the generation of open, relaxed chromatin, which is crucial for the processing and repair of the DSB (Xu and Price, 2011).

One of the proteins that is recruited to sites of DSBs after H2AX phosphorylation is the mediator of DNA damage checkpoint protein 1 (MDC1), which binds to γH2AX through the BRCA1 C-terminal repeat (BRCT) domains (Figure 1.1) (Stewart *et al.*, 2003, Stucki *et al.*, 2005). MDC1 is constitutively phosphorylated by casein kinase 2 (CK2) within SDT repeats in its N-terminus and these sites have been shown to bind to the FHA domain of NBS1, therefore tethering the MRN complex to sites of damaged chromatin (Melander *et al.*, 2008, Spycher *et al.*, 2008). The binding of MDC1 to γH2AX also facilitates the recruitment of phosphorylated ATM to the damaged chromatin; therefore the accumulation of both ATM and the MRN complex through MDC1 amplifies the ATM-dependent DDR via the spreading of phosphorylated H2AX to the extended chromosomal regions surrounding DSBs (measured in megabases) (Lou *et al.*, 2006).

In addition, MDC1 provides a docking site whereby other protein complexes can accumulate at the area surrounding the DSB. ATM-mediated phosphorylated sites of MDC1 have been found to interact with the E3 ubiquitin ligase, RNF8, which, in concert with another E3 ligase, HERC2 and the E2-conjugating enzyme, UBC13, ubiquitylates H2A-type histones that surround the DNA lesion (Figure 1.1) (Bekker-Jenson *et al.*, 2010, Huen *et al.*, 2007, Kolas *et al.*, 2007). Ubiquitylated histones are recognised by another E3 ubiquitin ligase, RNF168,

which, together with UBC13, ubiquitylates H2A-type histones and, therefore, further amplifies the ubiquitin signal (Stewart *et al.*, 2009).This leads to the coordinated assembly of BRCA1 and 53BP1 at sites of DNA damage and the consequent activation of cell cycle checkpoints and DNA repair pathways. 53BP1 is recruited to sites of damage via the interaction between its tudor domain and Lys79-methylated histone H3 and Lys20-methylated histone H4, which are unmasked by histone ubiquitylation (Botuyan *et al.*, 2006, Huyen *et al.*, 2004). The recruitment of BRCA1 to DNA breaks is mediated through the interaction between poly-ubiquitylated histones and RAP80, which tethers the BRCA1-binding protein, Abraxas, and therefore recruits the BRCA1 complex (Kim *et al.*, 2007, Sobhian *et al.*, 2007).

1.2.2 ATR-mediated DNA damage response

ATR primarily responds to ssDNA structures, which rapidly become coated by replication protein A (RPA), which is required to protect ssDNA (Fanning *et al.*, 2006). The recognition of these ssDNA structures is one way in which ATR is able to respond to a range of DNA lesions or replication fork blockages. The localisation of ATR to RPA-coated ssDNA sites depends on the interaction between ATR and ATR-interacting protein (ATRIP), the latter being able to indirectly recognise ssDNA through its interaction with the large RPA (70K) subunit (Ball *et al.*, 2007, Ball *et al.*, 2005, Cortez *et al.*, 2001). Once the ATR-ATRIP complex is localised to RPA-ssDNA, RPA recruits a DNA damage-specific clamp loader, RAD17-replication factor C (RFC), which consequently loads a RAD9-RAD1-HUS1 (9-1-1) checkpoint clamp onto 5'ends adjacent to the area of RPA-ssDNA (Figure 1.2) (Zou *et al.*, 2003). This checkpoint clamp is needed for the recruitment of the ATR activator protein, topoisomerase-binding protein-1 (TOPBP1). TOPBP1 is brought to the vicinity of ATR-

ATRIP through the interaction between the two BRCT domains on TOPBP1 and a phosphorylated C-terminal tail of RAD9 – a component of the 9-1-1 complex (Lee *et al.*, 2007). The binding of TOPBP1 with ATR-ATRIP utilises the activation domain of TOPBP1 and a region on ATRIP. This results in the activation of ATR kinase, enabling it to phosphorylate numerous downstream targets, including its effector kinase, the S/T kinase checkpoint kinase-1 (Chk1). The activation of Chk1 requires its phosphorylation by ATR on two serine residues, S317 and S345 (Zhao and Piwnica-Worms, 2001). Two protein complexes found at replication forks mediate the activation of Chk1 by ATR; Claspin and a complex composed of Timeless (Tim) and Tim-interacting protein (Tipin) (Figure 1.2) (Liu *et al.*, 2006, Unsal-Kacmaz *et al.*, 2007). Upon its phosphorylation, Chk1 targets and phosphorylates the CDC25 phosphatases to inhibit their activity and as a result, prevent CDK activation. This culminates in cell cycle arrest.

The activities of ATR extend to the maintenance of replication fork integrity by promoting replication fork stability and mediating the recovery of stalled forks. Downstream targets of ATR such as components of the MCM2-7 replicative helicase, MCM2 and MCM3, promote replication fork stabilisation and to allow the completion of replication (Cimprich and Cortez, 2008, Cortez *et al.*, 2004). Once these MCM proteins are activated, they recruit Polo-like kinase-1 (PLK1), which facilitates the completion of replication at sites near the stalled fork (Trenz *et al.*, 2008).

1.2.3 ATM and ATR cross-talk

Evidence suggests that a degree of cross-talk exists between the ATM- and ATR-mediated signalling pathways despite the different DNA structures to which each kinase responds. This

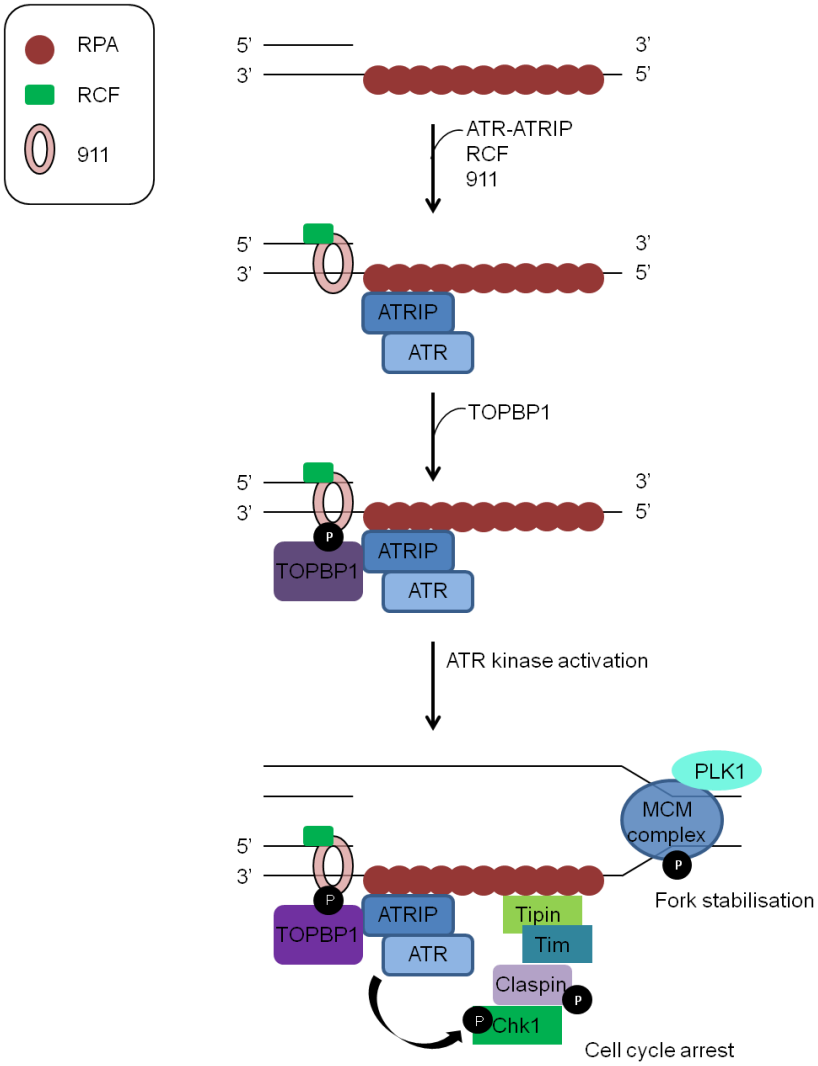

Figure 1.2 ATR-mediated DDR
The generation of ssDNA after UV damage or replication stress leads to the accumulation of RPA. RPA-caoted ssDNA recruits the ATR/ATRIP complex, followed by the loading of the 9-1-1 complex in an RCF-dependent manner. TOPBP1 recruitment to 9-1-1 activates ATR kinase activity. ATR phosphorylates Claspin, which, together with another mediator protein, Tipin, mediates the phosphorylation and activation of Chk2 by ATR. Once activated, Chk1 is able to phosphorylate key targets and consequently leads to cell cycle arrest (adapted from Cimprich and Cortez, 2008).

phenomenon is partly explained by the fact that these structures are partially interchangeable during different forms of DNA repair (Cimprich and Cortez, 2008). DNA-end resection is one example since it is initially ATM-dependent but eventually leads to ATR activation via the generation of ssDNA (1.3.1.5) (Jazayeri *et al.*, 2006). In addition, stalled replication forks can collapse in the absence of ATR and DSBs often form at these sites, which results in the activation of ATM (Brown and Baltimore, 2003). However, the role of ATM at sites of collapsed forks where no DSBs are generated has yet to be elucidated. There is also the observation that a subset of substrates, such as p53 and BRCA1, can be phosphorylated by both kinases, which again suggests interplay between ATM and ATR signalling pathways (Cortez *et al.*, 1999, Tibbetts *et al.*, 1999, Tibbetts *et al.*, 2000)

There is evidence to suggest that ATR is able to phosphorylate ATM after replication fork stalling or after treatment with UV; this results in the activation of ATM, indicating that ATR can function upstream of ATM in certain signalling pathways (Stiff *et al.*, 2006). Furthermore it has also been shown that ATR and ATM can both phosphorylate another member of the PIKK family, DNA-PK (Chen *et al.*, 2007, Yajima *et al.*, 2006). Therefore it is clear that an interdependent relationship exists between members of the PIKK family throughout the network of interacting pathways that together execute the DDR.

1.2.4 Cell cycle checkpoints

One of the most fundamental outcomes of the DDR is the activation of cell cycle checkpoints. These function to delay or arrest cell cycle progression to allow the cell time to repair the damage or if the damage is too severe, to initiate apoptosis. The components of the DDR can mediate checkpoints at different phases of the cell cycle: gap 1 (G1), DNA replication (S) or

gap 2 (G2). The predominant mechanism for control of cell cycle progression is through the regulation of cyclin-dependent kinases (CDKs) and their regulatory subunits, cyclins (Elledge and Harper, 1994). These exist in complexes and are activated by phosphatases that remove the inhibitory phosphorylation on CDKs, which allows the cell to progress through the cell cycle. This process is mediated by the CDC25 family of phosphatases, which consist of three mammalian isoforms: CDC25A, -B and -C and specifically regulate the activities of CDK1 and CDK2 (Boutros *et al.*, 2007). Effector kinases phosphorylate the CDC25 phosphatases in response to DNA damage and inhibit their activity thereby preventing CDK activation and consequently cell cycle progression (Figure 1.3).

1.2.4.1 The G1 phase checkpoint

Damage encountered in G1 results in the activation of the G1/S checkpoint, which prevents cells with DNA damage from undergoing DNA replication in S phase. The ATM/Chk2 and ATR/Chk1 pathways primarily mediate cell cycle arrest in G1 and the kinases within these pathways mainly target CDC25A and p53 during this phase. ATM can directly phosphorylate p53 within its N-terminal region serine 15 (S15) residue and together with the ATM-dependent Chk2 phosphorylation of p53 on serine 20 (S20) and threonine 18 (T18) this results in the stabilisation of the p53 transcription factor (Banin *et al.*, 1998, Canman *et al.*, 1998, Shieh *et al.*, 2000). Phosphorylation of these amino acids inhibits the p53-MDM2 interaction and disrupts the MDM2-induced nuclear export and degradation of p53 (Dumaz and Meek, 1999, Schon *et al.*, 2002). There is evidence that ATR can directly phosphorylate p53 on S15 as well, although ATM appears to be the dominant PIKK that mediates G1 arrest (Tibbetts *et*

al., 1999). The increased stabilisation of p53 through its phosphorylation not only results in its accumulation after DNA damage; its activity as a transcription factor is enhanced as well. One of the main transcription targets of p53 is the cyclin-dependent kinase inhibitor (CKI), p21, which inhibits CDK4/cyclinD1 and CDK2/cyclin E (Figure 1.3) (He *et al.*, 2005a). The activation of p21 inhibits the phosphorylation of pRB (Retinoblastoma protein), which leads to the stabilisation of the Rb/E2F complex and therefore, inactivation of E2F-dependent transcription of pro-proliferation genes that are normally required for progression through G1(Delavaine and La Thangue, 1999, Dimri *et al.*, 1996). In late G1, the expression of ATR and Chk1 increases and, in response to DNA damage, Chk1 is activated by ATR-mediated phosphorylation. A key target of Chk1 is CDC25A, the phosphorylation of which leads to the inhibition of the CDK2/cyclin E complex (Figure 1.3) (Zhao *et al.*, 2002).

1.2.4.2 The S phase checkpoint

The S phase checkpoint is the term used to describe the cell cycle arrest that occurs during DNA replication in response to genotoxic stress. The integrity of the genome is most vulnerable during S phase since spontaneous lesions can occur during the process of replication as well as from DNA damaging agents (Bartek *et al.*, 2004). Therefore three S phase checkpoints exist: the replication-independent intra-S phase checkpoint (intra-S phase checkpoint), the replication-dependent intra-S phase checkpoint (replication checkpoint) and the S/M checkpoint (Bartek *et al.*, 2004). The latter two checkpoints respond to stresses during DNA replication whereas the intra-S phase checkpoint does not require replicating DNA and is activated by DSBs. The replication checkpoint functions through the ATR-mediated damage response pathways in response to a stalled replication fork as described

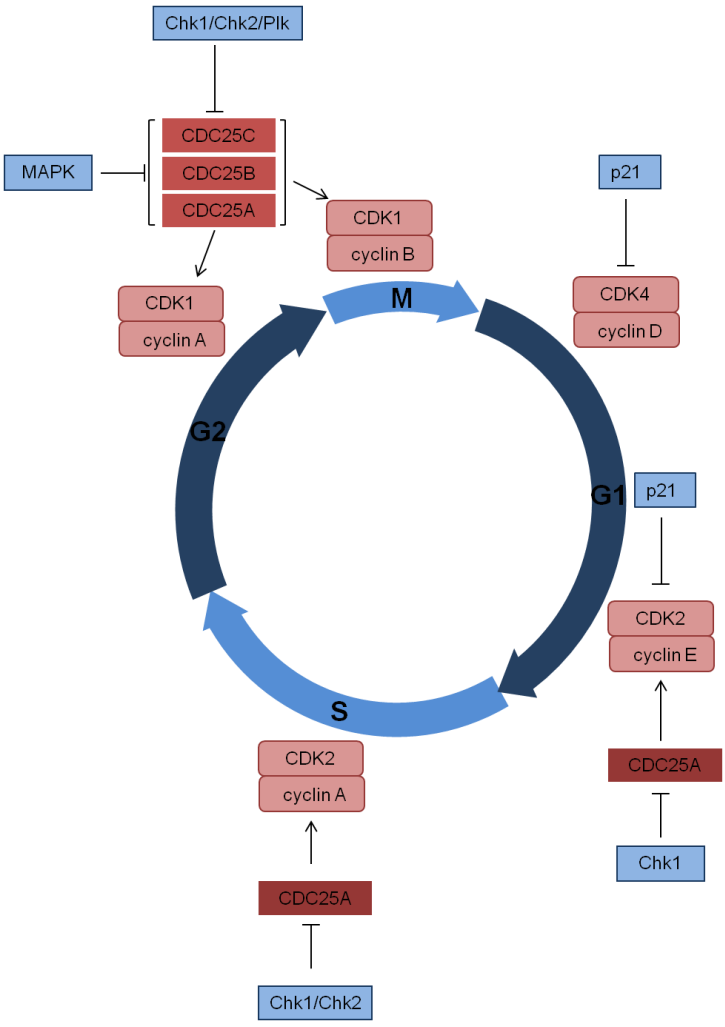

Figure 1.3 Cell cycle checkpoints
Regulation of cell-cycle progression is primarily mediated through CDK and cyclin complexes, which are in turn regulated by CDC25 phosphatases. Progression through G1/S is medated by CDK4/cyclin D and CDK2/cyclin E. p21 inhibitis both of these complexes. Chk1 phosphorylates CDC25A, inhibiting its activity and preventing its activation of CDK2. Similarly, CDC25A is inhibited in S phase, disrupting its dephosphorylation and activation of CDK2. All 3 CDC25s function in G2/M to activate CDK1/cyclin A and CDK1/cyclin B. All CDC25s are inhibited by DNA damage-inducible kinases in this phase.

previously in 1.2.2; this ATR-dependent pathway is the primary regulator of the progression from S to M phase when replication forks stall. The S/M checkpoint is an ATR-independent pathway that ensures that replication has accurately completed before cells divide and involves the inhibition of CDK1/cyclin B although the mechanism is not fully understood (Brown, 2003).

The intra-S phase checkpoint responds to DSBs during S phase and is mediated through the ATM/Chk2 pathway as described previously, the outcome in this phase of the cell cycle being the S phase-dependent degradation of CDC25A. There is evidence to suggest that this process does depend on the underlying activity of the ATR/Chk1 pathway that also phosphorylates CDC25A but the exposure to DSBs enhances the proteolysis of this phosphatase during S phase (Falck *et al.*, 2001, Zhao and Piwnica-Worms, 2001). Therefore both Chk1 and Chk2 phosphorylate CDC25A leading to the consequent inhibition of CDK2/cyclin A activity (Figure 1.3). This blocks the loading of the CDC45 initiation factor onto chromatin, which would otherwise mediate the recruitment of DNA polymerase α (Pol α) to pre-replication complexes (Kastan and Bartek, 2004). The inhibition of CDK2 therefore prevents the firing of new replication origins.

Another branch of the intra-S phase checkpoint is the ATM-dependent phosphorylation of SMC1 (structural maintenance of chromosomes-1) and NBS1, although the significance of the phosphorylation of these ATM targets in relation to the intra-S phase checkpoint is currently unknown (Kim *et al.*, 2002, Yazdi *et al.*, 2002).

1.2.4.3 The G2/M phase checkpoint

The G2/M checkpoint is activated when damage occurs in the G2 phase of the cell cycle or if cells progress into G2 phase with unrepaired damage from G1 or S phase; this halts progression into mitosis and inhibits segregation of damaged chromosomes (Kastan and Bartek, 2004). The mode of regulation within this checkpoint is predominantly by the inactivation of CDK1/cyclin B1 through phosphorylation of CDC25s. CDC25C can be phosphorylated by Chk1 and Chk2, which allows the binding of 14-3-3 proteins to this phosphatase and renders it inactive and/or sequesters it to the cytoplasm (Abraham, 2001). Another mechanism involved in the G2/M checkpoint utilises the p38 mitogen-activated protein kinase (MAPK) pathway that has been shown to target all three CDC25s (Bulavin *et al.*, 2001, Reinhardt *et al.*, 2007). In addition, the Polo-like kinase, Plk3, has been found to play a role in this checkpoint via inhibitory phosphorylation of CDC25C (Figure 1.3) (Bahassi *et al.*, 2004). The p53-dependent transcription of cell cycle inhibitors such as p21, 14-3-3 sigma proteins and GADD45a (growth arrest and DNA damage-inducible 45 alpha) also maintains the G2 checkpoint. Other checkpoint mediators include BRCA1 and 53BP1 (Wang *et al.*, 2002, Xu *et al.*, 2001).

1.3 DNA DAMAGE REPAIR

1.3.1 Repair of double strand breaks

Double strand breaks (DSBs) are the most deleterious of DNA lesions and can be repaired by a network of DNA repair pathways. Failure to repair such lesions would be disastrous for the cell, leading to death (Jeggo and Lobrich, 2007). The inappropriate repair of DSBs can result

in chromosomal alterations that can subsequently lead to the onset of tumourigenesis. The repair of DSBs is conducted by at least four pathways: homologous recombination (HR), non-homologous end-joining (NHEJ), alternative non-homologous end-joining (alt-NHEJ) and single strand annealing (SSA).

1.3.1.1 Homologous Recombination

Homologous recombination (HR) repair is an important repair pathway of DSBs and utilises homologous sequences to repair damaged DNA. Because of this, HR repair primarily functions in late S and G2 phases of the cell cycle and is regarded as being an error-free repair pathway. The substrate for the initiation of HR repair is the ssDNA that arises from the processing of a DSB as in 1.3.1.5. HR is catalysed by the recombinase family of enzymes, namely Rad51, which polymerises on ssDNA to form a nucleoprotein filmament and, consequently, the presynaptic filament. Rad51 possesses a lower affinity for ssDNA than RPA; therefore different proteins act to mediate the displacement of RPA by Rad51 (Hartlerode and Scully, 2009). Rad52 is one example, as is BRCA2, which interacts directly with Rad51, recruits it to ssDNA and catalyses the nucleation of Rad51 (Sugiyama and Kowalczykowski, 2002, Yang *et al.*, 2005). Rad51 is also modified by Chk1, which mediates the phosphorylation of Rad51 and is required for its recruitment to sites of damage (Sørensen *et al.*, 2005).

The Rad51 nucleoprotein filament binds duplex DNA and, in concert with the accessory protein Rad54, invades an undamaged dsDNA template to locate homologous regions of DNA (Figure 1.4) (Mazin *et al.*, 2000). In the process of strand invasion, one strand becomes displaced and a displacement (D)-loop structure is formed (Jackson, 2002). Strand invasion is

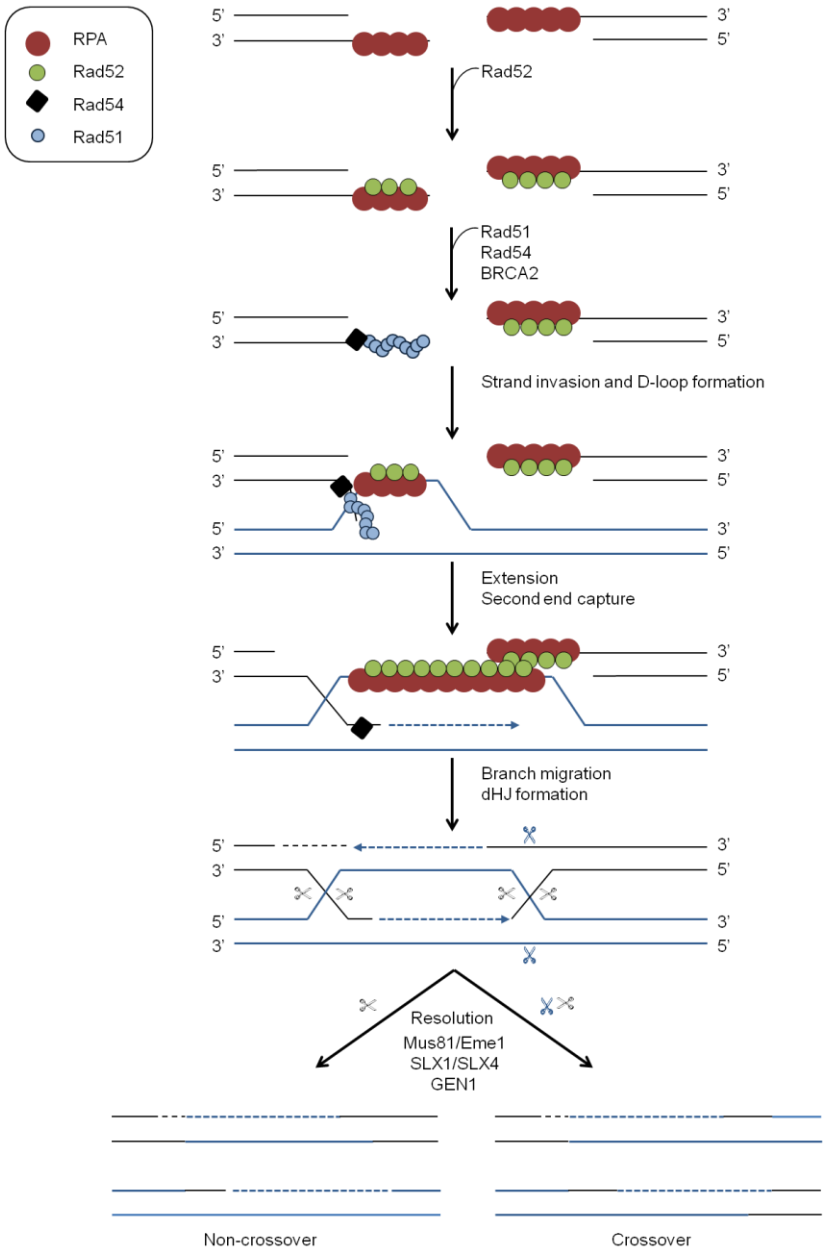

Figure 1.4 Homologous Recombination
ssDNA generated from DNA end-resection is initially bound by RPA. BRCA2 and Rad52 aid the formation of the Rad51 nucleoprotein filament. Together with Rad54, the Rad51 filament interacts with an undamaged DNA duplex, displacing one strand to form a D-loop. The second resected end is captured in a Rad52-dependent manner, after which DNA synthesis occurs to fill in both sides of resected DNA. Branch migration leads to the formation of a double Holliday junction. Crossover and non-crossover products are generated after resolution of the double Holliday junction (adapted from Ciccia and Elledge, 2010, Jackson, 2002).

followed by DNA synthesis, which is conducted by DNA polymerases such as Pol δ that use the homologous DNA as a template to replace the sequence of DNA that was previously on the damaged strand (Maloisel *et al.*, 2008). At this point the reaction can proceed through the synthesis-dependent strand annealing pathway (SDSA) where the newly synthesised strand is displaced by the RTEL helicase and anneals with the ssDNA on the other side of the break.

The SDSA pathway of HR explains the fact that crossover events in mitotic DSBR are rare and also that meiotic DSBR events do not result in crossovers (Allers and Lichten, 2001, Ferguson and Holloman, 1996). Alternatively, the reaction can occur via the DSBR pathway where the invading strand captures the second resected DNA break in a process known as second end capture, which is facilitated by Rad52 (Nimonkar *et al.*, 2009). DNA synthesis at either side of the structure follows, along with branch migration, and results in an intermediate known as a double Holliday junction (HJ).

These intermediates must then be resolved to separate the covalently-bound sister chromatids. Resolution of HJs is dependent on the activities of the Mus81/Eme1 and SLX1/SLX4 endonuclease complexes as well as the HJ resolvase, GEN1 (Ciccia *et al.*, 2003, Constantinou *et al.*, 2002, Fekairi *et al.*, 2009, Ip *et al.*, 2008). The generation of non-crossover events prevent the loss of heterozygosity and genomic rearrangement and occur if there is no exchange of DNA sequences between the sister chromatids. Conversely, crossover events occur if DNA is exchanged between the two chromosomes. Double HJs can also be processed through dissolution and this is reliant on the Bloom syndrome protein (BLM) helicases in complex with topoisomerase IIIα (TOP3α), which together catalyse the HJ dissolution to yield non-crossover products (Sung and Klein, 2006).

1.3.1.2 Non-homologous end-joining

Despite it being an error-prone pathway, the prevailing repair pathway in the G1 phase of the cell cycle is non-homologous end-joining (NHEJ). Initially, broken ends of the DNA molecule are tethered in a process that requires the Ku70/Ku80 heterodimer to bind to the DNA broken end first. The complex consisting of the Ku heterodimer and DNA mediates the recruitment of DNA-PKcs (Gottlieb and Jackson, 1993). The association of DNA-PK_{CS} with the Ku heterodimer-DNA complex results in a molecular bridge being formed and causes the two ends of the DNA molecule to be brought closer together (Figure 1.5) (Spagnolo *et al.*, 2006). The unphosphorylated form of DNA-PKcs acts to block and protect the DNA ends from premature degradation or ligation until both ends of the broken DNA molecule are brought into closer proximity (Calsou *et al.*, 1999). Once this occurs, DNA-PKcs becomes activated by autophosphorylation and undergoes a conformational change at the synaptic complex that relieves the blockage and allows processing enzymes and ligases to access the DNA ends (Reddy *et al.*, 2004).

Often, non-compatible DNA ends will be produced by a DSB and these cannot be directly ligated because at least one of the strands will possess a 3' or 5' single strand overhang. These overhangs need to be processed before ligation can occur and it is this stage of NHEJ that renders it an imprecise pathway since loss of nucleotides at the site of the break often occurs during processing of the DNA ends (Smith *et al.*, 2001). Artemis is the only known nuclease to date that is involved in NHEJ and is required to process the DNA overhangs by resection at the position where the single strand extends from the double-strand helix (Weterings and Chen, 2008). The modes of activation of Artemis have been the cause of controversy; although it is known that Artemis becomes phosphorylated by both DNA-PK and ATM after

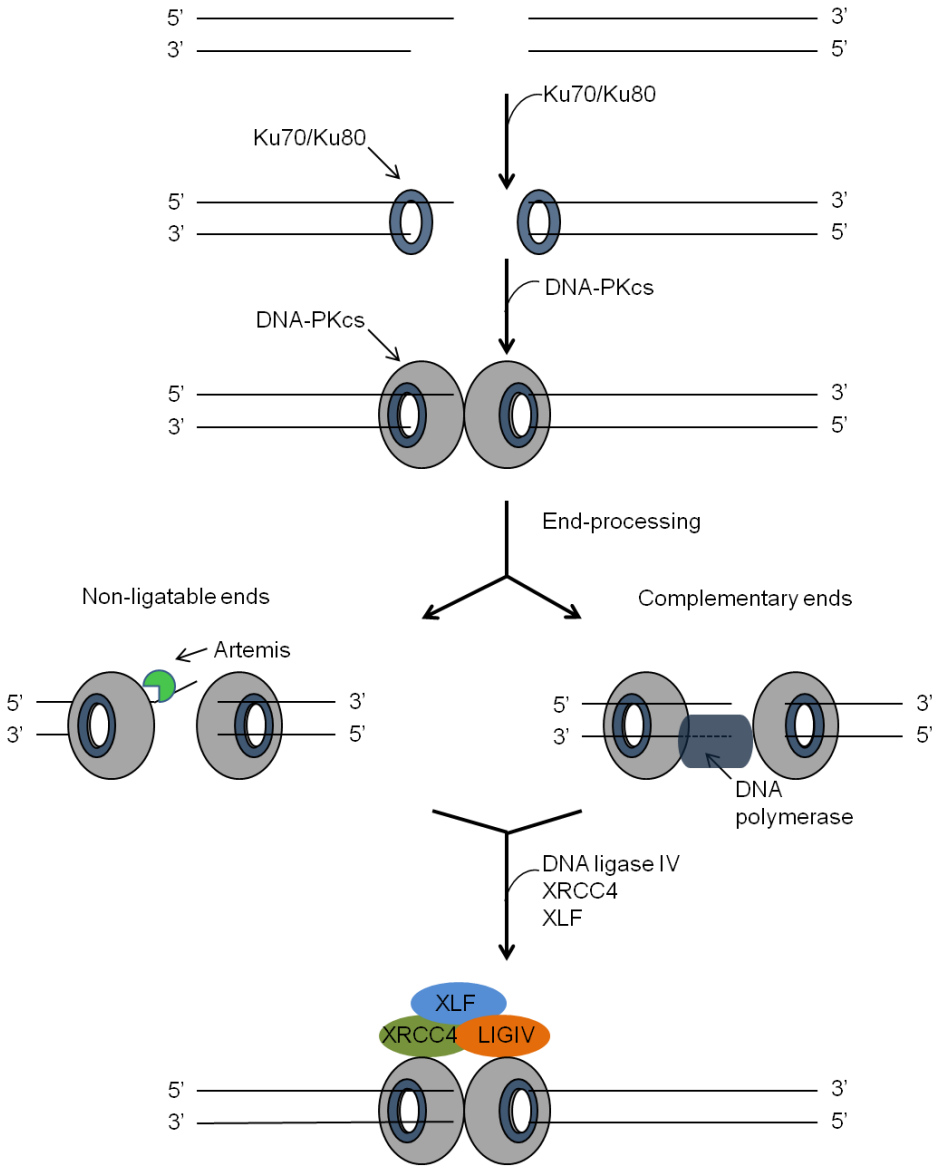

Figure 1.5 Non-homologous end-joining
The Ku70/Ku80 heterodimer binds to broken DNA ends and recruits DNA-PKcs to tether the two ends together. Autophosphorylation of DNA-PKcs results in a conformational change that makes the DNA ends accessible to processing enzymes. Non-ligatable ends are processed by Artemis, while polymerases synthesis nucleotides at compatible ends. Ligase IV/XRCC4, in concert with XLF, mediates re-ligation of DNA ends (adapted from Ciccia and Elledge, 2010, Weterings and Chen, 2008).

IR, it is not known whether phosphorylation is needed for its nuclease activity (Goodarzi *et al.*, 2006, Niewolik *et al.*, 2006). While Artemis is responsible for processing non-compatible ends by resection, polymerases are required to synthesise complementary nucleotides in another process to remove single-strand overhangs. Such polymerases include DNA polymerase μ (Pol μ), DNA polymerase λ (Pol λ) and the human terminal deoxynucleotidyltransferase (TdT) (Hartlerode and Scully, 2009).

Once DNA ends have been sufficiently processed and non-compatible ends converted into 5' ligatable ends, re-ligation can occur. This stage of NHEJ is mediated by DNA ligase IV and XRCC4, which exist in a complex and are recruited together to the DNA-Ku scaffold. It has been suggested that the interaction between the ligase IV/XRCC4 complex and Ku70/Ku80 increases the ligase activity of the former complex (Nick McElhinny *et al.*, 2000). Studies have also shown that another protein, XRCC4-like factor or Cernunnos (XLF/Cernunnos) can stimulate the ligation ability of ligase IV/XRCC4 (Ahnesorg *et al.*, 2006). Together, these proteins are able to mediate the repair of the DSBs.

1.3.1.3 Alternative non-homologous end-joining

Studies have implicated another pathway in the repair of DSB, and this is more error-prone than the classical NHEJ pathway. This is known as alternative NHEJ (alt-NHEJ) or microhomology mediated end-joining (MMEJ) since junctions that are repaired by this pathway are characterised by deletions, insertions and relatively large regions of microhomology. Alt-NHEJ requires limited DSB resection, which occurs in G1 and is facilitated by CtIP and the MRN complex (Yun and Hiom, 2009). Other components involved in alt-NHEJ have not been fully characterised, yet several proteins have been implicated.

Poly(ADP-ribose) polymerase 1 (PARP1) has been shown to bind to resected DSBs, particularly in the absence of Ku (Wang *et al.*, 2006). The mammalian polynucleotide kinase, PNK, has been implicated in the processing of the DNA ends (Audabert *et al.*, 2006). Finally, the DNA ligase IIIα/XRCC1 complex has been found to possess the end-joining activity required in this pathway (Della-Maria *et al.*, 2011).

1.3.1.4 Single-strand annealing

A DSB that occurs between closely-repeated sequences can also be repaired during HR using a process called single-strand annealing (SSA). The first step in this pathway is DNA resection, which utilises the same components as in HR, after which, RAD52 binds the 3'ssDNA ends to promote the annealing of two repeated sequences (Ciccia and Elledge, 2010). It is interesting that the N-terminal region of RAD52 is responsible for promoting SSA *in vitro*, a region which is unable to stimulate the recombinase activity of RAD51 in HR (Singleton *et al.*, 2002). The non-homologous 3'ssDNA ends that are left are cleaved by the mammalian ERCC1/XPF endonuclease complex and, incidentally, this process does not require strand invasion since the DSB ends are processed so they can anneal with each other (Al-Minawi *et al.*, 2008).

1.3.1.5 Regulation of DSB repair

The balance between DSBR pathways shifts during the cell cycle since HR, largely an error-free process, relies on the presence of an intact sister chromatid and, therefore, occurs primarily during S/G2 phases of the cell cycle whereas NHEJ predominates mainly during G0, G1 and early S phase and is an error-prone repair pathway (Takata *et al.*, 1998). One process known to influence which repair pathway to use is resection of DNA ends

surrounding the break. HR-mediated repair is dependent on DSB resection during the S/G2 phases of the cell cycle, which generates ssDNA through the digestion of 5'ends of DNA.

Initially, MRN is recruited to a DSB and binds broken DNA ends. MRN alone is not sufficient for DNA-end resection and requires additional proteins, including CtIP, which associates with the MRN complex (Figure 1.6) (Takeda *et al.*, 2007). The role of the MRN complex in DNA-end resection relies on the endonuclease activity of MRE11, although, interestingly, this function is not required for the ability of the MRN complex to sense DSBs as described in 1.2.1 (Buis *et al.*, 2008). There is evidence to suggest that binding of CtIP to MRN could influence the function of MRN such that it is directed toward end resection (Sartori *et al.*, 2007).

It is important that resection and HR occur at the same stage of the cell cycle since HR is dependent on end resection; therefore the activity of CtIP in end resection is highly regulated at the post-translational level. Both phosphorylation and ubiquitylation play a role in this and these processes are mediated by CDK2 and BRCA1, respectively (Chen *et al.*, 2008, Huertas and Jackson, 2009, Yu *et al.*, 2006). This occurs in the S/G2 phases of the cell cycle to promote the association of CtIP with DSBs, which subsequently promotes end resection. Limited resection can occur in G1, which is facilitated by CtIP, indicating that CtIP is able to promote DSB resection in the absence of its activation by CDK and BRCA1 in S phase (Yun and Hiom, 2009). The MRN complex initiates the unwinding of DNA ends; CtIP and the MRN complex are then thought to promote resection by endonucleolytic cleavage of 5'ssDNA ends.

Extensive resection is further conducted by the concerted activities of exonuclease-1 (EXO-1), DNA replication helicase 2 (DNA2) and BLM, after which, RPA is recruited to the

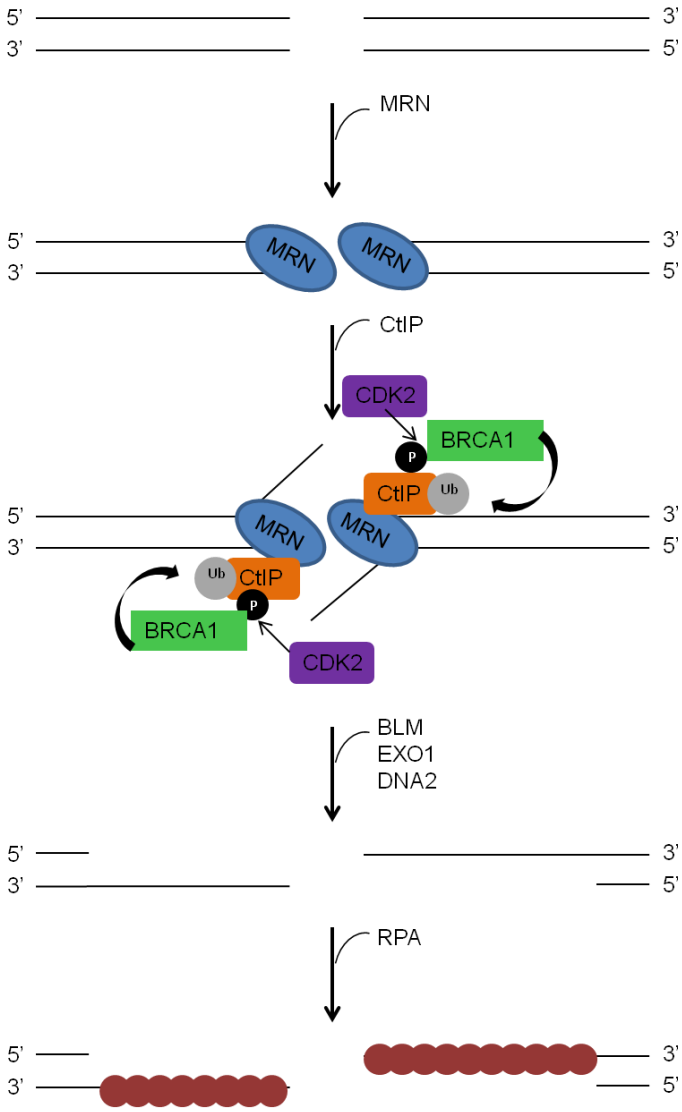

Figure 1.6 DNA-end resection
The MRN complex is recruited to DSBs and binds to DNA ends where is becomes bound by CtIP. CDK2-dependent phosphorylation of CtIP mediates its association with BRCA1, which consequently ubiquitinates CtIP. Both phosphorylation and ubiquitylation of CtIP facilitates its association with DSBs. MRN promotes the initial unwinding of DNA. Endonucleolytic cleavage of 5' ends is then mediated by CtIP and MRN before extensive DNA processing is conducted by EXO1, DNA2 and BLM. ssDNA from DSB resection is bound by RPA, which activates DNA repair by HR (adapted from Ciccia and Elledge, 2010).

ssDNA ends generated by DNA resection and prevents these ends from annealing or from forming secondary structures (Ciccia and Elledge, 2010, You and Bailis, 2010, Zhu *et al.*, 2008). During NHEJ, binding of the Ku complex and DNA-PK to DNA ends inhibits HR repair since, in their absence, more DSBs undergo end resection (Pierce *et al.*, 2001). In addition, the phosphorylation of particular residues of DNA-PKcs has an inhibitory effect on HR repair through the prevention of end resection (Cui *et al.*, 2005).

1.3.2 Single-strand break repair

Single-strand breaks (SSBs) are the most common type of damage that occurs in cells and these can arise directly or indirectly. Direct SSBs can occur from genotoxic insults such as IR or reactive oxygen species (ROS), the latter resulting in the ROS-induced disintegration of oxidised deoxyribose (sugar damage). SSBs are also generated indirectly during the process of base excision repair (BER) or from erroneous TOP1 activity (Caldecott, 2008). If SSBs are left unrepaired in proliferating cells they can cause replication forks to stall or collapse, which may generate DSBs and induce genomic instability (Kuzminov, 2001).

The main sensor of SSBs in the process of single-strand break repair (SSBR) is PARP1, which becomes activated upon binding to SSBs and facilitates a number of processes that are fundamental to SSBR. The binding of PARP1 to sites of SSBs results in its activation, which causes the synthesis of poly(ADP-ribose) (PAR) chains on itself and other target proteins. Chromatin structure is also regulated by PARP1 since the latter assembles PAR chains onto histones H1 and H2B, a modification that serves to recruit other components of the SSBR pathway to SSB sites (Schreiber *et al.*, 2006). This process is transient since PAR glycohydrolase (PARG) rapidly degrades PAR chains to allow PARP1 to be restored for

further SSB detection (Davidovic *et al.*, 2001). PAR chains serve as a signal for the recruitment of other factors to promote SSBR (Ciccia and Elledge, 2010). X-ray repair cross-complementary protein (XRCC1) is recruited to SSBs in a PARP1-dependent manner and promotes SSBR through the recruitment of XRCC1-interacting proteins (Masson *et al.*, 1998). End-processing follows the detection of such lesions and this restores damaged 3'-termini to the proper hydroxyl state. This stage of SSBR is conducted by many enzymes and is dependent on the type of SSB that arises. End-processing enzymes include DNA polymerase β (Pol β), polynucleotide kinase 3'-phosphatase (PNKP) and the nucleases, apurinic-apyrimidinic endonuclease 1 (APE1) and aprataxin (APTX). These are all recruited to sites of SSBs through interaction with XRCC1 (Caldecott *et al.*, 1996, Clements *et al.*, 2004, Whitehouse *et al.*, 2001, Winters *et al.*, 1994).

After end-processing, gap-filling of the missing nucleotides must occur. The insertion of one nucleotide is termed short-patch repair, whereas gap-filling that needs to be extended for 2-12 nucleotides constitutes long-patch repair. Pol β mediates both types of gap-filling but may require additional accessory proteins for long-patch repair such as flap endonuclease 1 (FEN1) (Liu *et al.*, 2005). The final step of SSBR is DNA ligation, which is catalysed by DNA ligase 3 (LIG3) for short-patch repair and DNA ligase 1 (LIG1) for long-patch repair (Caldecott, 2007).

1.3.3 Repair of DNA base adducts

DNA adducts are generated by exposure to alkylating agents or through endogenous compounds. Small alterations of DNA bases can be removed and repaired before they encounter replication forks by base excision repair (BER) or nucleotide excision (NER) repair

pathways. The mismatch repair (MMR) pathway replaces mis-repaired DNA bases with correct bases during DNA replication.

1.3.3.1 Direct repair

Repair of a damaged base can be conducted without removal of the damage. This occurs by the action of enzymes such as alkyltransferases and DNA dioxygenases that revert the damaged base back to its unmodified form (Sedgwick, 2004). The main alkyltransferase to function in direct reversal is O^6-methylguanine-DNA methyltransferase (MGMT), which demethylates the mutagenic lesion O^6-methylguanine: the methyl group of the lesion is transferred to a cysteine residue in the active site of MGMT (Sedgwick *et al.*, 2007). Alpha-ketoglutarate-dependent dioxygenase alkB homologues 2 and 3 (ALKBH2 and ALKBH3) catalyse the oxidative demethylation of N-alkyl lesions such as 1-methyladenine and 3-methylcytosine (Duncan *et al.*, 2002). Incidentally, these are the main lesions that are induced in ssDNA by alkylating agents.

1.3.3.2 Base excision repair

BER acts to remove a damaged DNA base through a series of coordinated steps. Simple alkylation adducts are recognised by lesion-specific DNA glycosylases, which catalyse the hydrolysis of the *N*-glycosyl bond thus resulting in the removal of the base in question. The loss of a pyrimidine or purine base leaves an apyrimidinic or apurinic (AP) site, respectively, which is recognised by the AP endonuclease (APE1). This enzyme incises the DNA backbone of the AP site to generate a 3'hydroxyl and a 5'deoxyribose phosphate (5'-dRP) group. The latter is removed by the 5'-dRP lyase activity of Pol β, which also mediates the single

nucleotide gap filling, followed by re-ligation of the nicked DNA strand catalysed by LIG1 or LIG3 (Matsumoto and Kim, 1995).

1.3.3.3 Nucleotide excision repair

Larger DNA adducts or lesions that are able to distort the double-stranded DNA helix are removed and repaired by the NER pathway. Lesions that are recognised by NER include cyclobutane pyrimidine dimers (CPDs) and 6,4 photoproducts (6,4PPs), both of which are induced by UV damage. Defects in components of NER display the same photosensitivity and predisposition to skin cancer observed with the repair syndrome Xeroderma Pigmentosum (XP) (de Laat *et al.*, 1999). There are two types of NER: global genome NER (GG-NER), which is used to repair lesions over the entire genome, and transcription-coupled NER (TC-NER) that targets transcription-blocking lesions in actively-transcribing DNA (de Laat *et al.*, 1999). The two types of NER also differ in the manner of substrate lesion recognition; otherwise a common set of steps exist to complete the repair process.

In TC-NER, lesions are detected by the elongating RNA polymerase II, whereas the primary sensor of damaged DNA in GG-NER is the complex of XPC and hRAD23B (hR23B) (de Laat *et al.*, 1999, Masutani *et al.*, 1994, Sugasawa *et al.*, 1998). RPA and XPA are also known as damage recognition proteins in NER and both are required for the recruitment of other repair factors (de Laat *et al.*, 1999, Evans *et al.*, 1997). The initial recognition of helix-distorting lesions is via the XPC-hR23B complex, which then recruits the transcription factor, TFIIH, to the damaged DNA; indeed, both TFIIH and XPC-hR23B have been found to be essential for catalysing an open formation around the lesion, which is consequently required for dual incision on either side of the lesion (Evans *et al.*, 1997). Incidentally, TFIIH

functions in both types of NER and contains XPB and XPD DNA helicases that allow TFIIH to generate an open complex around the lesion (Roy *et al.*, 1994, Schaeffer *et al.*, 1993). The unwinding of the damaged DNA region results in a junction between ssDNA and dsDNA, which is recognised by the endonucleases XPG and ERCC1-XPF for the dual incision of the 3' and 5' ends respectively (Matsunaga *et al.*, 1995, O'Donovan *et al.*, 1994).

After the removal of the damaged strand of DNA, various enzymes function to catalyse the gap-filling repair synthesis and ligation, which constitute the later steps of NER. The replicative polymerases Pol δ and Pol ε are responsible for NER repair synthesis and require the processivity factor PCNA, although evidence has shown that Pol κ may also play a role (de Laat *et al.*, 1999, Ogi *et al.*, 2010). The nick sealing step that allows the ligation of the 5'end of the new strand to the original DNA is conducted by LIG1 (de Laat *et al.*, 1999).

1.3.3.4 Mismatch Repair

The MMR pathway functions during S phase to detect and repair mismatched base pairs that arise during DNA replication. Mismatches during replication include base mis-pairing that arise from errors of DNA polymerases as well as insertion/deletion (ID) mis-pairs that occur when primer and template strands re-anneal incorrectly; these are present in microsatellite sequences (Kunkel, 1993). The MMR pathway occurs at replication fork sites themselves and is, therefore, different to BER and NER in that MMR cannot repair damage before replication (Helleday *et al.*, 2008). Whilst defects in MMR can lead to an increase in the spontaneous mutation rate and can result in resistance to some anti-cancer agents, the MMR process itself can mediate the toxicity of some compounds.

In humans, the MutS ATPase complexes recognise mismatched DNA. Two such complexes exist: MutSα, which is a heterodimer of MSH2 and MSH6, and MutSβ, a heterodimer consisting of MSH2 and MSH3. MutSα is responsible for recognising mismatched base pairs and very small ID mispairs that are 1 or 2 nucleotides long, whereas longer ID mis-pairs are sensed by MutSβ (Kunkel and Erie, 2005). The binding of one of the MutS complexes to the mismatch leads to the recruitment of a second heterodimer, MutLα, a complex of MLH1 and PMS2 that possesses ATPase activity and facilitates mismatch recognition (Figure 1.7) (Jiricny, 2006). Excision has a bi-directional capacity that is dependent on the presence of a 'nick' or incision in the daughter strand; this can be located 3' or 5' to the mis-paired base (Modrich, 1997). This allows the excision of the area between the nick and the mispair to occur in a strand-specific manner to direct repair to the new strand. EXO-1 mediates the hydrolysis of the aforementioned DNA and requires the presence of MutSα/MutSβ and RPA to conduct 5' nick-directed excision, whereas the concerted activities of MutLα, PCNA and RCF are needed for EXO-1 to be able to take part in 3' nick-directed excision (Genschel and Modrich, 2003).

DNA re-synthesis and ligation occur once the mismatch has been removed and this process is catalysed by replicative polymerases such as DNA polymerase δ (Pol δ), which requires the processivity factor, PCNA and LIG1 respectively. It is clear that PCNA does not only function at the re-synthesis step of MMR: it is also thought to play a role in the first steps of MMR since it is able to interact with both MutS complexes (Umar *et al.*, 1996). Another protein that appears to act throughout MMR is RPA since it not only protects the ssDNA that is generated during excision, it also binds to 'nicked' DNA before MutS and MutLα (Guo *et al.*, 2006).

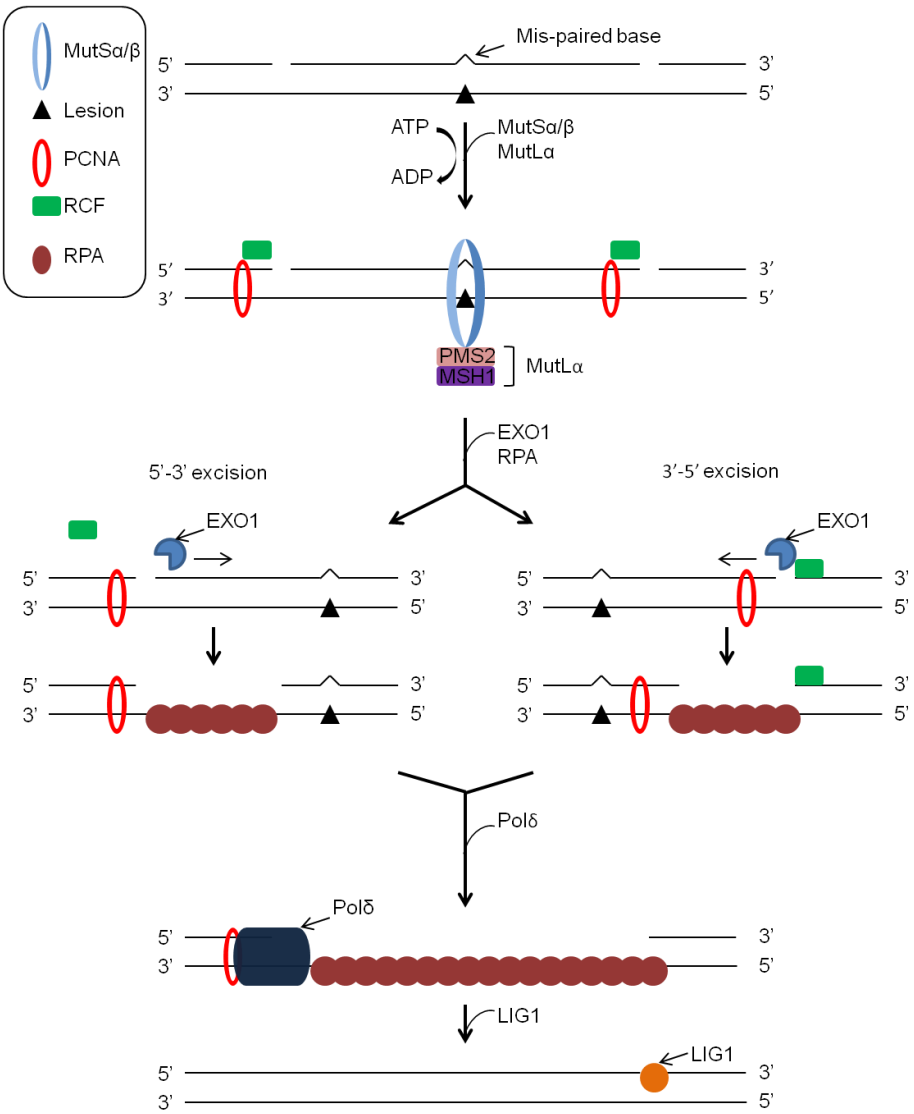

Figure 1.7 Mismatch Repair
The mis-paired base or nucleotides are recognised by MutSα or MutSβ respectively. MutS binding recruits MutLα, leading to an ATP-dependent conformational change to allow excision to occur. The daughter strand is distinguished by nicks and MMR can be directed from a nick 5' or 3' of the mismatch. EXO-1 mediates the excision of the mismatch in a 5' directed manner, whereas it requires PCNA and RCF to conduct 3' directed excision (adapted from Jiricny, 2006, Li, 2008).

1.3.4 DNA damage tolerance and repair of ICLs

1.3.4.1 Translesion synthesis

Mechanisms must exist to overcome lesions such as unrepaired breaks that could block the replication machinery and lead to the stalling of replication forks. Cells have developed tolerance pathways to respond to this potentially catastrophic damage during replication to either avoid the lesion or to replicate past it. One of these damage tolerance mechanisms is known as translesion DNA synthesis (TLS), where non-replicative DNA polymerases are used to bypass damaged bases during replication (Friedberg, 2005). The TLS polymerases include several from the Y family of polymerases: Rev1, Pol κ, Pol η, Pol ι; and a member of the B family of polymerase, Pol ζ. They all have different substrate specificities, which enable them to mediate replication past a variety of base lesions (Lehmann, 2006).

The TLS polymerases can accommodate damaged bases within their active sites, unlike the more stringent replicative polymerases (Lehmann, 2005). The homotrimeric processivity factor, PCNA, acts as a platform for the TLS polymerases after damage through its mono-ubiquitylation by Rad6/Rad18 (Hoege *et al.*, 2002). The mono-ubiquitylation of PCNA occurs after damage such as UV or other agents that stall replication forks and this post-translational modification allows PCNA to preferentially bind TLS polymerases over replicative polymerases since the former possess ubiquitin-binding motifs. It has been proposed that when a fork stalls, the mono-ubiquitylation of PCNA increases its affinity for TLS polymerases, thereby enabling efficient displacement of replicative polymerases (Lehmann, 2007). However, there is also evidence to suggest that ATR-induced phosphorylation of Pol η plays a role in TLS, therefore hinting at another level of regulation of this process (Gohler *et*

al., 2011). Once the TLS polymerases are recruited to the stalled replication fork by mono-ubiquitylated PCNA, they are able to insert nucleotides across the DNA lesion and extend the DNA strand. DNA synthesis across replication-blocking lesions can result in the generation of mutations since many TLS polymerases are characterised by low fidelity. Therefore, mono-ubiquitylation of PCNA is tightly regulated to ensure that the lesion-bypass activity is controlled (Avkin *et al.*, 2006).

1.3.4.2 Interstrand Crosslink Repair

Lesions that result in interstrand crosslinks (ICLs) cause two replication forks to converge at the crosslink; therefore both strands of the DNA helix are affected. The generation of an ICL activates the Fanconi anaemia (FA) pathway and involves the coordination of different repair pathways such as TLS, NER and HR.

Core FA proteins, FANCM and FAAP24, recognise blocked replication forks and bind to ssDNA regions to recruit the core FA complex to sites of damage (Ciccia *et al.*, 2007, Kim *et al.*, 2008). This core complex consists of 12 FA proteins (FANCA, B, C, E, F, G, L, M, FAAP24, FAAP20, MHF1 and MHF2) to form an E3 ubiquitin ligase and, once associated with a stalled replication fork, this ligase ubiquitylates the FANCD2-FANCI complex (Garcia-Higuera *et al.*, 2001, Smogorzewska *et al.*, 2007). Mono-ubiquitylation of FANCI-FANCD2 is required for its retention at sites of crosslinks as well as for the recruitment of further nucleases and polymerases that promote ICL repair.

Recently, Fanconi-associated nuclease 1 (FAN1) was shown to interact with mono-ubiquitylated FANCD2 and to excise DNA that is adjacent to a stalled replication fork (Kratz *et al.*, 2010, MacKay *et al.*, 2010). This type of incision is termed ‘unhooking’ and can also

be conducted by Mus81-Eme1 and the NER endonuclease, ERCC1-XPF (Kuraoka *et al.*, 2000, Niedernhofer *et al.*, 2004). After unhooking, the nascent strand is extended past the lesion by TLS polymerases, which are recruited to mono-ubiquitylated PCNA (Moldovan and D'Andrea, 2009).

Proteins involved in HR are recruited at later stages in ICL repair to repair DSBs that are generated during this process. One example is FANCD1 (BRCA2), which is essential for HR as described in 1.3.1.1 (Wang *et al.*, 2004). Another downstream FA protein is FANCJ (also known as BRIP1 or BACH1), which can unwind D-loop structures and is, therefore, involved in Rad51 resolution in HR (Cantor *et al.*, 2001). FANCN (or PALB2), a BRCA2 interaction partner, also functions to promote HR downstream of the FA pathway (Xia *et al.*, 2006). Once the lesion has been repaired and the cells commence cycling, the FANCD2-FANCI complex becomes de-ubiquitylated by the USP1/UAF1 complex (Cohn *et al.*, 2007, Nijman *et al.*, 2005).

1.3.5 Repair Syndromes

The crucial role of the DDR is highlighted by the fact that genes encoding many of the proteins that are involved in these pathways are mutated in a variety of cancers and in a number of hereditary disorders. These are generally autosomal recessive genetic syndromes and can be a result of both SSBR and DSBR pathway defects.

1.3.5.1 Ataxia-Telangiectasia

Classical Ataxia-Telangiectasia (A-T) is caused by a loss of the ATM protein that generally arises from two truncating mutations in the *ATM* gene (Gilad *et al.*, 1996). However, milder forms can be caused by missense mutations of splice site mutations (Stewart *et al.*, 2001,

Teraoka *et al.*, 1999). The clinical features of A-T include: cerebellar ataxia, cell-mediated and humoral immunodeficiency, oculocutaneous telangiectasia (dilated blood vessels), an increased predisposition toward developing tumours, an increased sensitivity to IR and speech impediment (Taylor *et al.*, 1975).

1.3.5.2 Ataxia-Telangiectasia-like disorder

Another recessive neurological disorder with features that are similar to A-T is Ataxia-Telangiectasia-like disorder (ATLD). ATLD is caused by mutations in the *MRE11* gene (Stewart *et al.*, 1999). There are different forms of ATLD, corresponding to the homozygous and heterozygous mutations in *MRE11* that lead to the expression of low-levels of truncated or partially active MRE11 protein respectively (Stewart *et al.*, 1999). Apart from a lack of conjunctival telangiectasia, overt immunodeficiency and a tumour predisposition, the other clinical features of ATLD are difficult to distinguish from A-T patients.

1.3.5.3 Nijmegen breakage syndrome

Nijmegen breakage syndrome (NBS) is caused by mutations in the *NBS1* gene, which is another member of the MRN complex (Varon *et al.*, 1998). This chromosomal instability disorder is characterised by a hypersensitivitiy to IR, microcephaly, intellectual impairment, dysmorphic facial features, growth retardation, immunodeficiency, developmental delays and a susceptibility to lymphoid tumours, particularly lymphoma .This syndrome is also characterised by a defect in the intra-S phase DNA damage checkpoint, which is consistent with the fact that the phosphorylation of NBS1 by ATM plays a role in this branch of the checkpoint.

1.3.5.4 Nijmegan breakage syndrome-like disorder

Mutation in the third component of the MRN complex, the *RAD50* gene, was found to result in a clinical phenotype, termed Nijmegan breakage syndrome-like disorder (NBSLD) (Waltes *et al.*, 2009). The only identified NBSLD patient presented with clinical features, comparable to those of patients with NBS, which include microcephaly, a hypersensitivity to IR and chromosomal instability, although immunoglobulin levels appeared to be normal (Waltes *et al.*, 2009).

1.3.5.5 Seckel syndrome

Mutations in *ATR* are much rarer than mutations in *ATM* since the disruption of the *ATR* gene severely compromises genomic integrity and results in early embryonic lethality in mice (Brown and Baltimore, 2000). However, mutations in the *ATR* gene have been identified in a single family with Seckel syndrome (O'Driscoll *et al.*, 2003). This homozygous mutation results in abnormal splicing of exon 9 of *ATR*, leading to the reduced expression of ATR. The characteristics of Seckel syndrome include proportional dwarfism, microcephaly, a 'bird-headed' facial appearance and mental retardation. Recently, mutations in *ATR* have been reported to occur in an autosomal dominant manner and were reported to be present in a cancer syndrome (Tanaka *et al.*, 2012). Non-ATR Seckel patient cell lines have also been found, indicating that the mutations of other genes can also cause this syndrome. Mutations in the *PCNT* gene that encodes for pericentrin (a structural and centrosomal protein) were found to result in a Seckel-like syndrome (Griffith *et al.*, 2008).

1.3.5.6 Fanconi Anaemia

Fanconi Anaemia (FA) is also a recessive disorder that is very rare and is characterised by the following: a predisposition to both haematological and solid cancers, progressive bone marrow failure, congenital malformations and a hypersensitivity to cross-linking agents. At least 20 proteins have been identified to function in the repair of DNA ICLs, 15 of which have been found to be mutated in FA. Of the known 20 ICL repair proteins, 12 constitute the core E3 ubiquitin ligase complex of the FA pathway, which ubiquitylates the FANCI-FANCD2 complex. The 12 FA genes that form the E3 ubiquitin ligase are: *FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FANCM, FAAP24, FAAP20, MHF1, MHF2,* with the majority of mutations residing in *FANCA* (Moldovan and D'Andrea, 2009). Other FA genes known to be mutated in FA include *FANCJ, FANCN, RAD51C (FANCO), SLX4 (FANCP)* and *FANCD1 (BRCA2)* (Kim *et al.*, 2011).

1.3.5.7 Xeroderma Pigmentosum

Xeroderma Pigmentosum (XP) is caused by mutations in genes that are involved in the repair of UV-induced DNA lesions. XP is an autosomal recessive disorder and was, incidentally, the first DNA repair disorder to be discovered. The phenotype of XP includes a severe sensitivity to sunlight and sun-induced skin abnormalities that can range from extensive freckling to malignant skin neoplasia. The majority of XP patients have defects in NER genes; there are 7 complementation groups that are deficient in NER. These include *XPA, XPB* (also known as *ERCC3*), *XPC, XPD* (also known as *ERCC2*), *XPE* (also known as *DDB2*), XPF (also known as *ERCC4*) and *XPG* (also known as *ERCC5*). Another hereditary photosensitive disorder arises from mutations in *XPV*, which is known as XP variant and encodes DNA Pol η. XP

variant cells exhibit a functional NER pathway but show a delay in post-replication recovery after UV exposure (Lehmann *et al.*, 1975). XPA individuals have the most severe clinical features that develop at an early age and are completely deficient in NER, whereas XPD patients are less sensitive to UV than XPA patients although still more sensitive than cells of other complementation groups (Lehmann, 2001).

1.4 P53

One of the most important proteins that is involved in the complex array of pathways of the DDR is p53, the 'guardian of the genome' (Lane, 1992). p53 is at the centre of these pathways and orchestrates numerous responses that determine cell fate. The fact that more than 50% of sporadic tumours harbour a mutation or deletion in the p53 gene underscores its crucial role in coordinating the cellular response to genotoxic stress.

1.4.1 Structure of p53

Wildtype (WT) p53 contains 393 amino acids and has an apparent molecular weight of 53 kDa. In an active form it exists as a tetramer where each monomer consists of 3 functional domains: the N-terminal domain, a core DNA binding domain (DBD) and a C-terminal tetramerisation domain. The N-terminal region of p53 contains its transactivation domains (TAD) and also comprises a proline-rich domain (Figure 1.8).

The TAD contains 2 subdomains, denoted as TAD1 and TAD2, which are responsible for interacting with components of the basal transcription machinery and also comprise the binding site of mouse double minute protein 2 (MDM2), the main E3 ubiquitin ligase for p53. In addition, the N-terminal TAD contains the binding site for MDMX, another negative

regulator of p53. The proline-rich domain (PRD) within the N-terminal domain contains four copies of a PXXP motif, where P denotes proline and X denotes any amino acid. This motif has been shown to mediate binding of proteins that contain Src-homology-3 (SH3) domains (Yu *et al.*, 1994).

The central DBD allows p53 to bind to its target genes in a sequence-specific manner. The DNA consensus binding site for WT p53 in these target genes was found to consist of two repeated decameric sequences of the following: PuPuPuC(A/T)(A/T)GPyPyPy, where Pu denotes a purine base and Py denotes a pyrimidine (El-Deiry *et al.*, 1992). Most cancer-associated p53 mutations are found within this central conserved domain since such proteins exhibit defective sequence-specific binding, which highlights the importance of p53 transactivation. Unusually for a transcription factor, p53 contains a second DNA binding site; this is located within its C-terminal region. This second binding site allows p53 to bind DNA, not in a sequence-specific, but rather a structure-specific manner where ssDNA, dsDNA and mismatched DNA are all recognised (Javaraman and Prives, 1995, Lee *et al.*, 1995). The non-specific DNA binding domain of p53 has been shown to positively regulate its specific DNA binding activities (McKinney *et al.*, 2004). Therefore, this region undergoes many post-translational modifications that render it able to influence the efficiency of p53 to act as a transcription factor.

1.4.2 Post-translational modifications of p53

While it has a short half life and is maintained at a low level in unstressed cells, p53 must be activated in response to genotoxic stress. Its stability and activation are under tight regulation, mediated, in part, by various post-translational modifications; indeed, both the C- and the N-

terminal regions of p53 are subject to extensive post-translational modifications following DNA damage (Figure 1.8) (Kruse and Gu, 2009a). Many of these covalent modifications can occur on the same residues; therefore, an element of cross-talk exists. This adds a further level of complexity to the regulation of p53 through post-translational modifications and ensures that in the absence of specific residues, other mechanisms are in place to regulate p53 function.

1.4.2.1 Ubiquitylation

The regulation of its protein level (half life) through ubiquitylation is one of the key mechanisms whereby p53 activity is modulated. Upon genotoxic stress, the levels of p53 are greatly elevated and become stabilised, leading to the activation of its transcriptional activity (Brooks and Gu, 2006). It is vital that p53 is maintained at low basal levels to prevent abnormal activity. In unstressed cells, p53 protein levels are largely controlled by two functions of MDM2. Firstly, MDM2 binds to the N-terminal TAD of p53 and blocks the interaction between p53 and members of the transcriptional machinery to inhibit p53-mediated transactivation (Chen *et al.*, 1993, Oliner *et al.*, 1993).

The second function by which p53 stabilisation is regulated by MDM2 is through the latter's E3 ubiquitin ligase activity that targets the C-terminal region of p53 (Haupt *et al.*, 1997). Under stress-free conditions, MDM2-mediated ubiquitylation predominantly occurs within six C-terminal lysine (K) residues: on p53: K370, K372, K373, K381, K382 and K386 (Figure 1.8) (Lohrum *et al.*, 2001). However, a study by Feng *et al.* in 2005 showed that the mutation of these lysine residues did not affect p53 stabilisation. This indicates that additional

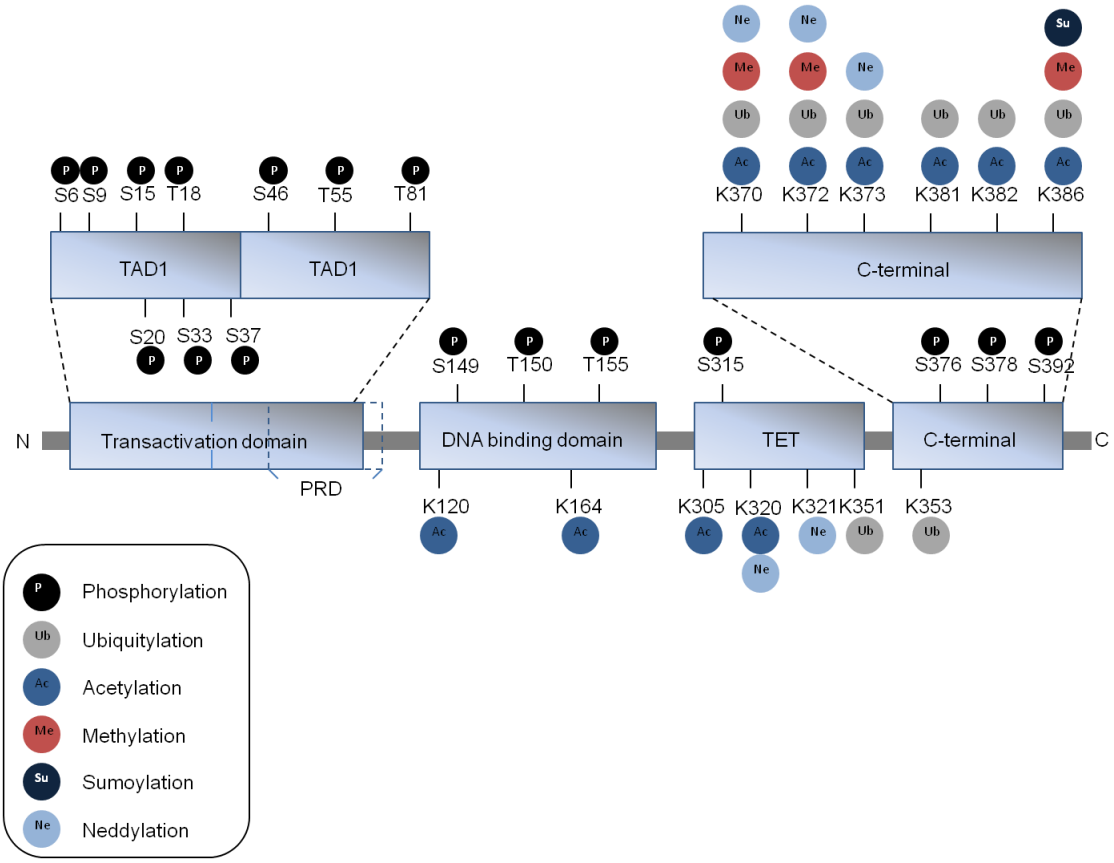

Figure 1.8 p53 domains and post-translational modifications
The main domains of the tumour suppressor protein, p53, comprise the transactivation domain (TAD), the DNA binding domain (DBD), the tetramerisation domain (TET) and the C-terminal domain. Many sites within these domains are modified by different enzymes. Major phosphorylation, acetylation and ubiquitylation sites are shown. In addition, sites for methylation, neddylation and sumoylation are depicted (adapted from Kruse and Gu, 2009a).

lysines within p53 may be targeted by MDM2 or that other E3 ligases exist for p53 (Feng *et al.*, 2005). The discovery of E3 ubiquitin ligases such as ARF-BP1, COP1 and Pirh2 lends support to this idea and indicates that p53 stability can be regulated in an MDM2-independent manner (Chen *et al.*, 2005, Dornan *et al.*, 2004, Leng *et al.*, 2003).

There is evidence to suggest that the MDM2-mediated mono-ubiquitylation of p53 promotes its nuclear export compared to poly-ubiquitylation, which leads to its degradation (Li *et al.*, 2003). While the former leads to the inhibition of p53 transcriptional activity, the accumulation of cytoplasmic p53 may actually benefit transcriptionally-independent functions of p53, including the induction of apoptosis and the inhibition of autophagy. In addition, other E3 ligases have been shown to drive the cytoplasmic localisation of p53, including MSL2, which ubiquitylates p53 at lysine residues, K351 and K357 (Kruse and Gu, 2009b).

The control of MDM2 expression itself is critical to control p53 stability and this process involves numerous proteins. MDM2 is regulated by p53 itself and both proteins function in an autoregulatory feedback loop; therefore, whilst p53 positively regulates *MDM2* gene expression, functional MDM2 protein negatively regulates p53 stability (Wu *et al.*, 1993). One positive regulator of MDM2 is MDMX (also known as MDM4), which exists in a complex with MDM2 to form a dimer. MDMX does not possess E3 ligase activity itself but appears to promote that of MDM2 and, therefore, negatively regulates p53 stability (Okamoto *et al.*, 2009).

Interestingly, another member of the MDM family of proteins actually functions positively towards p53. In addition to the full length MDM2 protein ($p90^{MDM2}$), the *MDM2* gene also codes for a smaller protein, $p76^{MDM2}$, which has been shown not only to inhibit the interaction between MDM2 and MDMX, but also to ubiquitylate MDMX and target it for

proteasomal degradation (Giglio *et al.*, 2010). Another regulator of MDM2 is p14ARF, a tumour suppressor whose levels are increased during genotoxic stress. p14ARF is able to bind to MDM2 and disrupts the interaction between p53 and MDM2, resulting in the stabilisation of p53 upon DNA damage (Sherr, 2006).

It is clear that the ubiquitylation of p53 plays a major role in determining its stability. The process of deubiquitylation has also been shown to regulate the stabilisation of p53, which is mediated by deubiquitylases (DUBs). The ubiquitin-specific protease, HAUSP (also known as USP7), was first shown to stabilise p53 by directly deubiquitylating it. However, it was later found to directly target MDM2 rather than p53 for deubiquitylation (Li *et al.*, 2004, Li *et al.*, 2002a). Brooks *et al.* (2007) proposed that the preferred substrate for HAUSP in unstressed cells is MDM2, whereas under conditions of DNA damage, the binding of HAUSP to MDM2 is reduced to favour p53 stabilisation (Brooks *et al.*, 2007). Another DUB that regulates p53 stability is USP10, which was found to deubiquitylate p53 in the nucleus after genotoxic stress (Yuan *et al.*, 2010).

1.4.2.2 Phosphorylation

A number of stress-activated kinases mediate the phosphorylation of p53 on key residues (Figure 1.8). The main sites that undergo phosphorylation reside in the N-terminal of p53 and include serine residues (S6, S9, S15, S20, S33, S36, S37 and S46) as well as threonine residues (T18, T55 and T81) (Kruse and Gu, 2009a). Those kinases primarily involved in N-terminal phosphorylation include ATM, ATR and DNA-PK, which phosphorylate S15 of p53 (Banin *et al.*, 1998, Canman *et al.*, 1998, Lees-Miller *et al.*, 1992, Tibbetts *et al.*, 1999). The downstream kinases, Chk1 and Chk2, also target N-terminal serine residues, namely S20

(Shieh *et al.*, 2000). The phosphorylation of N-terminal residues of p53 disrupts the p53-MDM2 interaction and, therefore, allows p53 to associate with its co-factors for transactivation of its target genes. While the phosphorylation of S15 and S20 have been shown not to disrupt the binding of MDM2 to p53, the phosphorylation of T18 does reduce this interaction, indicating that the latter residue is important for determining the interaction between MDM2 and p53 (Dumaz and Meek, 1999, Schon *et al.*, 2002). However, the importance of the other N-terminal residues cannot be discounted because of this. The phosphorylation of S15 and S20 stimulates the recruitment of p53 co-activators, p300/CBP, which results in the acetylation of C-terminal lysines that enhance specific DNA binding of p53 (Dumaz and Meek, 1999, Lavin and Guevan, 2006). In addition, S15-phosphorylation is a prerequisite for phosphorylation of T18 and S20 (Saito *et al.*, 2003). This highlights the level of interdependency that exists among the N-terminal phosphorylation sites of p53.

Another N-terminal serine phosphorylation site, S46, has been shown to be phosphorylated by a number of kinases including homeodomain interacting protein kinase 2 (HIPK2), ATM, dual-specificity tyrosine-phosphorylation-regulated kinase (DYRK) and protein kinase C δ (PKC δ) (D'Orazi *et al.*, 2002, Kodama *et al.*, 2010, Taira *et al.*, 2007, Yoshida *et al.*, 2006). This particular residue contributes to the selectivity of p53 activation since its phosphorylation by the specific kinases is linked to the up-regulation of pro-apoptotic target genes.

Phosphorylation is not restricted to the N-terminal region and some serine sites in the DBD (S149, S150 and S155) and some in the C-terminal region (S315, S376, S378 and S392) are also targeted by various kinases. The C-terminal serine sites, S376 and S378, are constitutively phosphorylated by PKC in unstressed cells and the dephosphorylation of S376

after IR causes p53 to have an increased affinity for sequence-specific DNA (Waterman *et al.*, 1998). Therefore, it is clear that phosphorylation of C-terminal residues does not necessarily enhance the DNA binding ability of p53. In contrast, the phosphorylation of the other C-terminal residue, S392, stimulates sequence-specific binding of p53, by promoting its tetramerisation (Sakaguchi *et al.*, 1997). This site is phosphorylated by casein kinase 2 (CK2) in response to genotoxic stress (mainly in the form of UV), although the exact nature of this modification is not fully understood (Cox and Meek, 2010).

If phosphorylation of p53 is important, the process of dephosphorylation cannot be overlooked. Although less is known about the dephosphorylation of p53, a few phosphatases have been found to target certain serine residues within p53. One of these enzymes is dual-specificity phosphatase 26 (DUSP26), which has been shown to dephosphorylate serine residues S20 and S37 and to consequently inhibit p53 activity in response to DNA damage (Shang *et al.*, 2010a). Another p53 phosphatase is protein serine/threonine phosphatase-1 (PP-1), which has been demonstrated to dephosphorylate S37, as well as S15, to negatively regulate pro-apoptotic p53 functions (Li *et al.*, 2006). WT p53-induced phosphatase 1 (WIP1 or PPM1D) controls p53 levels by dephosphorylating MDM2 at S395 and thus stabilising this negative regulator of p53 (Lu *et al.*, 2007).

1.4.2.3 Acetylation

The acetylation of histones is a well accepted mechanism of regulating transcription but p53 was the first non-histone protein that was shown to undergo acetylation and deacetlyation (Gu and Roeder, 1997). The levels of acetylated p53 increase upon damage, which is consistent with an increase in p53 activation in response to stress. The acetylation of p53

comprises an extremely important element in the regulation its activity and is mediated by a number of histone acetyltransferase (HAT) enzymes, the main ones being its transcriptional co-activators, p300/CREB-binding protein (CBP) (Gu and Roeder, 1997, Lill *et al.*, 1997). p300, together with its family member, CBP, predominantly target the cluster of six C-terminal lysine residues, K370, K372, K373, K381, K382 and K386 (Kruse and Gu, 2009a). Although these are the main sites of acetylation, two lysine residues in the DBD are also targeted, which imparts a level of redundancy to the activation of target genes since only the mutation of all 8 lysine sites abolishes the ability of p53 to activate the *p21* promoter (Figure 1.8) (Tang *et al.*, 2008). The sites in the DBD include K120 and K164, which are acetylated by another histone acetyltransferase, Tip60/hMof, and p300/CBP respectively (Sykes *et al.*, 2006, Tang *et al.*, 2006, Tang *et al.*, 2008). Tip60/hMof-mediated acetylation of K120 is enhanced after genotoxic stress; mutation of this site disrupts p53-mediated pro-apoptotic functions but does not have a significant effect on cell cycle arrest (Sykes *et al.*, 2006, Tang *et al.*, 2006). This indicates that K120 acetylation may provide a mechanism by which p53 can preferentially activate a subset of its target genes. Another histone acetyltransferase, PCAF, also acetylates p53 following DNA damage, at a site distant to the cluster of six C-terminal lysines, on the lysine residue, K320, which resides in the tetramerisation domain (Figure 1.8) (Liu *et al.*, 1999). Another lysine residue targeted by acetyltransferases that is distinct from the C-terminal lysines is K305, which is modified by p300 (Wang *et al.*, 2003).

The interplay between the different post-translational modifications of p53 is again evident, this time regarding acetylation and ubiquitylation. Acetylation in the C-terminal region of p53 has been shown to inhibit MDM2 ubiquitylation of these residues and, therefore, increases the

stability of p53 (Li *et al.*, 2002b). This is unsurprising considering the C-terminal lysines can be targeted by both acetyltransferases and E3 ubiquitin ligases.

Like many post-translational modifications, acetylation is reversible. Consequently, up-regulation of activated p53 can be reversed by deacetylation, which is catalysed by histone deacetylases (HDACs) (Luo *et al.*, 2001, Luo *et al.*, 2000, Vaziri *et al.*, 2001).

1.4.2.4 Methylation

p53 is one of the few transcription factors that is regulated by protein methylation and demethylation. Lysine methyltransferases target certain C-terminal lysine residues of p53 and there exists an element of crosstalk between methylation of these residues and other post-translational modifications of p53 (Figure 1.8). The transfer of a methyl group onto lysine residue, K372 by the lysine methyltransferase, Set7/9, was shown to positively regulate p53 stability and to promote its activity (Chuikov *et al.*, 2004). The relevance of Set7/9-mediated methylation is underscored by the fact that it is required for p53 acetylation and that Set7/9 is involved in p53-mediated G2/M arrest following DNA damage (Ivanov *et al.*, 2007). In contrast, the methylation of K370 by Smyd2 lysine methyltransferase represses p53 activity and, interestingly, methylation at this site is inhibited by Set7/9-mediated methylation of K372 (Huang *et al.*, 2006). The third lysine methyltransferase, Set8, catalyses the mono-methylation of the K382 residue; this enzyme acts in a similar manner to Smyd2 and represses p53-mediated transactivation (Shi *et al.*, 2007). Interestingly, the depletion of Set8 leads to an increase in p53 pro-apoptotic and checkpoint functions, which is consistent with the observation that methylation at K382 prevents p53 binding to promoters of the *PUMA* and *p21* genes respectively (Shi *et al.*, 2007).

The lysine residue, K370, is also subject to di-methylation although the enzyme that mediates this is, as yet, unknown (Huang *et al.*, 2006). Lysine methylation is also subject to its reversible process, demethylation, which, in p53, is catalysed by the demethylase, LSD1; however, LSD1 only demethylates K370 if it has been di-methylated (Huang *et al.*, 2006).

Arginine methyltransferases also play a role in the regulation of p53; this is achieved by the activity of protein arginine methyltransferase 5 (PRMT5) (Jansson *et al.*, 2008). The majority of arginine residues in p53 reside within its DBD. Indeed, common 'contact site' hotspot mutations (i.e. those that affect the interaction between p53 and DNA) include arginine residues, R248 and R278 in the central DBD of p53 (Harms and Chen, 2006).

1.4.2.5 Sumoylation and Neddylation

The C-terminal sites of p53 are also subject to other post-translational modifications such as sumoylation and neddylation. The sumoylation of p53 occurs at the C-terminal lysine residue, K386 (Figure 1.8) (Wu and Chiang, 2009). Less than 5% of p53 is sumoylated *in vivo* and as such, it is difficult to assess the relevance of SUMO-conjugated p53 (Melchior and Hengst, 2002). Indeed, there have been conflicting reports on the effect that sumoylation has on p53 activity. While some results show that the sumoylation of p53 enhances its transcriptional activity, others report that this modification by SUMO-1 inhibits p53 function (Rodriguez *et al.*, 1999, Wu and Chiang, 2009).

The mechanism of neddylation of p53 is also unclear. Both FBXO11 and MDM2 have been shown to mediate the conjugation of Nedd8 to p53 on C-terminal lysine residues, K320/K321 and K370/K372 respectively (Figure 1.8) (Abida *et al.*, 2007, Xirodimas *et al.*, 2004). Interestingly, modification by both proteins inhibits the transcriptional activity of p53.

1.4.3 Co-factors of p53

A number of co-factors, comprising co-activators and co-repressors, are known to influence p53 activity without post-translationally modifying it and some of these co-factors play a role in the promoter-specific activity of p53 (Figure 1.9, Table 1.1). The main co-factors of p53 have already been mentioned: MDM2, MDMX and p300/CBP. These all bind directly to p53 to influence its stability and transcriptional activity. Conversely, indirect co-factors, such as stress-responsive activator of p300 (Strap), can act by affecting the recruitment of other p53 co-factors (Demonacos *et al.*, 2001). Interestingly, a number of p53 co-activators are themselves tumour suppressors since their loss can increase the onset of tumourigenesis. Conversely, co-repressors could be classed as oncogenes because they inhibit the tumour suppressive function of p53.

1.4.3.1 Co-factors that affect cell cycle arrest gene expression

Recently, a loss-of-function screen identified bromodomain-containing 7 (BRD7) as a p53-interacting protein that is required for the transcriptional activation of a subset of cell cycle genes, including *p21*, but not pro-apoptotic genes (Drost *et al.*, 2010). BRD7 is an example of a co-factor that can also indirectly influence p53 since BRD7 is able to interact with p300 and enhance p300-mediated acetylation of p53 as well as that of the histones surrounding specific target promoters (Drost *et al.*, 2010). This correlates with the fact that p300-mediated acetylation is crucial for the activation of p21.

Another indirect p53 co-factor, Strap, facilitates the recruitment of the p53 co-activator p300 to enhance p53 activation and also indirectly inhibits MDM2-mediated degradation of p53 (Demonacos *et al.*, 2001).

BRCA1 is also a p53 co-factor and is itself a tumour suppressor. By interacting directly with the C-terminus of p53 it drives p53-mediated transcription (Ouchi *et al.*, 1998, Zhang *et al.*, 1998). BRCA1 is another co-factor that promotes the selective expression of cell cycle arrest genes over pro-apoptotic genes (MacLachlan *et al.*, 2002). In addition, BRCA1, in conjunction with its binding partner BARD1, is thought to act as an adaptor for p53 to enable ATM/ATR-mediated phosphorylation of serine residue S15 of p53 (Fabbro *et al.*, 2004). The phosphorylation of this residue of p53 is known to be required for the recruitment of the tumour suppressor's acetyltransferases (Dumaz and Meek, 1999).

The haematopoietic zinc finger protein, Hzf, is a p53-responsive gene and selectively promotes the p53-dependent expression of cell cycle arrest genes (Das *et al.*, 2007). Similarly, the membrane glycoprotein, MUC1, is also able to associate with p53 and act as a co-activator to induce its transcription of the *p21* gene (Figure 1.9, Table 1.1) (Wei *et al.*, 2005).

1.4.3.2 Co-factors that affect apoptotic gene expression

The apoptosis-stimulating proteins of p53 (ASPP) family are ankyrin-repeat-SH3-domain and proline-rich-region-containing proteins and are important co-factors that regulate p53 activity, specifically its apoptotic functions (Samuels-Lev *et al.*, 2001). This family includes two pro-apoptotic co-factors, ASPP1 and ASPP2, and an anti-apoptotic co-factor, iASPP. The latter functions as a p53 co-repressor and interacts with the PRD and DBD of p53 to prevent it from binding to pro-apoptotic promoters (Bergamaschi *et al.*, 2003). In contrast,

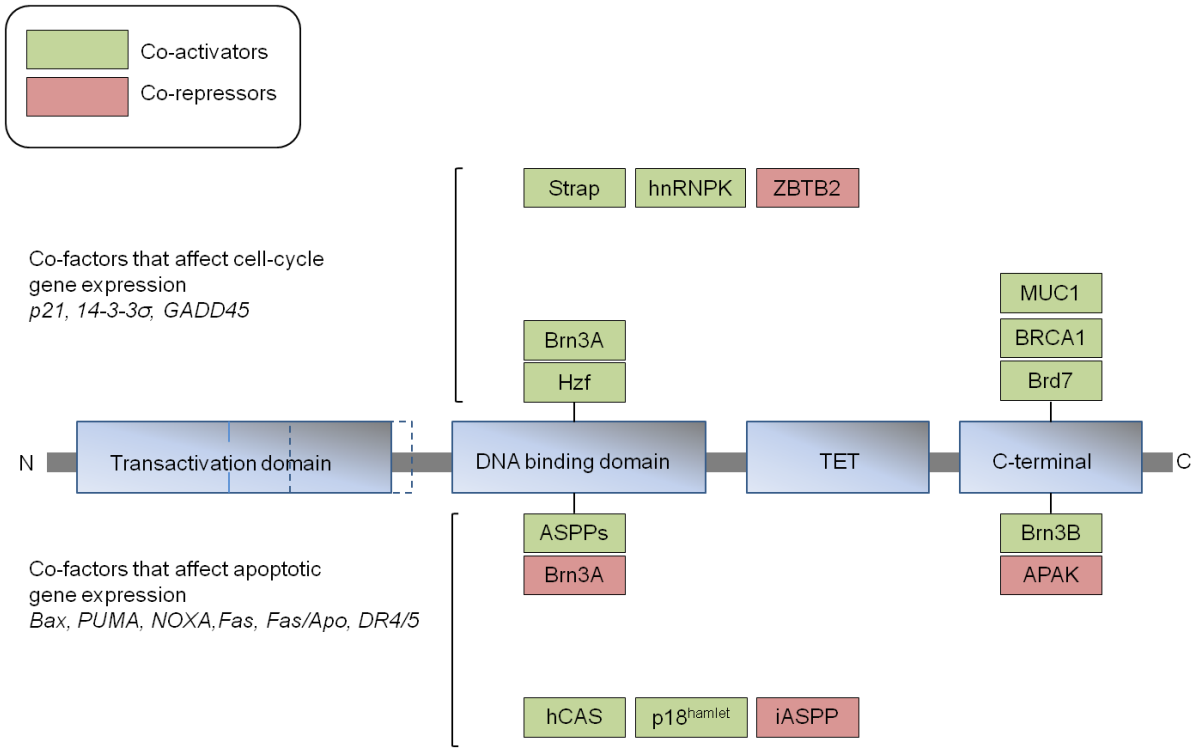

Figure 1.9 p53 co-factors exert selective influences on p53 responses
Co-factors can influence p53's ability to activate or repress its target genes in a non-covalent manner. Some co-factors can bind directly to p53 to promote different p53-dependent cellular responses. These direct co-factors interact with the p53 DBD or C-terminal domain as shown. Indirect co-factors that facilitate either p53-dependent cell cycle arrest or apoptosis are also shown. Co-activators are depicted in red, whereas co-repressors are depicted in red (adapted from Vousden and Prives, 2009).

ASPP1 and ASPP2 selectively promote p53 binding to pro-apoptotic promoter regions by mediating the dissociation of iASPP (Samuels-Lev *et al.*, 2001). However, there are conflicting reports regarding the function of cytoplasmic ASPP1 since it has also been shown to inhibit apoptosis, thus highlighting an oncogenic role of cytoplasmic ASPP1 compared to the tumour suppressive properties of nuclear ASPP1 (Vigneron *et al.*, 2010). Recently however, new findings have suggested additional roles of cytoplasmic ASPP1 in which it increases the activity of YES-associated protein (YAP) to indirectly inhibit the ability of p53 to stimulate expression of p21 (Vigneron and Vousden, 2011). This indicates that the subcellular localisation of these proteins is also important for their function.

The ATM and p53-associated KRAB-type zinc-finger (KZNF) protein, APAK, was identified as a co-repressor of p53 whereby it binds to p53 in unstressed cells and regulates the modification of p53 to enable selective repression of pro-apoptotic genes (Tian *et al.*, 2009). Recently, additional mechanisms of APAK have come to light where it has been shown that it selectively binds to the intron of the p53 pro-apoptotic target, p53AIP1, which also happens to overlap the p53 binding site within this gene (Yuan *et al.*, 2012). Therefore, by blocking p53 binding to the *p53AIP1* gene, APAK prevents p53AIP1 transcription and inhibits p53-mediated apoptosis.

Another co-factor that can determine a different cellular outcome through promoter-selective transactivation of p53 is human cellular apoptosis susceptibility protein (hCAS). The down-regulation of hCAS results in the significant reduction of p53 induction of pro-apoptotic genes such as *PIG3* and *p53AIP1*, whereas little effect was observed for *p21* (Tanaka *et al.*, 2007). This implicates hCAS as being able to predispose p53 toward its apoptotic function rather than mediating its cell cycle arrest effects.

The p38 mitogen-activated protein kinase (MAPK) family constitute an important signalling pathway in response to cellular stress and can interact with a number of proteins. One of these interacting proteins, $p18^{Hamlet}$ was found to bind to p53 upon DNA damage and activate the p53 pro-apoptotic genes, *NOXA* and *PUMA* (Figure 1.9, Table 1.1) (Cuadrado *et al*., 2007).

1.4.3.3 Other p53 co-factors

Brn3A is an unusual p53 co-factor in that it acts to stimulate and repress p53-mediated responses. It has been shown that Brn3A represses the apoptotic function of p53 by inhibiting its ability to activate pro-apoptotic promoters of genes such as *Bax* and *NOXA* (Table 1.1) (Budhram-Mahadeo *et al.*, 2002, Hudson *et al.*, 2005). Conversely, co-expression of p53 and Brn3A has a stimulatory effect on the p21 promoter, thus indicating that Brn3A can activate the cell cycle effects of p53 (Budhram-Mahadeo *et al.*, 2002). Therefore, it seems that Brn3A can co-operate with p53 to determine the fate of those cells that co-express both proteins.

The Brn3B transcription factor, although related to Brn3A, displays strikingly different cellular properties. While Brn3A antagonises Bax expression and positively regulates p21 expression, Brn3B interacts with p53 and stimulates the expression of Bax but not p21, therefore functioning in the opposite manner to Brn3A (Budhram-Mahadeo *et al.*, 2006).

Ribosomal proteins also play a role in regulating p53, mainly by functioning in the MDM2-p53 feedback loop. The ribosomal proteins L5, L23 and L11 have all been found to inhibit MDM2 and prevent its ubiquitylation of p53 (Dai and Lu, 2004, Jin *et al.*, 2004, Lohrum *et al.*, 2003).These proteins are therefore co-activators of p53.

In addition, a member of the heterogeneous nuclear ribonucleoprotein (hnRNP) family has been shown to function as a p53 transcriptional co-activator. Heterogeneous nuclear

ribonucleoprotein K (hnRNPK) was found to interact with p53 before and after DNA damage and is recruited along with p53 to p53-responsive promoters of *p21* and *MDM2* (Moumen *et al.*, 2005). Since the effect of hnRNPK on apoptotic genes has not been investigated, it is unclear whether this co-activator functions to confer specificity to the p53 response to genotoxic stress.

Through over-expression studies, Barral *et al.* (2005) revealed that increasing exogenous levels of another hnRNP member, hnRNPUL-1, results in the repression of p53-dependent transcriptional activity although the exact mechanism by which this occurs remains unknown (Barral *et al.*, 2005).

1.4.4 Additional factors that promote specific activation of target genes

The responses that are elicited upon p53 activation can be divided into those that result in the survival of the cell through the inhibition of its proliferation and subsequent repair of damage, and those that lead to cell death. These outcomes contribute to the role of p53 as a tumour suppressor since the onset of oncogenicity would be halted as a result of both responses.

There are numerous mechanisms that exist to promote the specific transactivation of certain p53-regulated genes, many of which have already been discussed. The abundance of p53, as well as its various post-translational modifications and the plethora of co-factors, determine whether a given p53-dependent promoter is activated or repressed.

An additional factor that contributes to the specificity of the p53 response is the core promoter structure of actual p53 target genes. Target promoters display structural differences such that higher levels of elements of the basal transcriptional machinery are found at promoters of cell cycle arrest genes such as *p21* compared to those promoters belonging to

apoptotic targets (Espinosa *et al.*, 2003). This is further supported by the observation that there is a rapid formation of pre-initiation complexes (PIC) at the *p21* promoter, which is critical for gene activation; however, re-initiation is intrinsically inefficient at this particular promoter (Morachis *et al.*, 2010). In contrast, the *Fas* promoter undergoes slow PIC formation but once trancriptionally engaged, becomes very efficient at re-initation (Morachis *et al.*, 2010). This phenomenon is consistent with the observations that cell cycle arrest genes are activated more rapidly than apoptotic genes and confirms that timing of the regulation of p53 is critical during the course of the stress response. The latter fact also lends support to the concept that promoters of cell cycle arrest genes contain high-affinity sites for p53 compared to the promoters of apoptotic genes, which contain lower-affinity binding sites (Chen *et al.*, 1996). However, this is not always the case since p53 can bind to the promoter of *PUMA* with a similar binding affinity to that of *p21* and *MDM2* (Vousden and Lu, 2002).

1.4.5 Functions of p53

p53 functions to efficiently mediate cell cycle arrest and to promote apoptotic cell death or senescence. The ability of p53 to act as a transcription factor allows it to bind to specific regions in the promoters of p53-responsive genes to mediate the response to genotoxic stress. Principal p53-responsive genes include those involved in inhibiting cell cycle progression and also a subset of apoptotic genes (Table 1.1). These properties define it as a tumour suppressor.

1.4.5.1 Cell cycle arrest

Cell cycle progression is regulated by the cell cycle checkpoints at G1, S and G2/M, which become activated upon DNA damage as described in 1.2.4. The activation of p53-induced cell cycle checkpoints after genotoxic stress preferentially promotes cell survival rather than cell

death; therefore many genes that become activated to co-ordinate cell cycle arrest also function to inhibit apoptosis (Table 1.1) (Vousden and Prives, 2009).

The primary regulator of p53-mediated G1 arrest in response to DNA damage is $p21^{WAF1/CIP1}$, a CKI and a p53 target gene (El-Deiry *et al.*, 1993). p21 primarily inhibits the kinase activity of CDK2 and CDK4, thus disrupting the CDK2/cyclin E and CDK4/cyclin D1 complexes, respectively (Sherr and Roberts, 1995). This inhibits both Rb phosphorylation and the consequent activation of E2F, which would otherwise mediate the expression of proliferative genes required for progression through G1 (Delavaine and La Thangue, 1999, Dimri *et al.*, 1996). In addition, p21 possesses anti-apoptotic effects, which allows the cell to repair or remove any damage without the death of the cell (Dotto, 2000). However, this additional function of p21 may also be viewed as oncogenic rather than tumour suppressive if tumour cells are allowed to resume proliferation rather than being eliminated (Roninson, 2002).

Although there are reports that a p53 isoform, Δp53, is involved in the transactivation of p21 during the ATR-mediated intra-S checkpoint, there appears to be no role for full length p53 (Rohaly *et al.*, 2005). During this phase of the cell cycle, however, p53 primarily mediates DNA repair, which is vital to protect the integrity of the genome at a stage when the cell is particularly vulnerable (Helton and Chen, 2007).

The product of the 14-3-3σ gene, which is induced by p53 following cellular stress, sequesters CDK1/cyclin B1 complexes away from their substrates in the nucleus into the cytoplasm (Hermeking and Benzinger, 2006). Since the inhibition of the progression of cells through G2 is dependent on the disruption of the CDK1/cyclin B1 complex, 14-3-3σ is involved in p53-mediated cell cycle arrest in G2 and is transactivated by p53 in response to DNA damage (Hermeking *et al.*, 1997). Another gene that is involved in controlling p53-mediated G2/M

arrest is GADD45, which is induced by p53 after exposure to IR (Kastan *et al.*, 1992). In a manner similar to 14-3-3σ, GADD45 has also been shown to negatively regulate the levels of nuclear cyclin B1, therefore inhibiting cellular progression through the G2 phase of the cycle (Jin *et al.*, 2002).

1.4.5.2 Apoptosis

Programmed cell death, or apoptosis, is an essential process for multi-cellular organisms and must be stringently regulated. Apoptosis is activated in response to a variety of signals through two major pathways; the extrinsic pathway and the intrinsic pathway. p53 participates in both of these pathways in response to high levels of DNA damage that cannot be repaired. Therefore, the extent of DNA damage affects the p53 response and the subsequent activation of apoptotic genes (Table 1.1). Interestingly, it is not only the transcriptional function of p53 that plays a role in promoting apoptosis; it may also mediate this process in a direct manner (Mihara *et al.*, 2003).

1.4.5.2.1 Role of p53 in the extrinsic apoptotic pathway

The extrinsic pathway or death receptor pathway is so named because it is stimulated by binding of death ligands to cell surface death receptors (Jin and El-Deiry, 2005). This causes receptor oligomerisation and the consequent recruitment of the adaptor molecule, Fas-associated death domain (FADD), which when bound to the death domain of the activated receptor, forms the death-inducing signalling complex (DISC). Procaspase 8 is sequestered to the DISC where, upon its autocatalytic activation, caspase 8 is released, resulting in the processing of downstream effector caspases, caspase 3, 6 and 7. These effector caspases

DNA damage	Function	Genes	Co-activators	Co-repressors
	Cell cycle arrest	*p21* *14-3-3σ* *GADD45*	p300/CBP BRD7 Hzf Brn3A MUC1 Strap hnRNPK	ZBTB2
	DNA repair	*XPC* *MSH2* *MLH1* *PCNA* *PMS2*		
	Apoptosis	*Bax* *PUMA* *NOXA* *Fas/APO-1* *DR4/5*	ASPP1/2 hCAS Brn3B P18^{hamlet}	Brn3A iASPP APAK

Table 1.1 p53-activated genes and p53 co-factors
At low levels of DNA damage, p53 promotes cell survival by activating cell cycle arrest and DNA repair. At high levels of damage, p53 promotes apoptosis (adapted from Helton and Chen, 2007).

cleave specific substrates to induce cell death. The extrinsic apoptotic pathway can be promoted in a p53-dependent manner since p53 has been found to transactivate genes that code for some of the death receptors. These include death receptor 4 (DR4) and death receptor 5 (DR5 or KILLER) (Liu *et al.*, 2004, Takimoto and El-Deiry, 2000). p53 also upregulates the expression of the Fas death receptor and, interestingly, also increases the expression of its death ligand, Fas ligand/APO-1 (Owen-Schaub *et al.*, 1995).

1.4.5.2.2 Role of p53 in the intrinsic apoptotic pathway

The intrinsic apoptotic pathway is also known as the mitochondrial pathway and can be activated by intracellular stresses, such as growth factor withdrawal, direct DNA damage and hypoxia. In response to these stress signals, pro-apoptotic Bcl-2 family members disrupt the mitochondrial membrane, resulting in the release of apoptogenic factors such as cytochrome *c*. The latter, together with apoptosis protease-activating factor 1 (Apaf-1) and procaspase 9, form the apoptosome. This is an intracellular multi-protein DISC-like complex and its formation leads to the activation of caspase 9, which consequently cleaves and activates the effector caspase, caspase 3.

p53 also induces the expression of the BH3-only Bcl-2 family members and therefore activates the intrinsic apoptotic pathway in a transcription-dependent manner. The BH3-only Bcl-2 family members promote apoptosis by binding to and displacing pro-apoptotic Bcl-2-like proteins such as Bax and Bak from inhibitory complexes with pro-survival proteins like Bcl-2 and Bcl-xL (Jin and El-Deiry, 2005). One such pro-apoptotic BH3-only protein is p53 upregulated modulator of apoptosis or Bcl-2 binding component 3 (PUMA or bbc3) and is a direct transcriptional target of p53(Nakano and Vousden, 2001). Similarly, *NOXA* encodes another BH3-only pro-apoptotic protein and is also a p53 target gene (Oda *et al.*, 2000). Upon

their activation, these BH3-only proteins allow the other pro-apoptotic Bcl-2 family members to permeabilise the outer mitochondrial membrane and to trigger the onset of the caspase cascade, leading to mitochondrial apoptosis.

p53 also functions in a transcriptional-independent manner in the mitochondrial apoptotic pathway. It is known that a significant amount of p53 is translocated to the mitochondria upon DNA damage where it can initiate apoptosis without the transactivation of pro-apoptotic target genes (Mihara *et al.*, 2003). It has been shown that regulatory factors can aid p53 localisation to the mitochondria to trigger apoptosis independent of its role as a transcription factor. One such 'chaperone' protein is Tid1, which interacts with p53 in response to hypoxic stress and mediates its translocation to the mitochondria (Ahn *et al.*, 2010). Once p53 is retained at the mitochondria, it is able to directly activate Bax and Bak, allowing the release of cytochrome *c* (Chipuk *et al.*, 2004, Leu *et al.*, 2004). In addition, p53 is able to directly interact with pro-survival proteins, Bcl-2 and Bcl-xL, which leads to the release of pro-apoptotic proteins (Mihara *et al.*, 2003).

1.4.5.3 DNA repair

The onset of DNA repair is critical for the completion of the cell cycle; therefore, p53 plays a vital role in modulating DNA repair processes in response to a plethora of genotoxic insults. Many p53 target genes participate in DNA repair pathways but p53 is also directly involved in these processes via transcriptionally-independent mechanisms.

The NER gene, *XPC*, is expressed following p53 activation after DNA damage, indicating a transcriptional role for p53 in the regulation of NER (Adimoolam and Ford, 2002). However, there are also reports that p53 can regulate NER via protein-protein interactions such as direct

binding to the helicases, XPB and XPD (Wang *et al.*, 1995). Therefore, p53 has both a transcriptional and a direct impact on the process of NER.

Another example of the direct involvement of p53 in repair processes can be seen in its ability to interact with AP endonuclease and Pol β, both of which are vital to BER pathway (Zhou *et al.*, 2001). Several components of the MMR pathway are p53-regulated genes, including the crucial *MSH2* gene, which forms the core of two complexes involved in the recognition of mismatches (Warnick *et al.*, 2001). In addition, *MLH1* and *PMS2* have also been shown to be upregulated by p53 transactivation (Chen and Sadowski, 2005).

In addition to its role in replication, the processivity factor, PCNA, participates in many repair processes. p53 binds to the promoter of PCNA and modulates its expression; interestingly, this depends on the concentration of p53 and occurs in a damage-dependent manner (Table 1.1) (Morris *et al.*, 1996, Xu and Morris, 1999).

1.5 HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN U-LIKE-1

1.5.1 The heterogeneous nuclear ribonucleoprotein family

In eukaryotes, precursor mRNA transcripts produced by RNA polymerase II are called pre-mRNAs or heterogeneous nuclear RNAs (hnRNAs) (Dreyfuss *et al.*, 1988a). The proteins that bind these primary transcripts are known as the heterogeneous nuclear ribonucleoproteins (hnRNPs) and remain associated with this mRNA through the entire process of eukaryotic gene expression. The family of hnRNPs were first identified in a complex of RNA-binding proteins (Choi and Dreyfuss, 1984). To date, at least 20 major hnRNPs have been identified in humans (designated hnRNPA to hnRNPU) and it is interesting that most of these appear to be highly conserved among vertebrates (Dreyfuss *et al.*, 1993). Many of these proteins are

involved in processes such as pre-mRNA processing, nucleocytoplasmic mRNA transport and mRNA localisation, translation and stability (Dreyfuss *et al.*, 2002). Notably, there are many isoforms of these major proteins and less abundant hnRNPs exist as well (Dreyfuss *et al.*, 2002).

Most, if not all, hnRNP proteins are able to bind to RNA and, therefore, possess an RNA binding motif (Dreyfuss *et al.*, 1993). The most common motif of this kind in the hnRNP family is the RNP consensus RNA-binding domain (RNA motif) (Dreyfuss *et al.*, 1988b). This domain is approximately 90 amino acids in length and consists of two consensus sequences that are located 30 amino acids apart: RNP1 and RNP2 (Dreyfuss *et al.*, 1988b). RNP1 is a highly conserved octapeptide sequence consisting of the following: Lys/Arg-Gly-Phe/Tyr-Gly/Ala-Phe-Val-X-Phe/Tyr; whereas the hexapeptide, RNP2 is less conserved and is rich in aromatic and aliphatic amino acids (Adam *et al.*, 1986, Dreyfuss *et al.*, 1988b).

1.5.1.1 hnRNPs and DNA damage

It has been reported that a number of hnRNP proteins are implicated in mediating DNA repair, and are, consequently involved in the DDR. The hnRNP family member, hnRNPB1, interacts with DNA-PK and inhibits its activity *in vitro,* implying that this member of the hnRNP family is a negative regulator of NHEJ in DNA repair (Iwanaga *et al.*, 2005). There is also evidence that hnRNPC1/C2 is implicated in DNA repair since both bind to chromatin in a DNA damage-dependent manner and both isoforms are phosphorylated by DNA-PK in an RNA-dependent manner (Lee *et al.*, 2005, Zhang *et al.*, 2004). Similarly, hnRNPU/SAF-A, is phosphorylated by DNA-PK in response to DNA damaging agents that cause DSBs, and is implicated as a marker of DNA-PK activity (Berglund and Clarke, 2009).

Interestingly, hnRNPA18 levels are induced upon exposure to UV, which is unusual since most hnRNPs are constitutively expressed (Yang and Carrier, 2001). Another isoform of hnRNPA, hnRNPA1, plays a role in the stress response to oxidative damage, osmotic or heat shock. It localises to the cytoplasm following these extracellular stresses whereupon it mediates the recruitment of translationally-arrested mRNA to stress granules (SGs) (Guil *et al.*, 2006).

1.5.1.2 hnRNPs and transcriptional regulation

Previous work has implicated other members of the hnRNP family in the regulation of transcription, which is unsurprising considering that many proteins belonging to the hnRNP family are able to bind ssDNA (Dreyfuss *et al.*, 1993). hnRNPU has been found to inhibit the basal transcription machinery by hindering the phosphorylation of RNA Polymerase II, thus inhibiting the process of transcriptional elongation (Kim and Nikoderm, 1999). There are also studies that implicate hnRNPU in the repression of glucocorticoid receptor (GR)-driven transcription (Eggert *et al.*, 2001, Eggert *et al.*, 1997). Both hnRNPU and hnRNPD have been found to interact with the transcriptional co-activator, p300, implicating these proteins in the regulation of transcription (Martens *et al.*, 2002, Tolnay *et al.*, 2002). hnRNPU also binds to the promoter of *Oct4*, a transcription factor whose activation is required for the formation of the inner cell mass (ICM) where embryonic stem cells originate from (Vizlin-Hodzic *et al.*, 2011). Another transcription factor, WT1, directly associates with hnRNPU; its transcriptional activity is reduced upon this interaction (Spraggon *et al.*, 2007). Furthermore, hnRNPU constitutively binds to IKKβ, which is a component of the IκB kinase (IKK) complex that phosphorylates IκB to allow the activation of the transcription factor, NF-κB

(Tsuchiya *et al.*, 2010). In addition, another hnRNP family member, hnRNPK, serves as a transcriptional co-activator for p53 (Moumen *et al.*, 2005). In addition to its role in the cellular response to stress, hnRNPA1 is implicated in transcription through its interaction with IκBα, an inhibitor of NF-κB; this is required for the activation of NF-κB (Hay *et al.*, 2001).

1.5.2 Heterogeneous nuclear ribonucleoprotein U-like-1

A novel member of the hnRNP family was first identified as adenovirus 5 (Ad5) E1B-55K-associated protein 5 (E1B-AP5 or AP5) (Gabler *et al.*, 1998). Because of its striking sequence homology to hnRNPU/scaffold attachment factor A (SAF-A), this protein was also known as heterogeneous nuclear ribonucleoprotein U-like-1 (hnRNPUL-1) (Gabler *et al.*, 1998). For the purpose of this study this protein will be referred to as hnRNPUL-1.

1.5.2.1 Structure of hnRNPUL-1

The predicted molecular mass of hnRNPUL-1 is 95,805 Da, although it migrates at a molecular weight of 120 k under SDS-PAGE, which may be due to post-translational modifications or to structural idiosyncrasies such as the polyproline region (Gabler *et al.*, 1998). In total, hnRNPUL-1 consists of 856 amino acids and possesses several domains, most of which are shared with hnRNPU/SAF-A but not all (Figure 1.10) (Gabler *et al.*, 1998). The N-terminal region of hnRNPUL-1 contains a scaffold attachment factor (SAF) box, which is present in many proteins involved in mRNA metabolism. hnRNPU/SAF-A was found to have a high binding specificity for specialised AT-rich regions of DNA called scaffold attachment regions (SAR) (Fackelmayer *et al.*, 1994). Later, the SAF-box was shown to be the domain responsible for binding to these regions (Kipp *et al.*, 2000). This interaction is critical for nuclear architecture since it allows chromatin to be organised into discrete structures. The

SAF-box of hnRNPUL-1 is considered to be the region that allows this protein to bind to dsDNA. Notably, the SAF domain is found in several proteins that are involved in the DDR: it is also known as SAF-A/B, Acinus and PIAS (SAP) regions since these proteins were found to contain a similar domain (Aravind and Koonin, 2000). Another DNA damage protein that contains a SAF-box is PARP1, which is unsurprising considering it is an abundant nuclear protein that is involved in the regulation of chromatin structure and in transcription (Aravind and Koonin, 2000). The SAF-box has also been found in the C-terminal regions of Ku70 and in Rad18, which allows the latter protein to bind to DNA and leads to its consequent recruitment to stalled replication forks (Aravind and Koonin, 2000, Tsuji *et al.*, 2008). SAR DNA has been found to reside near enhancer elements, which may implicate proteins that bind to SAR in the regulation of gene expression (Gasser and Laemmli, 1986).

Another domain present in hnRNPUL-1 is the spore lysis A/ryanodine receptor (SPRY) domain. The SPRY domain is responsible for the recognition of a specific protein partner rather than a consensus sequence motif (Woo *et al.*, 2006). The central region of hnRNPUL-1 comprises a predicted kinase domain, clusters of orthologous groups of proteins 4639 (COG4639). This central region was found to contain sequence homology to a guanine nucleotide-binding motif, suggesting that it possesses a potential GTP/ATP binding site (Gabler *et al.*, 1998). Interestingly, this domain also corresponds to the kinase domain of PNK, imparting sequence homology between hnRNPUL-1 and this enzyme (Bernstein *et al.*, 2005, Wang and Shuman, 2001). Whether hnRNPUL-1 actually has DNA kinase activity is a very interesting and unanswered question that will have to await further investigation. It has also been demonstrated that the central region of hnRNPUL-1 contains a BRD7 binding site (BBS) (Kzhyshkowska *et al.*, 2003).

Like many members of the hnRNP family, hnRNPUL-1 contains an RNA binding domain. Unlike the common RNA motif that exists in numerous hnRNPs, the RNA-binding domain of hnRNPUL-1 resides in an arginine/glycine-rich region termed the RGG box and this is the same domain that is used by hnRNPU to bind RNA (Kiledjian and Dreyfuss, 1992). Interestingly, hnRNPUL-1 has been found to be methylated within this RGG box by the arginine methyltransferase, PRMT2 (Kzhyshkowska *et al.*, 2001). hnRNPUL-1 also possesses a polyproline (PP) region, which is widely believed to mediate protein-protein interactions and is also characteristic of many other RNA-binding proteins.

The hnRNPUL-1-related protein, hnRNPUL-2 (also known as scaffold attachment factor A2, SAF-A2), is structurally and functionally similar to hnRNPUL-1 (Polo *et al.*, 2012). Like hnRNPUL-1, hnRNPUL-2 contains a SAF domain, a SPRY domain, a putative kinase domain and an RGG box. However, it lacks a PP region and is smaller than hnRNPUL-1, consisting of 747 amino acids. Its functional similarity to hnRNPUL-1 will be discussed in 1.5.2.2.

1.5.2.2 The role of hnRNPUL-1 in DNA damage

The functions of hnRNPUL-1 are not limited to mRNA metabolism; this is, perhaps, to be expected since many studies that have shown that other members of the hnRNP family are involved in other pathways such as those that involve DDR components. It has been found that hnRNPUL-1 is required for the ATR-dependent phosphorylation of RPA32 during adenovirus infection, implicating hnRNPUL-1 in the ATR-dependent signalling pathway in response to DNA damage (Blackford *et al.*, 2008).

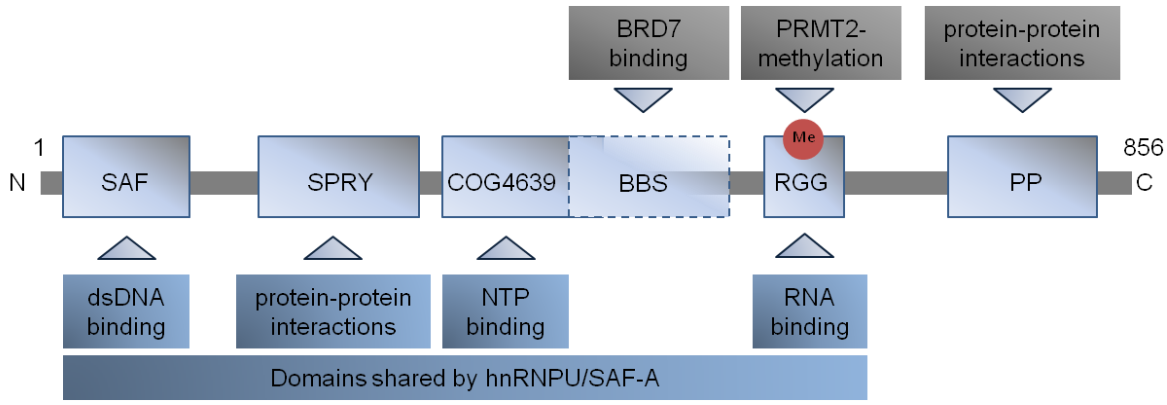

Figure 1.10 Schematic representation of the structure of hnRNPUL-1
The known domains of hnRNPUL-1 are depicted, including their function and the site of post-translational modification by PRMT2. Those domains that share sequence homology with hnRNPU/SAF-A are highlighted.

Both hnRNPUL-1 and hnRNPUL-2 have recently been identified as binding partners for the MRN complex, specifically binding to the C-terminal region of NBS1; they are recruited to sites of DNA damage in an MRN-dependent manner (Polo *et al.*, 2012). The binding site on hnRNPUL-1 that is required for the interaction with NBS1 was mapped to its central region and overlaps its BBS and RGG box (Polo *et al.*, 2012). In addition, hnRNPUL-1 and hnRNPUL-2 have been found to play a role in DNA-end resection and function downstream of the MRN complex and CtIP to mediate the recruitment of BLM helicase to sites of damage (Polo *et al.*, 2012). This suggests that both hnRNPUL proteins promote HR in the repair of DSBs. Interestingly, it was found that although the hnRNPUL proteins were recruited to sites of damage, both also moved away from sites of laser-induced damage, events that rely on the RGG box of hnRNPUL-1 (Polo *et al.*, 2012). It was hypothesised that this phenomenon allowed the hnRNPUL proteins to remove inhibitory proteins away from sites of damage (Polo *et al.*, 2012).

1.5.2.3 The role of hnRNPUL-1 in transcription

The involvement of hnRNPUL-1 at the level of transcriptional regulation has previously been shown to be mediated by its interaction with BRD7 (Kzhyshkowska *et al.*, 2003). The bromodomain is present in many proteins that are involved in transcriptional regulation and mutations in this domain contribute to the onset of carcinogenesis (Peng *et al.*, 2006). BRD7 itself has been implicated in the transcriptional regulation of a number of genes, including those involved in the important cell signalling pathways, such as those involving ras/MEK/ERK and Rb/E2F (Zhou *et al.*, 2004). In addition, BRD7 has been found to interact with the transcription factor, BRCA1, and mediates the recruitment of this protein to specific

promoter regions, which is important since BRCA1 itself cannot bind to DNA directly (Harte *et al.*, 2010). Notably, BRD7 can also bind to p53 and is required for the transcriptional activation of a subset of p53 target genes, not only via its ability to acetylate histones, but also through its recruitment of additional co-activators of p53 (Drost *et al.*, 2010).

It has been established that hnRNPUL-1 can bind directly to p53 via the C-terminal region of p53 and also that it has been implicated in regulating the p53-dependent transcriptional response (Barral *et al.*, 2005). The over-expression of hnRNPUL-1 was reported to inhibit the transcriptional activity of p53, although the exact mechanism by which this occurs is unknown (Barral *et al.*, 2005).

Interestingly, upon the inhibition of transcription, hnRNPUL-1 was still recruited to sites of DNA damage, indicating that two pools of the protein might exist: one that is involved in DSB repair and the other that is involved in RNA metabolism (Polo *et al.*, 2012).

1.6 CHEMOTHERAPEUTIC AGENTS

Cells are constantly exposed to environmental sources of DNA damage: for example, through UV irradiation from sunlight and IR from cosmic radiation and medical treatments involving x-rays (Ciccia and Elledge, 2010).

The mechanisms that exist to protect the integrity of the genome are numerous and intricate; they are capable of sensing DNA damage and transducing the signal to activate cell cycle arrest and repair or, if the damage is too great, initiate cell death. Importantly, however, the intentional generation of DNA damage in proliferating cells is the most common mechanism of action of chemotherapeutic drugs and has been the basis of such treatment for decades (Kastan and Bartek, 2004). Therapeutically, the optimal outcome of treatment with anti-

cancer agents is the death of cancer cells through apoptosis whilst sparing normal cells. Indeed, the efficiency of a chemotherapeutic agent is defined by the therapeutic index, which is the ratio of tumour cure:normal tissue complications. Although apoptosis is the preferred outcome of many cancer treatments, other cell death responses such as autophagy and mitotic catastrophe, have emerged as cellular responses to agents (Al-Ejeh *et al.*, 2010).

Tumour cells harbour inherent defects in their response to DNA damage that sometimes render them particularly vulnerable to DNA damaging agents. However, these same defects can also lead to the enhancement of particular repair pathways, which enables the survival and continued, or even enhanced growth of cancer cells, which already exhibit damaged DNA. Therefore, an alternative approach to chemotherapy is the development of drugs that specifically target key components that are involved in the response to DNA damage (Lord and Ashworth, 2012). An understanding of the response to DNA damage is becoming increasingly important in the development of anti-cancer agents.

The number of anti-cancer treatments are numerous and can be divided into several different categories, namely alkylating agents, topoisomerase inhibitors (including some anthracyclines), platinum compounds, anti-metabolites and anti-tumour antibiotics (Aziz *et al.*, 2012). Particular attention will be paid here to alkylating agents and those compounds that inhibit the topoisomerase enzymes. However, other classes of anti-cancer agents will be described briefly. The mechanisms employed by alkylating agents share some similarities with other anti-cancer drugs such as the platinum-based compounds. In addition, various plant alkaloids and anthracyclines fall into the topoisomerase inhibitor category of chemotherapeutics. The combination of present anti-cancer drugs with those that target proteins of the DDR can sensitise tumour cells to DNA damage.

1.6.1 Alkylating agents

Agents that attach alkyl carbon groups onto nitrogen (N) and oxygen (O) atoms of DNA bases are known as alkylating agents. Many alkylating agents are among the most commonly prescribed chemotherapeutics (Helleday *et al.*, 2008). These drugs can be categorised in terms of their mechanism of action and are often used in combination therapy to enhance the killing of tumour cells. The structure of the covalent alkyl lesion that forms depends on the type of alkylating agent that reacts with the DNA. As such, the response to these agents is variable and complex, using different detection and repair pathways. Alkylating agents can be loosely classified into different subtypes, which are dependent on the following properties: whether the agent is monofunctional or bifunctional, whether it follows a first or second order nucleophilic substitution (S_N1 or S_N2) and the type of alkyl group that is transferred to DNA. Monofunctional agents target single atoms on DNA bases using their one reactive site, in contrast to bifunctional alkylating agents that contain two reactive sites and can therefore generate interstrand crosslinks by targeting different DNA bases.

1.6.1.1 Alkylating damage to DNA

DNA contains many nucleophilic sites in the form of its N- and O-atoms that are prone to attack by electrophilic alkylators (Shrivastav *et al.*, 2010).These sites display varying degrees of reactivity and are differentially targeted by alkylating agents. Those atoms most prone to modification by alkylating agents include: N^1 and N^3 of adenine (where 1 and 3 depict nitrogen at position 1 and 3 respectively), O^6 and N^7 (where 6 represents oxygen at position 6) of guanine and N^3 of cytosine (Figure 1.11) (Fu *et al.*, 2012). Minor lesions occur on O^2, O^4 and N^3 of thymine and N^7 of adenine (Figure 1.11).

Replication-blocking lesions are produced when N^1 and N^3 on adenine are methylated to form N^1-methyladenine (1meA) and N^3-methyladenine (3meA) respectively. The predominant methylation adduct occurs on N^7 of guanine to induce N^7-methylguanine (7meG). While this lesion is not toxic on its own, it is able to undergo spontaneous depurination and the subsequent AP site is mutagenic (Fu *et al.*, 2012). The alkyl lesions that form on O^6 of guanine are particularly detrimental due to the miscoding property of the resulting O^6-methylguanine (O^6meG). This lesion is able to mispair with thymine, thereby inducing mutagenesis.

Since alkylating agents are able to transfer alkyl groups onto many biological molecules, it is not just DNA that is targeted. Other molecules, including RNA, are also affected by alkylating lesions, which alter their biological structure and function (Drabløs *et al.*, 2004).

1.6.1.2 Chemotherapeutic alkylating agents

The two main types of agents that fall into the monfunctional class of alkylating agents are the triazenes and the chloroethylating agents. The main lesions generated by triazenes such as temozolomide are 7meG, 3meA and O^6meG, while O^6-chloroethylguanine (O^6Cl-ethylG) usually forms after treatment with the chloroethylating agents (Lord and Ashworth, 2012).

Bifunctional agents include the nitrogen mustards, such as cyclophosphamide, and aziridines like mitomycin C. Both classes of bifunctional alkylators primarily target N^7 of guanine to form interstrand crosslinks and bulky N-monoadducts (Table 1.2).

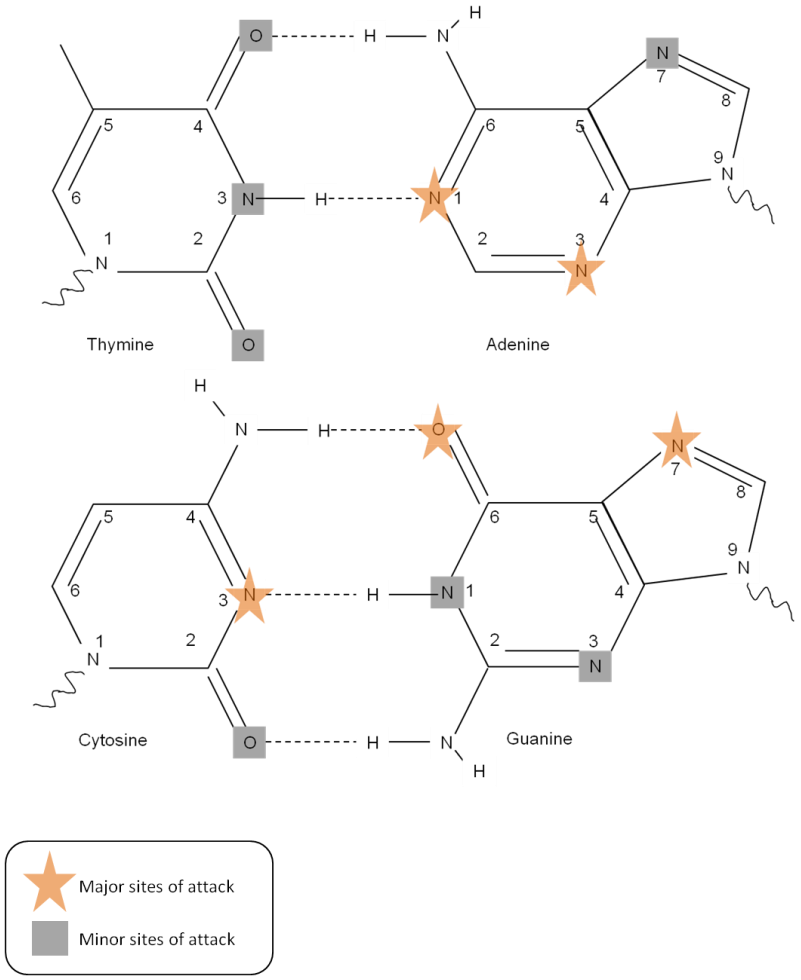

Figure 1.11 DNA bases and their sites of alkylation
Thymine, adenine, cytosine and guanine are depicted as schematics. The major sites of alkylating damage are: N^1 and N^3 of adenine; N^7 and O^6 of guanine and N^3 of cytosine. Minor sites of alkylation are: O^2, O^4 and N^3 of thymine; N^7 of adenine; O^2 of cytosine and N^1 and N^3 of guanine (adapted from Fu *et al.*, 2012).

1.6.1.3 Repair pathways involved in the response to alkylating damage

Alkyl lesions are recognised and repaired by a number of pathways, which are discussed in detail in 1.3.3. However, a brief overview is presented here to highlight the redundancy that exists between repair processes; a property that is increasingly being manipulated in cancer therapies (Table 1.2) (Lord and Ashworth, 2012).

Repair pathways that remove alkylation-induced damage before cells enter replication include direct reversal, BER and NER. Direct reversal is primarily catalysed by the alkyltransferase, MGMT, or the DNA dioxygenases, ALKBH2 and ALKBH3 (Duncan *et al.*, 2002, Lindhal *et al.*, 1982). The BER pathway removes simple alkylation adducts or a single damaged DNA base, whereas the NER pathway excises larger base adducts that distort the dsDNA helix. The MMR pathway has been implicated in the response to some alkylation-induced lesions, such as O^6meG (Table 1.2). The MMR pathway recognises and removes misincorporated nucleotides and it has been found that resistance to some alkylating agents occurs in cells that are defective in components of this pathway (Branch *et al.*, 1993, de Wind *et al.*, 1995). Numerous other repair processes can become activated in response to alkylating damage due to secondary lesions such as SSBs, DSBs and crosslinks. These include TLS, the FA pathway and HR (Fu *et al.*, 2012).

The inhibition of components of the DNA repair pathways that act on alkylation-induced damage is important in anti-cancer treatment to convert these lesions into fatal replication lesions to kill the tumour cell (Helleday *et al.*, 2008). Indeed, it has been found that the chemotherapeutic activity of temozolomide is effective in tumour cells where the promoter of the *MGMT* gene is methylated. Therefore, the expression of MGMT is greatly reduced, which causes alkyl lesions that would normally be reversed by the activity of the enzyme, to persist

(Lord and Ashworth, 2012). However, it is also important that the MMR pathway in these tumour cells is fully functional since the mismatches that are generated after temozolomide treatment are recognised by this pathway, which only targets the newly synthesised strand and does not repair the damaged base in the template strand. Therefore, DSBs are eventually generated, which results in the activation of the ATM/ATR pathway to promote cell cycle arrest and/or apoptosis in these tumour cells (Li, 2008).

1.6.2 Topoisomerase inhibitors

Another type of damaging agent that can be used as a chemotherapeutic drug is the class of compounds known as the topoisomerase inhibitors. These agents exploit DNA SSBs or DSBs that naturally arise within the cell (Helleday *et al.*, 2008). Human topoisomerases fall into two catergories: type I or type II. The type I family can be further sub-divided into types IA and IB. Similarly, the type II family consists of types IIA and IIB (Wang, 2002). Topoisomerase I (TOP1), topoisomerase IIIα (TOP3α) and topoisomerase IIIβ (TOP3β) belong to the type IB family, whereas topoisomerase IIα (TOP2α) and topoisomerase IIβ (TOP2β) fall into the type IIA category. Each family of topoisomerases has a slightly different biological role but as a group of enzymes, they generally resolve topological difficulties that occur during processes that require DNA-unwinding such as transcription and replication. By introducing transient breaks in DNA, these enzymes relax supercoiled DNA or allow the DNA strands to pass through each other (Wang, 2002). Here, the biological roles of TOP1, TOP2α and TOP2β and the function of their respective inhibitors will be discussed. Particular detail will be attached to the TOP2 enzymes. Specific drugs are used to target the different types of topisomerase enzymes.

Agent	Lesion	Repair
Monofunctional Triazenes Temozolomide	1meA	ALKBH2/ALKBH3
	3meA	BER/NER
	7meG	BER
	O^6meG	MGMT/NER
	O^6meG:T	MGMT/NER/MMR
Monofunctional chloroethylating Lomustine	O^6Cl-ethylG	MGMT/NER
	G-C crosslink	FA/NER
Bifunctional nitrogen mustards Cyclophosphamide **Bifunctional Aziridines** Mitomycin C	G-G crosslink	FA/NER

Table 1.2 DNA lesions caused by different alkylating agents.
The repair pathway for each lesion is shown. Secondary repair pathways for the DNA lesions include HR and NHEJ (adapted from Fu *et al.*, 2012).

Notably, topoisomerases can also be inhibited by endogenous sources such as base mismatches, pyrimidine dimers and oxidative stress (Lanza *et al.*, 1996, Li *et al.*, 1999, Pouquier *et al.*, 1997).

1.6.2.1 Biological role of topoisomerase I

TOP1 covalently binds to one DNA strand of the double helix and induces a SSB in the unbound strand. This allows the latter strand to rotate freely around the intact TOP1-bound DNA strand. Once the DNA is relaxed, TOP1 reverses the covalent binding to initiate the re-ligation of the break; this process is quicker than the cleavage step (Pommier *et al.*, 2003). The activity of TOP1, therefore, generates transient covalent TOP1-cleaved intermediates, which are known as TOP1 cleavage complexes (TOP1cc) (Pommier, 2006).

1.6.2.2 Topoisomerase I inhibitors

The cleavage complex that is generated during TOP1-mediated relaxation of DNA supercoils is a target for anti-cancer drugs. The main inhibitor of TOP1 is camptothecin, which exists in a naturally active form and binds reversibly to TOP1cc. By displacing the 5'hydroxyl group of the cleaved strand and preventing its re-ligation, camptothecin converts these cleavage complexes into DNA damage. However, continued exposure to camptothecin is required for DNA lesions to be generated since the drug rapidly diffuses away from the TOP1cc. The cleavage complexes are readily reversible once camptothecin is removed (Pommier *et al.*, 2003).

1.6.2.3 Biological role of topoisomerase II

TOP2 enzymes function in an ATP-dependent manner to remove DNA catenation and allow an intact DNA duplex to pass through another by generating a transient DSB in a second double-stranded DNA segment (Nitiss, 2009a). Two isoforms of the enzyme exist in mammalian cells - TOP2α and TOP2β. These are structurally similar; each one exists as a homodimer and they share 68% sequence homology (Austin *et al.*, 1993). The N-terminal domain and the central domain are similar between the two isoforms, whereas the C-terminal region differs slightly. The N-terminal region contains the site of ATP binding and hydrolysis that is vital for the cellular function of both isoforms. An active site tyrosine in the central domain drives the cleavage of the phosphodiester bond during TOP2 activity.

Despite their sequence homology, TOP2α and TOP2β possess different biological functions and these differences are often attributed to the differences in the C-terminal region of each enzyme (Gilroy and Austin, 2011, Linka *et al.*, 2007). TOP2α primarily functions in chromosome segregation and in replication and therefore acts in a cell cycle regulated manner. In contrast, TOP2β is the only TOP2 enzyme expressed in quiescent cells and does not play a vital role in proliferating cells. However, it has been shown that TOP2β-null mice are not viable and the defects observed led to the conclusion that TOP2β is implicated in transcription of essential genes involved in neuronal development (Ju *et al.*, 2006, Yang *et al.*, 2000). It is interesting that the expression of each isoform reflects their different roles: TOP2β appears to be constitutively expressed in all cells, whereas the expression of TOP2α is repressed in non-proliferating cells (Turley *et al.*, 1997).

1.6.2.4 Topoisomerase II inhibitors

The TOP2 enzymes are targets of a number of inhibitors, some of which have been exploited as the most effective clinical anti-cancer drugs. Inhibitors of these enzymes can be divided into two classes: the TOP2 poisons and the TOP2 catalytic inhibitors.

1.6.2.4.1 Topoisomerase II poisons

Drugs such as etoposide, doxorubicin and mitoxantrone belong to the class of TOP2 'poisons' (Nitiss, 2009b). Their common mode of action is the blocking of the re-ligation step of the cleaved DNA duplex following the passage of an intact DNA duplex (described as the G and T segments) through the transient DSB (Figure 1.12). By binding reversibly, these drugs effectively 'trap' TOP2α/TOP2β-DNA cleavage complexes (TOP2cc) where DNA strands are attached by their 5' ends through the TOP2 dimer and each end of the DNA is attached to a TOP2 monomer. In this way, these poisons convert the enzyme into a highly toxic DNA damaging agent. It is interesting to note, therefore, that TOP2 poisons are restricted to act at a certain stage of the TOP2 catalytic cycle.

Etoposide (also known as VP-16), belongs to the epipodophyllotoxin class of TOP2 poisons. Low doses of this drug result in the accumulation of TOP2cc that become cytotoxic at high concentrations. Therefore, etoposide treatment generates a number of TOP2-mediated DNA DSBs, which activate a DDR. However, the cellular processes that are involved in detecting and processing these cleavage complexes remain unclear. Although etoposide has been shown to induce the formation of both the TOP2α and TOP2β cleavage complexes, there have been studies to suggest that TOP2α rather than TOP2β is the mediator of etoposide-induced cytotoxicity (Azarova *et al.*, 2007). In addition, these studies showed that the development of secondary malignancies that arise from chromosomal rearrangements and DSBs after

etoposide treatment is mediated through TOP2β (Azarova *et al.*, 2007). The fact that these effects can be reversed by proteasomal degradation is supported by the findings that preferential degradation of TOP2β by the 26S proteasome pathway can occur after formation of TOP2cc (Mao *et al.*, 2001).

There are also reports that an isoform-specific poison exists in the form of NK314, a synthetic benzo[c]phenanthridine alkaloid. This agent was found to induce DSBs and exert anti-tumour effects in a TOP2α-dependent manner (Toyoda *et al.*, 2008).

1.6.2.4.2 Topoisomerase II catalytic inhibitors

The other class of agents are termed the TOP2 'catalytic inhibitors' and comprise a heterogeneous range of compounds that affect TOP2 activity at several different stages during the catalytic cycle of the enzyme. As their name suggests, these compounds exert their cytotoxicity by inhibiting the activity of TOP2, which results in abnormal mitosis and mitotic catastrophe (Jensen and Sehested, 1997, Larsen *et al.*, 2003). The mechanisms of action of catalytic inhibitors are diverse and are sometimes non-specific (Larsen *et al.*, 2003).

One mode of inhibition is the disruption of the binding between DNA and TOP2 by compounds such as the intercalator, aclarubicin (Figure 1.12). Incidentally, this compound is one of the few members of the class of catalytic inhibitors to be developed as a clinical agent. It has been found that aclarubicin inhibits the DNA poisons such as etoposide because it reduces the amount of TOP2 cleavage complex that is the substrate for etoposide (Peterson *et al.*, 1994). Thus, the rationale advanced by a number of studies is that the antagonistic behaviour of a catalytic inhibitor of TOP2 should selectively protect normal tissue and not tumour cells (Jensen and Sehested, 1997).

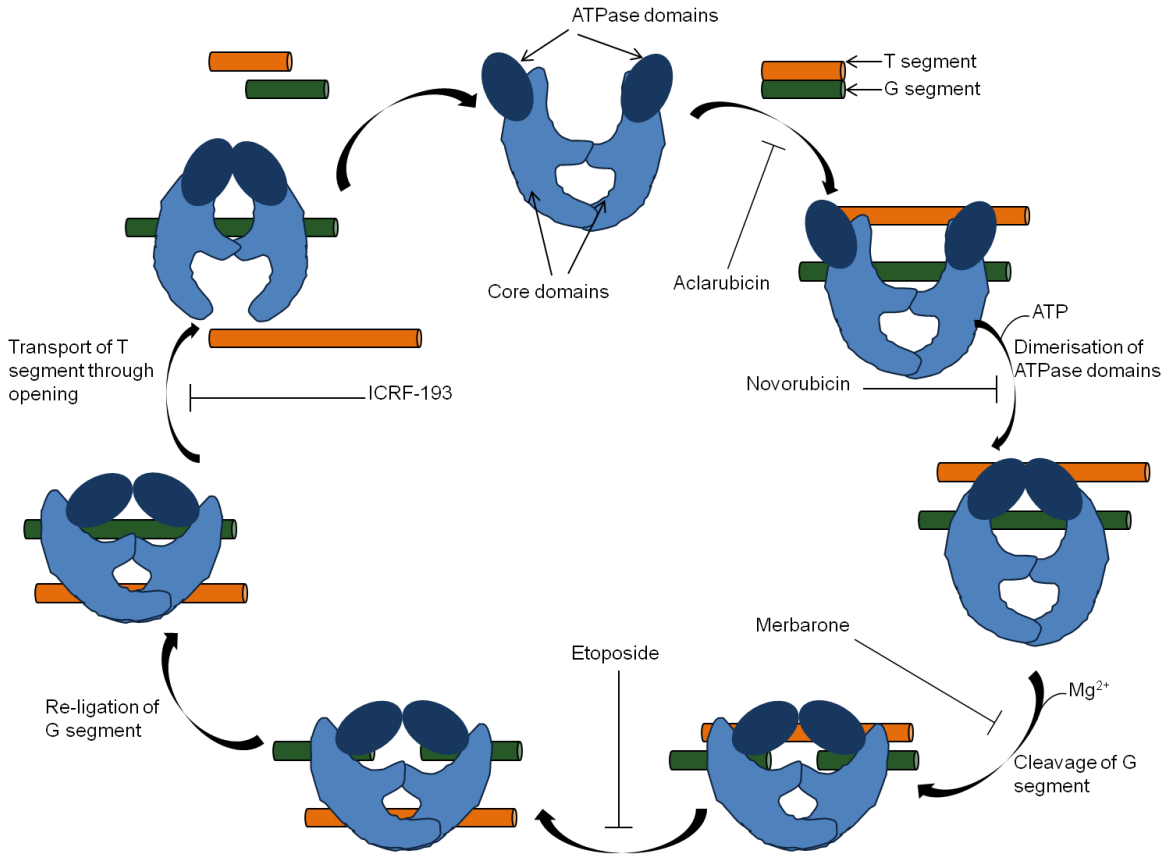

Figure 1.12 The catalytic cycle of TOP2 and its poisons and catalytic inhibitors
The TOP2 catalytic cycle is activated upon binding of the enzyme to two double-stranded DNA segments – the G and the T segments. Upon ATP binding, the two ATP domains dimerise and the G segment becomes cleaved. The T segment is transported through the break in the G segment and the latter is re-ligated. The T segment is then transported through the opening in the C-terminal region. Etoposide acts at the indicated step and traps TOP2 in complexes where the DNA strands of the cleaved segment are attached to each TOP2 monomer. Novobiocin blocks ATP binding; merbarone inhibits TOP2-mediated cleavage of the G segment and ICRF-193 stabilised a non-covalent form of TOP2 where it is closed around DNA (adapted from Wang 2002; Larson *et al*., 2003).

The inhibition of ATP binding is another mode of TOP2 catalytic inhibition. Novobiocin is an example of a drug that acts via this mechanism and blocks the ATP binding site on TOP2 (Figure 1.12) (Larsen *et al.*, 2003). However, the action of novobiocin is not restricted to TOP2; it can also disrupt the activity of certain ABC transporters and, interestingly, has been found to potentiate the action of etoposide via the inhibition of its transporter (Rappa *et al.*, 1993).

Finally, some agents belonging to this class of TOP2 inhibitors can also act to disrupt TOP2-mediated DNA cleavage and, therefore, stabilise TOP2-DNA complexes. In this way, drugs such as merbarone compete with TOP2 poisons like etoposide, which leads to the possibility that some catalytic inhibitors and some TOP2 poisons bind to the same site on TOP2. In addition, some bisdioxopiperazine derivatives like ICRF-193 can also act to inhibit TOP2 by stabilising a non-covalent form of the enzyme (Figure 1.12). Both merbarone and the bisdioxopiperazine derivatives show some selectivity towards TOP2α (Drake *et al.*, 1989, Perrin *et al.*, 1998).

1.6.3 Other classes of anti-cancer drugs

1.6.3.1 Platinum salts

Platinum-based drugs, notably cisplatin and carboplatin, are amongst the most commonly used chemotherapeutics. The platinum atom in these drugs specifically forms covalent bonds with N^7 of purine bases and results in platinum-DNA adducts, including DNA ICLs (Deans and West, 2011). In this respect, the mechanism of toxicity of cisplatin resembles that of bifunctional alkylating agents in 1.6.1 and similarly, the resulting adducts cause lethal disruption to replication and transcription.

The main pathways that recognise and repair platinum drug-induced lesions are a combination of NER and HR. Therefore, agents like cisplatin are effective in tumours that harbour mutations in either of these pathways. For example, in some high-grade ovarian tumours, the HR genes, *BRCA1* or *BRCA2* may be mutated. In addition, mutations in enzymes involved in the NER pathway are associated with an increased level of cisplatin-induced adducts and therefore exhibit a heightened apoptotic response to such drugs (Lord and Ashworth, 2012).

1.6.3.2 Anti-metabolites

Another class of anti-cancer drug is the chemotherapeutic anti-metabolites. These include methotrexate (MTX), 5-fluorouracil (5-FU) and hydroxyurea (HU). The general mechanism of action of this group of compounds is the inhibition of specific enzymes that catalyse reactions in the biosynthesis of purines and pyrimidines. The rationale behind such treatment is that tumour cells have a higher rate of proliferation than normal cells and may, therefore, be more sensitive to such drugs (Kinsella *et al.*, 1997). MTX is a specific inhibitor of dihydrofolate reductase and prevents the production of reduced folate, which is a co-factor for a number of enzymes involved in purine and pyrimidine synthesis. The pyrimidine antagonist, 5-FU, was specifically developed to resemble uracil. It rapidly enters the cell where it is converted into numerous metabolites, resulting in the fraudulent nucleotide, 5FdUMP that inhibits thymidylate synthase (TS). Thymidylate synthesis is subsequently blocked, disrupting DNA synthesis. Furthermore, the fraudulent nucleotide incorporates itself into DNA (Longley *et al.*, 2003).

1.6.3.3 Anti-tumour antibiotics

A number of compounds isolated from *Streptomyces* cultures were found to contain anti-tumour activity (Waksman and Woodruff, 1940). Therefore, drugs such as mitoxantrone and doxorubicin were designed from these natural precursors (Sparreboom *et al.*, 2005). Doxorubicin belongs to the anthracycline family of anti-tumour drugs, which are highly reactive molecules, possessing a high affinity for DNA (Sparreboom *et al.*, 2005). The main mode of cytotoxicity is their ability to bind TOP2, the implications of which are described in 1.6.2.4.1. Anthracyclines are limited in their use due to multi-drug resistance and the cardiotoxicity that often accompanies such treatment Therefore, the development of derivatives that are not substrates for resistance mechanisms and that have reduced toxicities is ongoing.

1.6.4 Resistance to chemotherapeutic agents

Many chemotherapeutics are limited in their effectiveness due to the problem of drug resistance. Drug resistance can occur through a number of mechanisms such as the over-expression of drug efflux transporters, increased drug inactivation and the enhanced repair of DNA damage that is induced by chemotherapeutics. The latter is often linked to secondary mutations that are acquired by the tumour cell to render it capable of repairing the drug-mediated damage. Therefore, resistance can be divided into those tumours that have an intrinsic resistance and those that acquire it throughout treatment. Indeed, acquired resistance is often associated with patients who are undergoing chemotherapy.

1.6.4.1 Over-expression of drug transporter proteins

The up-regulation of drug efflux ATP-binding cassette (ABC) transporters presents a serious obstacle to the use of chemotherapeutic drugs. The main transporters that are over-expressed in tumour cells are: P-glycoprotein (ABCB1/MDR1), multi-drug resistance protein (ABCC1/MRP1) and breast cancer resistance protein (ABCG2/BCRP) (Longley and Johnston, 2005). A large number of compounds make up the substrates of these efflux transporters, which is a concern considering the range of anti-cancer drugs that are available. Many strategies are in place to inhibit drug transporters. In addition, the need for designing drugs that are not substrates for such transporters is becoming increasingly important.

1.6.4.2 Increased drug inactivation

The inactivation of the reactive group of some anti-cancer drugs is often due to the over-expression of glutathione-S-transferase (GST), which catalyses the formation of conjugates between glutathione and some anti-cancer drugs (Townsend and Tew, 2003). This enzyme-mediated process is actually a mechanism that inhibits oxidative stress caused to DNA by endogenous and exogenous agents. However, since the rationale behind chemotherapy is the induction of DNA damage, the over-expression of GST is detrimental to the activity of such agents and causes resistance.

1.6.4.3 Secondary mutations

Drugs that target proteins involved in the DDR are often limited by resistance that is acquired through secondary mutations in other DDR genes. These secondary mutations can lead to an increase in the repair of damaging lesions. For example, resistance to PARP inhibitors is often

caused by secondary mutations in *BRCA2* that is vital to the process of HR (Edwards *et al.*, 2008, Sakai *et al.*, 2008). These mutations restore the open reading frame of these genes, which allows the generation of a partially functional protein; therefore, the damage resulting from PARP inhibition can be repaired. PARP inhibitors are usually particularly effective in tumours harbouring mutations in *BRCA1* or *BRCA2* (Bryant *et al*., 2005, Farmer *et al*., 2005). These mutations would normally render them defective and unable to repair DSBs.

1.7 AIMS AND OBJECTIVES

The main tumour suppressor activities of p53 include its ability to enforce growth arrest or apoptosis through the transcriptional activation or repression of various genes in response to differing degrees and types of stress. Many mechanisms exist to control this transcription factor, including co-factors that often exert discriminatory effects on the ability of p53 to regulate its targets. The involvement of hnRNPUL-1 in transcriptional regulation has previously been reported as well as the finding that it interacts with p53 and negatively regulates its transcriptional activity, although the mechanism by which this occurs was unknown (Barral *et al.*, 2005, Kzhyshkowska *et al.*, 2003). Taken together, these observations suggest that hnRNPUL-1, in addition to its RNA-processing properties, may also function as a transcriptional co-factor for p53. Therefore, the aims and objectives of this part of the study were:

- To investigate the site of interaction on hnRNPUL-1 for p53 and to confirm the inhibitory effects of hnRNPUL-1 on the transcriptional activity of p53.
- To determine which p53 target genes and consequent cellular responses are affected by the action of hnRNPUL-1.

p53 is the most commonly mutated tumour suppressor and is inactivated in nearly 50% of sporadic tumours (Levine *et al.*, 1991, Nigro *et al.*, 1989). Although a key event in the onset of tumourigenesis, p53 inactivation in tumours renders them particularly refractory to anti-cancer treatment since it is a mediator of chemosensitivity. Inactivating mutations in *TP53* are not the only means by which tumours become resistance to treatment: mutations in numerous repair genes and key damage sensors are commonly found in different types of tumours and are often the cause of treatment with anti-cancer agents being ineffectual. The need for agents to overcome such resistance mechanisms has led to the development of compounds derived from the anthracycline family. One such derivative, ALX, is a TOP2 inhibitor that also possesses an alkylating side-chain. Notably, ALX displays anti-cancer activity in treatment-resistant cell lines (Pors *et al.*, 2004, Pors *et al.*, 2003). However, any mechanistic insight into the mode of action of ALX remains poor. Therefore, the aims and objectives of this section of the study were:

- To identify the DDR pathway that becomes activated in response to ALX and to establish the mode of cell death.
- To determine which functions of ALX contribute to its anti-tumour activity.
- To determine whether the inactivation of key DDR components results in ALX resistance.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2 MATERIALS AND METHODS

2.1 TISSUE CULTURE TECHNIQUES

2.1.1 Cell Lines

All cell lines used in this study are summarised in (Table 2.1). DLD1 Seckel/Seckel Rescue cells were obtained from F. Bunz and tetracycline-inducible U2OS cells were obtained from E. Petermann. All other cell lines were commercially-available and originally obtained from the ATCC. All cell lines were checked routinely for mycoplasma.

2.1.2 Tissue culture media and solutions

The following cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen), supplemented with 10% fetal calf serum (FCS) (PAA), glutamine and penicillin/streptomycin (pen/strep) (Invitrogen): H1299, HeLa, HCT116 and hTERT-RPE1. U2OS osteosarcoma cells were grown in McCoy's medium (Invitrogen), supplemented with 10% FCS and pen/strep. Other cell lines that also required McCoy's medium included DLD1 parental, DLD1 Seckel, DLD1 Seckel Rescue and DLD1 Chk1 S317A.

2.1.3 Maintenance of cell cultures

The adherent cell lines above were maintained in 75 cm^2 tissue culture flasks (Corning) and subcultured in humidified incubators in a 95% air and 5% carbon dioxide environment at 37 ^oC. When cells reached confluency, the medium was removed and cells were subsequently washed in PBS (5.5 mL). Detachment of cells was performed by the addition of trypsin

Cell Line	Origin	Mutation/other comment
DLD1 parental	Colorectal carcinoma	*MSH6* mutant; *TP53* mutant
DLD1-Seckel	Colorectal carcinoma	Reduced ATR
DLD1-Seckel Rescue	Colorectal carcinoma	Engineered to re-express ATR
DLD1 Chk1 S317A	Colorectal carcinoma	*Chk1* mutant
H1299	Non-small cell lung carcinoma	*TP53* null
HCT116	Colorectal carcinoma	*TP53* WT; *MRE11* mutant; *MLH1* mutant
HeLa	Cervical carcinoma	Low levels of p53
RPE1	hTERT-immortalised retinal pigment epithelial	Immortal cell line with an extended replicative capacity
U2OS	Osteosarcoma	*TP53* WT
U2OS -Tet	Inducible osteosarcoma	Overexpresses Cyclin E (Tet-off)
U2OS +Tet	Inducible osteosarcoma	Normal levels of Cyclin E (Tet-on)
2008+vector	Ovarian carcinoma	*FANCF* deficient
2008+FANCF	Ovarian carcinoma	Engineered to re-express FANCF

Table 2.1 Cell lines used in this study
All cell lines orginate from humans unless otherwise stated.

(Invitrogen) (1.5 mL) before incubating cells for 5 min at 37 ^{o}C. After confirming detachment of cells under the microscope, the trypsin was inactivated by resuspending with appropriate media containing FCS (8 mL). Cells were then re-plated to the required density.

To synchronise hTERT-RPE1 cells in G0/G1 phase of the cell cycle, cells were grown to confluency and then maintained in media lacking FCS for 2 weeks prior to use. In the tetracycline-inducible U2OS cells, cyclin E expression was suppressed by the addition of 1 μg/mL tetracycline (Sigma) to growth media. To induce cyclin E expression, cells were maintained for 48 h in growth media lacking tetracycline.

2.1.4 Cryopreservation of cells

Cells were cultured to 80% confluency and trypsinised as before (2.1.3). The trypsin was inactivated by fresh media (5 mL) and centrifuged at 1200 rpm for 5 min. The media was gently removed and the remaining cell pellet was resuspended with 1 mL of freezing mixture containing 50% FCS, 40% media and 10% DMSO The cells were then transferred to a 1.5 mL cryovial (Nunc) and stored at -80 ^{o}C before being moved to storage in liquid nitrogen..

2.1.5 Cell Resuscitation

A 1.5 mL cryovial containing the cells was taken from liquid nitrogen tanks and opened under sterile conditions to let out any remaining liquid nitrogen. Once re-capped, the cryovial was warmed at 37 ^{o}C and a sterile Pasteur pipette was used to transfer the contents of the cryovial to a Falcon tube. The cells were resuspended in 10 mL of pre-warmed media, which was added drop-wise, and centrifuged at 1500 rpm for 5 min to remove any residual DMSO. The media was gently removed and the remaining cell pellet was resuspended in media before being transferred to a 75 cm^2 tissue culture flask to be incubated at 37 ^{o}C.

2.2 CYTOTOXIC AGENTS AND INHIBITORS

2.2.1 Chemotherapeutic drugs

The alkylating anthraquinone, Alchemix (ALX, 50 nM), was a gift from Klaus Pors (University of Bradford). A derivative of ALX lacking the alkylating side-chain, ZP275, was a gift from Klaus Pors and was used at a concentration of 50 nM. These cytotoxic agents were dissolved in dimethyl sulphoxide (DMSO) (Sigma) and aliquots were stored at -80 ^{o}C.

2.2.2 DNA damaging agents and kinase inhibitors

Cells were irradiated with ionising radiation (IR) to promote DNA double strand breaks at the following doses unless otherwise stated: 2, 5, 10 Gy. Ultraviolet light (UV) was used to promote the formation of intrastrand crosslinks (ICLs). Prior to UV irradiation, culture medium was removed and cells were exposed to UVC irradiation (254 nm) at a dose of 20 Joules m^{-2} unless otherwise stated. Camptothecin (CPT) inhibits the catalytic activity of mammalian DNA TOP1 and stabilises the TOP1cc. It was used at a concentration of 1 µM to induce DNA lesions at replication forks. An inhibitor of TOP2, Etoposide (Sigma Aldrich), was also used at a concentration of 1 µM. The ATM inhibitor, KU-55933 (Merck) was used at a concentration of 10 µM. The MRN-ATM pathway inhibitor, Mirin (Merck) was also used at a concentration of 10 µM. The DNA-PK inhibitor, DMNB was also obtained from Merck and used at a concentration of 50 µM. Cisplatin (EBEWE pharma, 10 µg/mL) was used to induce apoptosis. All inhibitors were reconstituted in DMSO and aliquots were stored at -20 ^{o}C.

2.3 PROTEIN CHEMISTRY TECHNIQUES

2.3.1 Protein extraction

Adherent cancer cell lines were cultured as appropriate to 80% confluency. After culture media was removed, the cells were washed in pre-warmed PBS and treated with trypsin as before (2.1.3). Once fresh media was added, cells were pooled and centrifuged at 1500 rpm for 5 min at room temperature. The cell pellet was resuspended in ice-cold PBS and centrifuged at 1500 rpm at 4 ^{o}C, after which the supernatant was removed. Whole cell extracts were prepared in a volume of UTB lysis buffer (8 M Urea, 50 mM Tris-HCl, [pH 7.5], and 150 mM β-mercaptoethanol), and transferred to a 1.5 mL microfuge tube. Samples were subsequently sonicated for 2 x 10 sec and then centrifuged at 16, 400 rpm for 20 min at 4 ^{o}C to remove the cellular debris. The supernatant containing the protein was transferred to a 0.5 mL microfuge tube and snap frozen in liquid nitrogen before being stored at -80 ^{o}C.

2.3.2 Bradford Assay

Protein extracts were prepared as described above (2.3.1). A standard protein curve was constructed using BSA (Sigma) made up in sterile distilled water: 0, 100, 200, 300, 400, 500 µg/mL. The BSA standards were aliquotted (10 µL), in quadruplicate, into a 96-well flat bottom plate (Iwaki). Protein samples were diuted 1:10 with distilled water and aliquotted (10 µL), in quadruplicate, into the same 96-well flat bottom plate. Bradford reagent (Bio-Rad) was diluted 1:5 with sterile distilled water and 200 µL was added to each well containing the protein sample and to wells containing the standards. The reaction was left at room temperature for 5 min before the absorbance was measured at a wavelength of 595 nm. A

standard curve was plotted from which protein concentrations of the samples were determined.

2.3.3 SDS-PAGE

Samples were separated on polyacrylamide gels consisting of 1M Tris/1M Bicine, 30% acrylamide (N, N'-methylene-bis-acrylamide) (Bio-Rad), 10% SDS solution (Sodium dodecyl sulphate w/v) (Bio-Rad), Temed (N, N, N'. N'-tetra-methyl-ethylenediamine) (Bio-Rad) and 10% APS (Ammonium Persulphate) (Bio-Rad). The polyacrylamide gels were made up to different percentages based on the volume of acrylamide used, which was dependent on the relative molecular weight of the protein of interest: >80 kDa – 6%, >40 kDa – 10% and <40 kDa - 12% (8 mL, 13.36 mL and 16 mL respectively). The polyacrylamide gel was diluted with sterile distilled water to give the desired final percentage with APS added last to initiate polymerisation. The gel apparatus was filled before inserting a well-forming plastic comb and then left to polymerise. The comb was removed once the gel had set and the wells were rinsed with running buffer (0.1M Tris/0.1M Bicine, 0.1% SDS). Protein samples were diluted 1:1 in 2x sample buffer (0.125 M Tris/HCl pH 6.8, 4% (w/v) SDS, 20% Glycerol, 0.2 M DTT, 0.02% Bromophenol blue) and denatured at 95 ^{o}C for 5 min. Samples were loaded (30 µg per well) onto the gel alongside a protein molecular weight marker (Fermentas). The gel was placed in the bottom reservoir containing running buffer before the top reservoir (also containing running buffer) was clamped on top of the gel. Samples were typically electrophoresed for 5-6 h at 20mA.

2.3.4 Biphasic gel

A biphasic polyacrylamide gel was used to separate very high and very low molecular weight proteins on the same gel. A 12% acrylamide gel (10 mL) was poured into the bottom half of the gel apparatus and the surface was overlaid with 1 mL water saturated butan-2-ol to ensure an even layer. Once the 12% gel had set, the water saturated butanol was removed and the gel plates rinsed in distilled water three times. A 6% acrylamide gel was poured on top of the 12% gel and the well-forming plastic comb was inserted as before.

2.3.5 Electrophoretic transfer of proteins

Following electrophoresis, cell lysates were electrophorectically transferred onto a nitrocellulose membrane (Thermo Scientific) using a blotting cassette consisting of electrode panels and foam sponges. The nitrocellulose membrane was pre-soaked in transfer buffer (0.02 M Tris, 0.19 M glycine and 20% v/v methanol) and placed onto a sheet of Whatman 3MM filter paper that had also been pre-soaked in transfer buffer. The gel was placed on top of the nitrocellulose membrane and then covered with another sheet of pre-soaked Whatman filter paper. All the air bubbles were removed to ensure even transfer. The complete blotting cassette was then placed in a Hoefer Transfer Gel Electrophoresis Unit and filled with transfer buffer. The cassette had to be placed in the correct orientation, with the nitrocellulose membrane facing the anode. The transfer of proteins was typically conducted overnight at 200 mA.

2.3.6 Immunodetection of proteins

After the proteins had transferred, the nitrocellulose membrane was stained with Ponceau S solution (Sigma) to visualise even loading and accurate transfer of proteins after electroblotting. Ponceau S solution was completely removed by washing the membrane in Tris-buffered saline with 0.1% Tween-20 (TBS-T). The membrane was then incubated in 5% skimmed milk powder (Marvel) at room temperature on a shaker to ensure that all hydrophobic binding sites were blocked with milk protein. The primary antibody was diluted to the appropriate concentration and the membrane was incubated in primary antibody in a sealed bag for 1 h at room temperature or overnight at 4 ^{o}C with gentle rocking (Table 2.2). Excess primary antibody was removed by 3 x 10 min washes in TBS-T, after which the membrane was incubated in a relevant secondary antibody (Table 2.2). The latter was conjugated with the horseradish peroxidase and directed against the IgG of the species that the primary antibody was raised from. After washing, the membrane was incubated with equal volumes of enhanced chemiluminescence system (ECL) (GE Healthcare) solutions 1 and 2 for 1 min. The horseradish peroxidise catalysed the oxidation of the chemiluminescent substrate, luminol. This resulted in an excited state product of luminol, which was consequently converted into a lower energy state, releasing photons of light in the process. The presence of a chemical enhancer increased the chemiluminescent signal, which was detected as explained next. Excess ECL reagents were removed and the membrane was then placed in an X-ray cassette and exposed to X-ray film (Kodak) for a varying amount of time, dependent on the strength of the signal, and consequently developed (Xograph Imaging Systems Compact X4).

2.3.7 Immunoprecipitation

U2OS cells were washed in PBS after removing culture media. Cells were then pooled into a pre-cooled Falcon tube and centrifuged at 1500 rpm for 5 mins. The pelleted cells were resuspended in NETN lysis buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl [pH 7.5] 1% Nonidet P-40) before being homogenised and cleared by ultracentrifugation at 40, 000 *x g* for 30 min at 4 ^{o}C. The lysates were immunoprecipitated with anti-hnRNPUL-1 antibody overnight at 4 ^{o}C (Table 2.2). Following this, the immunocomplexes were isolated by incubating with protein G-Sepharose (Sigma) for 1 h at 4 ^{o}C. Bead-bound immunoprecipitates were washed four times in NETN buffer and boiled in SDS sample buffer. Samples were separated by SDS-PAGE and analysed by immunoblotting as described before (2.3.3-2.3.6).

2.3.8 Immunofluorescence microscopy

Cells were cultured in 10 cm dishes on either 12 well poly-L-lysine coated glass slides (Hendley) or on poly-L-lysine coverslips (BD Biosciences) and grown to 70% confluency. Cells on slides were permeabilised in ice-cold extraction buffer (10 mM PIPES, 300 mM sucrose, 20 mM NaCl, 3 mM Mg Cl_2, 0.5% Triton X-100, pH6.8) for 7 min and then fixed in ice-cold paraformaldehyde (3.6% paraformaldehyde in PBS, pH 7.2) for 10 min. If cells were grown on coverslips, these were extracted using ice-cold methanol at -20 ^{o}C for 15 min. Cells on both slides and coverslips were subsequently washed in PBS (3 x 5 min) and then blocked in 10% FCS (that had been filter-sterilised) in PBS at room temperature for 1 h or at 4 ^{o}C overnight. Cells were then washed in PBS (3 x 5 min) and incubated in primary antibody for 1 h (Table 2.2). After this, excess antibody was removed by 3 x 5 min washes in PBS and cells were then incubated in secondary antibody in the dark for 1 h (Table 2.2). Following

incubation, excess secondary antibody was removed by 3 x 5 min washes in PBS. Cells on coverslips were mounted on glass slides (Surgipath) using a drop of Vectashield Mounting Medium (Vector Laboratories) that contained 4', 6-diamidino-2-phenylindole (DAPI). Cells on slides were protected by glass coverslips (Surgipath) and mounted using the same medium. Nail varnish was used to seal the edges and slides were kept in the dark at 4 ^{o}C until ready to visualise. Visualisation of cells was conducted using a Nikon Eclipse E600 Microscope. Images were recorded using the Hamamatsu C4742-95 digital camera, and analysed using the image analysis software, Velocity version 4.

2.3.9 GST-pull down assay with radioactive proteins

GST pull-down assays were conducted using GST-fusion proteins and [^{35}S]-labelled proteins (Table 2.3). [^{35}S]-labelled *in vitro* translated protein (5 µL) was incubated with GST-fusion protein (20 µg) that had been diluted with 300 µL GST lysis buffer (buffer A; 1mM EDTA in PBS, 1% Triton-X100) on ice for 2 h. Protein complexes were isolated by incubation with glutathione-agarose beads (50 µL) at 4 ^{o}C with rotation for 1 h. Beads were washed three times with buffer A and twice with GST wash buffer (buffer B; 1mM EDTA in PBS).

After washing, GST-protein complexes were released by the addition of GST elution buffer (25mM glutathione, 50mM Tris, pH 8.0) at 4 ^{o}C for 30 min. Samples were analysed by SDS-PAGE after being boiled with sample buffer. After electrophoresis, gels were stained with a solution of 0.1% (w/v) Coomassie Brilliant Blue R-250 (Sigma Aldrich), 10% glacial acetic acid and 20% methanol in deionised water at room temperature on a shaker for 30 min. To visualise protein bands, gels were placed in a destaining solution (10% glacial acetic acid and 20% methanol in deionised water) until bands were clearly visible. Destained gels were

Antibody (Species)	Use	Source
hnRNPUL-1 (rabbit)	WB, IP	Made in-house
HA (mouse)	WB	Sigma
β-Actin (mouse)	WB	Sigma
p53 (DO-1) (mouse)	WB	Dr R. Grand
p53 S15-P (rabbit)	WB	Cell Signalling
p21 (mouse)	WB	Cell Signalling
MDM2 (2A10) (mouse)	WB	Dr R. Grand
Caspase 3 (rabbit)	WB	New England biolabs
PARP1 (mouse)	WB	Santa Cruz
ATM (11G12) (mouse)	WB	Dr J. Last
ATM S1981-P (rabbit)	WB	R and D Systems
ATR (mouse)	WB	Santa Cruz
DNA-PK (mouse)	WB	Santa Cruz
DNA-PK S2056-P (rabbit)	WB	Abcam
KAP-1 (mouse)	WB	Bethyl Laboratories
KAP-1 S824-P (rabbit)	WB	Bethyl Laboratories
SMC1(rabbit)	WB	Bethyl Laboratories
SMC1 S966-P (rabbit)	WB	Bethyl Laboratories
NBS1 (mouse)	WB	Novus Biologicals
NBS1 S343-P (rabbit)	WB	Cell Signalling
Chk1 (mouse)	WB	Santa Cruz
Chk1 S345-P (rabbit)	WB	Cell Signalling
Chk2 (rabbit)	WB	Dr S. Elledge
Chk2 T68-P (rabbit)	WB	R and D Systems
RPA32 (mouse)	WB	NeoMarkers Fremont
RPA32 S4/8-P (rabbit)	WB	Bethyl Laboratories
H2A (rabbit)	WB	Millipore
γH2AX (mouse)	WB, IF	Millipore
TOP2α (rabbit)	WB	Bethyl Laboratories
TOP2β (rabbit)	WB	Bethyl Laboratories
PCNA (PC10) (mouse)	WB	Santa Cruz
FANCD2 (mouse)	WB	Santa Cruz
Lamin B (goat)	WB, IF	Santa Cruz
Cyclin A (mouse)/ Cyclin B (rabbit)	WB	Santa Cruz
Cyclin E (mouse)	WB	Santa Cruz
53BP1 (rabbit)	IF	Novus Biologicals
Histone H3 (rabbit)	WB	New England biolabs
Histone H3 S10-P (rabbit)	WB, IF	Santa Cruz, New England biolabs
Polyclonal swine anti-rabbit IgG-HRP	WB	DAKO
Polyclonal goat anti-mouse IgG- HRP	WB	DAKO
Alexa Fluor® 594 goat anti-mouse IgG	IF	Invitrogen
Alexa Fluor® 488 goat anti-rabbit IgG	IF	Invitrogen

Table 2.2 Antibodies used in this study

incubated in Amplify Reagent (GE Healthcare) at room temperature on a shaker for 30 min. Gels were dried under a vacuum at 80 ^{o}C for 2 h before being exposed to autoradiography using Kodak X-Ray film.

2.4 CELL BIOLOGY TECHNIQUES

2.4.1 Transient transfection of mammalian cells using Lipofectamine LTX

Cells were added to a 24-well plate at a density of 0.7 x 10^5 cells per well into a volume of 0.5 mL media or to a 6-well plate at 3.5 x 10^5 cells per well in a volume of 2 mL media. The next day, 500 ng DNA (Table 2.4) was diluted in 100 µL Optimem (Invitrogen) or 2.5 µg DNA was diluted in 500 µL Optimem for a 24-well and a 6-well plate respectively. The solution was gently mixed with 0.5 µL or 2.5 µL Plus™ reagent (Invitrogen) and left to incubate at room temperature for 5 mins. Lipofectamine™ LTX (Invitrogen) was added at a 1:4 DNA: Lipofectamine™ LTX ratio and the solution was mixed and left to incubate for 30 mins at room temperature. After this, 100 µL or 500 µL of the DNA-Lipid complex was added dropwise to the cells in the 24-well or 6-well plate respectively. The plate was rocked gently back and forth to mix and left to incubate for 4-6 h at 37 ^{o}C. Lipofectamine™ LTX was then removed from the cells and fresh media containing 10% FCS was added to each well.

2.4.2 RNA Interference

Duplexes of RNA (approximately 21-nucleotides in length) known as short-interfering RNAs (siRNAs) inhibit endogenous gene expression in a sequence-specific manner. siRNA duplexes used in this study are shown in Table 2.5 (Dharmacon).

Protein	Vector	Amino acids	Source
hnRNPUL-1 full length	pcDNA3	1-856	Prof. T. Dobner
hnRNPUL-1 N-terminal	pcDNA3	1-453	Prof. T. Dobner
hnRNPUL-1 Middle-terminal	pcDNA3	213-732	Prof. T. Dobner
hnRNPUL-1 C-terminal	pcDNA3	400-856	Prof. T. Dobner
hnRNPUL-1 ΔBBS	pcDNA3	Δ454-611	Prof. J. Kzhyshkowska
hnRNPUL-1 ΔBBS2a/b	pcDNA3	Δ456-594	Prof. J. Kzhyshkowska
hnRNPUL-1 ΔBBS3	pcDNA3	Δ456-570	Prof. J. Kzhyshkowska
hnRNPUL-1 ΔBBS4	pcDNA3	Δ456-495	Prof. J. Kzhyshkowska
hnRNPUL-1 ΔRGG	pcDNA3	Δ612-685	Prof. J. Kzhyshkowska
hnRNPUL-1 BBS-RGG	pcDNA3	445-695	Prof. J. Kzhyshkowska
hnRNPUL-1 splice variant	pcDNA3	100-856	Dr A. Rusch
hnRNPUL-2 full length	pcDNA3	1-747	Dr S. Polo
p53 full length	pBK-CMV	1-393	Dr E. Hammond
NBS1	pSG5	1-754	Dr M. Lavin

Table 2.3 Proteins expressed in *in vitro* transcription/translation assays in this study

Gene	Vector	Application	Source
hnRNPUL-1 full length (HA)	pcDNA3	DNA transfections	Prof. T. Dobner
hnRNPUL-1 full length (GST)	pGEX 5X-1	GST pull-down with radiolabelled proteins	Prof. T. Dobner
hnRNPUL-1 BBS-RGG (GST)	pGEX 4T-1	GST pull-down with radiolabelled proteins	Prof. J. Kzhyshkowska
hnRNPUL-2 full length (GST)	pGEX 4T-1	GST pull-down with radiolabelled proteins	Prof. J. Kzhyshkowska
GST	pGEX 4T-1	GST pull-down with radiolabelled proteins	Dr R. Grand
p53 full length	pBK-CMV	DNA transfections, luciferase reporter assays,	Dr E. Hammond
p53 full length (GST)	pGEX	GST pull-down with radiolabelled proteins	Dr A. Turnell
p53 luciferase reporter	PG13-Luc	luciferase reporter assays	Dr E. Hammond
p21 luciferase reporter	p21-Luc/WWP-Luc	luciferase reporter assays	Dr A. Turnell
MDM2 luciferase reporter	pGL3-Basic	luciferase reporter assays	Dr A. Turnell
Renilla	pRL-TK	luciferase reporter assays	Dr J. O'Neill

Table 2.4 Plasmids used in this study

siRNA	Sense sequence
Non-silencing	5'-CGUACGCGGAAUACUUCGAdTdT-3'
hnRNPUL-1	5'-GCAGUGGAACCAGUACUAUdTdT-3'
MSH2	5'-UCCAGGCAUGCUUGUCUUGAAdTdT-3'
TOP2α	5'-AAAGACUGUCUGUUGAAAGAAdTdT-3'
TOP2β	ON-TARGET plus SMART pool TOP2β

Table 2.5 siRNA sequences used in this study

2.4.2.1 Transfection of siRNA into H1299 cells

Twenty four hours prior to transfection, H1299 cells were added to 6 cm dishes at a density of 3.5 x 10^5 cells per dish in a volume of 3.5 mL. The dishes were rocked gently to establish an even distribution of cells. Transient transfection of siRNA (Dharmacon) was performed the next day using Oligofectamine (Invitrogen). The transfection mixture was prepared using the following amounts per well: a volume of 2 µL of 40 µM control or 5 µL of 75 µM hnRNPUL-1 siRNA was mixed with 10 µL Oligofectamine in 1 mL Optimem (Invitrogen) and left for 30 min. After this time, 1 mL of the solution was added to each 6 cm dish containing H1299 cells that had been washed with 2 mL Optimem. After 4-6 h incubation at 37 ^{o}C, 1 mL of media supplemented with 30% FCS was added to each dish. siRNA transfections were repeated 24 h later to bring about down-regulation.

2.4.2.2 Transfection of siRNA into U2OS cells

U2OS cells were plated in 6 cm dishes twenty four hours prior to transfection at a density of 4.0 x 10^5 cells per dish in a volume of 3.5 mL and rocked as above. The transfection mixture was prepared using the following amounts per well: 2 µL of 40 µM control or 5 µL of 75 µM hnRNPUL-1 siRNA was diluted in 368 µL or 365 µL Optimem respectively to a total volume

of 370 µL. In a separate 1.5 mL microfuge tube, the Oligofectamine (8 µL) was diluted in Optimem (22 µL), mixed and left for 5-10 min at room temperature. The diluted oligofectamine (30 µL) was added to the diluted siRNA to make a total volume of 400 µL, mixed gently and left to incubate for 20 min at room temperature. After this time, 400 µL of transfection mixture was added to each 6 cm dish containing U2OS cells that had been washed with 2 mL Optimem and left with 1.6 mL Optimem. After 4-6 h incubation at 37 ^{o}C, 1 mL of media supplemented with 30% FCS was added to each dish. Transfections were repeated 24 h later to bring about down-regulation.

2.4.3 Luciferase Reporter Assay

Luciferase expression was determined using a Dual-Luciferase® Reporter (DLR™) Assay System (Promega) according to the protocol provided. This system allows the co-transfection of two different luciferase vectors, firefly and *Renilla* (Table 2.4).

H1299 cells were seeded in a 24-well plate (2.4.1) and transfected with the appropriate reporter construct, plus the *Renilla* luciferase gene as a transfection control and an untagged p53 expression plasmid in pcDNA3. Transfection of a total of 500 ng DNA in a volume of 0.5 mL media was performed using Lipofectamine™ LTX according to the manufacturer's instructions (Invitrogen). After 4-6 h incubation at 37 ^{o}C, culture media was removed from each well and replaced with 0.5 mL fresh media supplemented with 10% FCS before being incubated overnight at 37 ^{o}C.

After culture media was removed from each well, cells were washed in 0.5 mL PBS and lysed using 100 µL 1X passive lysis buffer (PLB) (Promega) with gentle rocking for 15 min at room temperature. Lysates were transferred into sterile 1.5 mL microfuge tubes and stored at -

20 ^{o}C. The activities of firefly and *Renilla* luciferases were both measured sequentially from a single sample. Cell lysates (20 µL) were added to each well of a white 96-well flat-bottomed plate (Nunc.) in which the DLR™ Assay was conducted. The activity of the firefly luciferase reporter was measured first by the addition of 50 µL Luciferase Assay Reagent II (LARII) to each well containing the lysate. The luminescent signal generated was quantified by a plate reader (Victor 1420) and the reaction was subsequently quenched by the addition of 50 µL Stop and Glo® Reagent to the same samples. This simultaneously initiated the *Renilla* luciferase reaction, generating a luminescent signal, which was also measured using the plate reader. Relative luciferase units were calculated by dividing the firefly luciferase activity by the corresponding *Renilla* luciferase assay. This resulted in normalised values since the *Renilla* reporter provided the internal control. The DLR™ Assay was performed 3 times to ensure reproducibility.

2.4.4 Flow Cytometric Analysis by Propidium Iodide (PI) staining

Flow cytometry was used to assess the cell cycle distribution of cells either transfected with hnRNPUL-1 siRNA (2.4.2) or treated with drugs (2.2.1). This technique involved passing a constant thin stream of fluid containing individual cells that had been labelled with a fluorescent dye through a laser beam. The amount of light that was scattered from the beam was recorded and was an indication of cell size. The beam also caused the fluorescent dye to emit light at various frequencies, which was also recorded and was a measure of DNA content. In these studies, the DNA-binding dye, propidium iodide (PI) (Sigma Aldrich) was used, which is a stoichiometric dye that enters the nuclei of cells and intercalates with

dsDNA. Therefore, in combination with the light scatter, PI can be used to quantify the relative DNA content during the cell cycle.

Cells were trypsinised and washed as before (2.1.3). After washing with ice-cold PBS, cells were resuspended in 3 mL ice-cold PBS and fixed by the addition of 7 mL ice-cold ethanol whilst vortexing. Fixation in alcohol enabled the uptake of PI dye, which would otherwise be pumped out of cells. Samples were stored at -20 ^{o}C until required. On the day of analysis, samples were centrifuged at 1300 *x g* at 4 ^{o}C for 5 min, after which the supernatant was discarded. The cell pellet was rehydrated with 20 mL ice-cold PBS and centrifuged again at 1300 *x g* at 4 ^{o}C for 5 min. This process was repeated and the resulting cell pellet was resuspended in 1 mL PI/RNase solution (25 µg/mL PI, 0.1 mg/mL RNase to ensure the dye intercalated to DNA only), transferred to FACS tubes (Sarstedt) and incubated at 37 ^{o}C in the dark until analysis. DNA profiles were produced by running the samples through a Coulter XL-MCL flow cytometer at a wavelength of 488 nm. The proportion of cells in G1, S and G2 phases of the cell cycle were calculated using Windows Multiple Document Interface flow cytometry application (WinMDI version 2.9). A sub-G1 peak was also seen in certain samples and these were taken as 'dead cells' (Figure 2.1).

2.5 MOLECULAR BIOLOGY TECHNIQUES

2.5.1 Antibiotics, Bacterial strains and Media

Ampicillin and kanamycin were prepared as stock solutions in sterile distilled water (100 mg/mL) and stored at -20 ^{o}C until required. Ampicillin was used at a final concentration of 50 µg/mL and kanamycin was used at a final concentration of 25 µg/mL. Bacterial cultures were grown in Luria broth (LB) (Sigma). LB (50 g) was dissolved in 2 L distilled water (pH 7.5)

and sterilised before use. LB-agar was used to make up LB-agar plates, which were made by adding 1.2% agar (Sigma) to LB and sterilising the mixture prior to use. The LB-agar was boiled and allowed to cool before the addition of the appropriate antibiotic, after which the liquid LB-agar was poured into sterile 9 cm diameter plastic Petri dishes (Corning). The newly poured plates were allowed to set at room temperature before being used for bacterial transformation.

2.5.2 Transformation of bacteria

Bacterial transformations were conducted in competent *Escherichia coli* (*E. coli*) cells. MAX efficiency Stbl2 and DH5α competent cells (Invitrogen) were used for plasmid production, and BL21-Gold competent cells (Stratagene) were used to generate recombinant proteins. For each transformation, competent cells were initially thawed on ice and a 50 µL aliquot was then combined with approximately 50 ng DNA and mixed gently. Following incubation on ice for 30 min, samples were heat-shocked at 42 ^{o}C for either 20, 25 or 30 sec (for BL21, Stbl2 and DH5α cells respectively), and cooled on ice for 2 min. After the addition of 450 µL S.O.C Medium (Invitrogen), the transformation mixture was incubated at 30 ^{o}C for either 90 min (Stbl2), or at 37 ^{o}C for 1 h (DH5α and BL21), with shaking (225 rpm). Cells were then spread onto L-agar plates, containing the appropriate antibiotic (2.5.1), which were then incubated at 37 ^{o}C overnight.

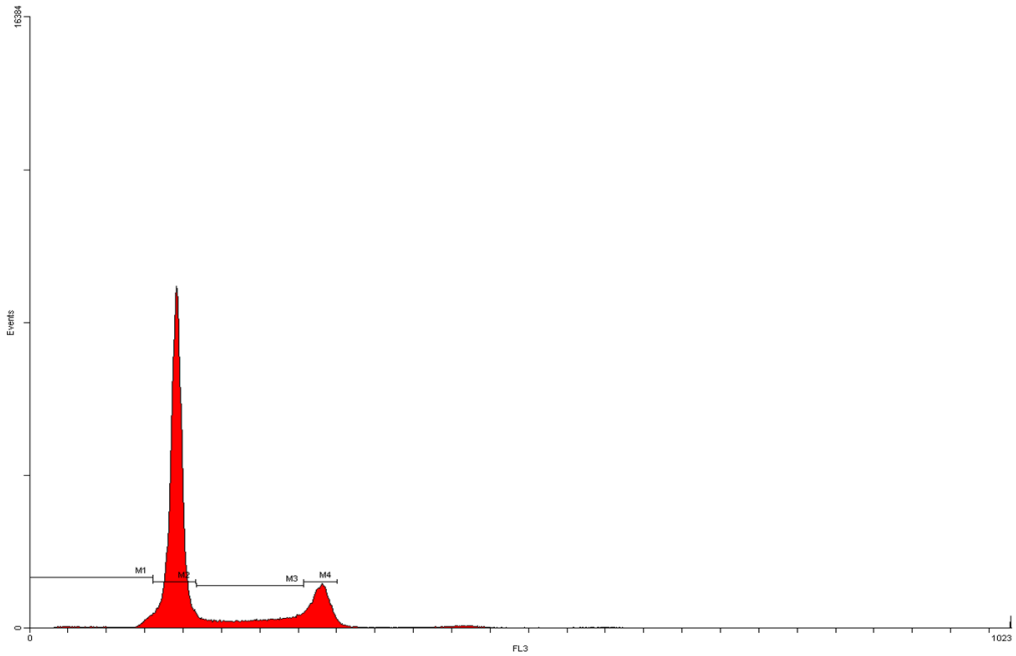

Figure 2.1 Representation of a cell cycle profile
M1 represents cells in sub-G1, M2 represents cells in G1, M3 represents cells in S and M4 represents cells in G2/M.

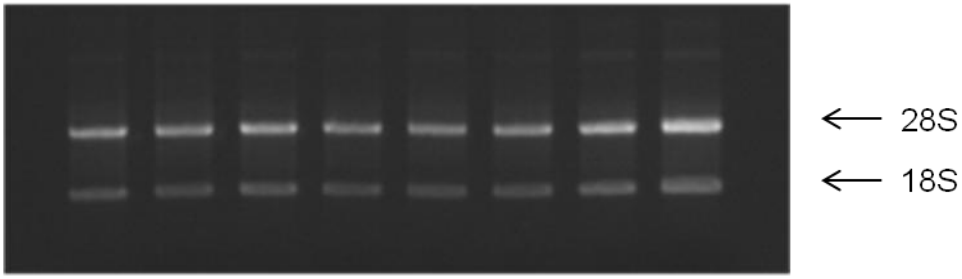

Figure 2.2 Agarose gel to determine integrity of RNA
Intact RNA exhibits 28S and 18S rRNA bands with the 28S band being approximately twice as intense as the 18S band.

2.5.3 Growth of overnight cultures from colonies

Colonies of competent cells from the L-agar plates above (2.5.2) were picked using a sterile tip and transferred to a well of a 96-well flat-bottomed plate containing 150 µL LB with appropriate antibiotic. The plate was sealed and incubated for 2 h at 37 ^{o}C, after which time it was stored at 4 ^{o}C until required.

2.5.4 Preparation of plasmid DNA

2.5.4.1 Small Scale preparation

Plamid DNA was extracted using the GenElute™ Plasmid Miniprep Kit (Sigma) according to the protocol provided. Twenty four hours prior to extraction, a single colony (10 µL of each well of the 96-well flat-bottomed plate in 2.5.3containing the desired plasmid was used to inoculate 5 mL of LB supplemented with appropriate antibiotic (2.5.1). This was incubated at 37 ^{o}C overnight with shaking (225 rpm). The following day, 3 mL of the culture was pelleted at 13, 000 *x g* for 1 min (the remaining culture was stored at -20 ^{o}C). Bacterial pellets were completely resuspended in 200 µL Resuspension Solution with RNase A. The resuspended pellets were then lysed with 200 µL Lysis Solution for 5 min. The solution was neutralised with 300 µL Neutralisation/Binding Solution and pelleted at 13, 000 *x g* for 10 min. The supernatant was transferred to a GenElute Miniprep Binding Column that had been prepared with 500 µL Column Preparation Solution. Transferred lysate was centrifuged at 13, 000 *x g* for 1 min. Following washing with 750 µL Wash Solution and centrifugation at 13, 000 *x g* for 30 sec, the DNA was eluted into a fresh collection tube with 100 µL Elution Solution and centrifugation at 13, 000 *x g* for 1 min. The eluted DNA was subsequently stored at -20 ^{o}C.

2.5.4.2 Large Scale preparation

Plasmid DNA was purified using the PureLink™ HiPure Plasmid Filter Maxiprep Kit (Invitrogen) according to the protocol provided. Initially, a single colony (10 µL of each well of the 96-well flat-bottomed plate in 2.5.3) containing the desired plasmid was used to inoculate 5 mL of LB supplemented with appropriate antibiotic (2.5.1). This was incubated at 37 ^{o}C overnight with shaking (225 rpm). Sixteen hours prior to purification, 250 µL of this was used to inoculate 200 mL LB broth supplemented with appropriate antibiotic (2.5.1) and incubated at 37 ^{o}C for 16 h with shaking. After this, the culture was transferred to a sterile centrifuge tube and centrifuged at 4000 *x g* at 4 ^{o}C for 10 min. The pellet was completely resuspended in 20 mL Resuspension Buffer with RNase (R3) before being lysed in 20 mL Lysis Buffer (L7). The cell suspension was left at room temperature for 5 min and neutralised with 10 mL Precipitation Buffer (N3). The precipitated lysate was transferred to a HiPure Filter Maxi Column, which had already been primed for adsorption of DNA by the addition of 30 mL Equilibration Buffer (EQ1), which was allowed to run by gravity flow. Bacterial lysate in the HiPure Filter Maxi Column was washed with 50 mL Wash Buffer (W8) and allowed to drain by gravity flow. Elution Buffer (E4) (15 mL) was added to elute the DNA into a fresh tube. The DNA was precipitated by the addition of 5.2 mL isopropanol (Fisher Scientific) and the purified DNA was then centrifuged at 5000 *x g* at 4 ^{o}C for 1 h. DNA pellets were resuspended in 5 mL 70% ethanol (Fisher Scientific) and centrifuged at 5000 *x g* at 4 ^{o}C for 1 h. The pellets were air dried, resuspended in 200-500 µL TE Buffer and stored at -20 ^{o}C.

2.5.5 Determination of DNA purity and concentration

DNA concentration and purity was determined using a nanodrop spectrophotometer. The concentration of DNA was quantified by measuring the absorbance at OD_{260nm} and calculated against 1 OD_{260nm} unit being an equivalent of 50 µg of DNA in 1 mL. The DNA was considered pure and free from protein and RNA contamination if the ratio of $OD_{260nm/280nm}$ was more than 1.8.

2.5.6 Preparation of GST-fusion protein

Plasmids encoding GST-tagged proteins were used to transform BL21 bacteria cells as described in 2.5.2. A 10 mL culture of the successful transformation was grown overnight at 37 ^{o}C overnight with shaking (200 rpm). The following day, this was divided between 2 flasks, each containing 500 mL LB with the appropriate antibiotic (2.5.1). These bacterial cultures were incubated at 37 ^{o}C overnight with shaking (200 rpm) until the optical density at 600 nm had reached 0.6-0.8. Isopropyl-β-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM to induce the expression of the GST-fusion protein. After incubation at 30 ^{o}C for 3 h with shaking (200 rpm), cultures were centrifuged at 5000 rpm for 15 min. The supernatant was discarded and the bacterial pellet stored at -80 ^{o}C until required.

To purify the GST-fusion protein, the pellet was thawed, resuspended in 50 mL ice-cold buffer A (2 mM EDTA, 1% Triton X-100 in PBS) and sonicated on ice for 3 x 30 sec. Cell debris was removed by centrifugation at 18000 rpm for 30 min. Glutathione-agarose beads (3 mL of a 50% slurry, Sigma) were added to the clarified supernatant and rotated at 4 ^{o}C for 3 h. Following this, the beads were pelleted by centrifugation at 1500 rpm for 5 min and the supernatant was incubated with a second batch of glutathione-agarose beads as before. The

beads were washed three times with buffer A and once with buffer B (2 mM EDTA in PBS). GST-fusion proteins were eluted from the washed beads by the addition of 5 mL GST elution buffer (25 mM glutathione pH 8.2) and rotation for 1.5 h. After this time, the beads were removed by centrifugation at 1500 rpm for 5 min. The supernatant containing the eluted GST-fusion protein was dialysed overnight at 4 ^{o}C against a solution of 0.15 M NaCl, 20 mM Tris pH 7.4, 2 mM DTT). The following day, dialysis was repeated for 3 h in fresh buffer and GST-fusion proteins were collected, aliquoted and stored at -80 ^{o}C until required.

2.5.7 *In-vitro* transcription/translation

Eukaryotic *in vitro* transcription/translation was conducted using the TNT® Coupled Reticulocyte Lysate System according to the manufacturer's instructions (Promega). Each reaction mixture contained 25 µL TNT® rabbit reticulocyte lysate, 2 µL TNT® reaction buffer, 1 µL RNasin® ribonuclease inhibitor (Promega), 1 µL amino acid mixture minus methionine, 2 µL [^{35}S]-methionine (1000Ci/mmol at 10mCi/mL; MP Biomedicals), 0.5 µg/ µL DNA template, 1 µL TNT® T7 RNA polymerase and nuclease-free water to a final volume of 50 µL. The reaction mixture was incubated at 30 ^{o}C for 90 min. To determine the expression of *in vitro* translated protein, 1-2 µL of the sample was subjected to SDS-PAGE, stained with Coomassie blue, destained, incubated in Amplify and dried under vacuum to visualise proteins as described in 2.3.9.

2.5.8 Site directed mutagenesis

2.5.8.1 Primer design

To generate hnRNPUL-1 constructs resistant to hnRNPUL-1 siRNA, silent mutations were introduced in hnRNPUL-1 siRNA sequence using site-directed mutagenesis (Stratagene) according to the manufacturer's instructions. The hnRNPUL-1 siRNA sequence is shown by sequence (A). Silent mutations were introduced (depicted as the underlined bases on the bottom sequence) as shown by sequence (B) using the forward primer 5'-AGTATGCCCAGCAATGGAATCAATACTATCAGAACCAGGG-3' and the reverse primer 5'- CCCTGGTTCTGATAGTATTGATTCCATTGCTCGGCATACT-3'. The hnRNPUL-1 siRNA-resistant construct was then generated following steps 2.5.8.2-2.5.8.8.

(A) 5'-GCAGUGGAACCAGUACUAU-dTdT-3'

(B) 5'-GCA<u>A</u>UGGAA<u>U</u>CA<u>A</u>UACUAU- dTdT -3'

2.5.8.2 QuikChange Site-Directed Mutagenesis parameters

The mutant strand synthesis reaction was performed in thin-walled tubes using Pfu Ultra DNA polymerase (Stratagene). A 50 µL reaction was made up containing 5 µL 10X Buffer, 1 µL DNA (40 ng), 1.25 µL forward primer (125 ng), 1.25 µL reverse primer (125 ng), 1 µL dNTP mix (each dNTP at 10 mM) and 40.5 µL injection water (Fannin). The Pfu Ultra DNA polymerase (1 µL) was added last and the reaction mixture was spun down. The tubes were placed in a thermal cycler (Applied Biosystems, Veriti 96-well thermal cycler) and the following cycling parameters were applied: 1 cycle (heatshock) of 95 ^{o}C for 30 sec; 18 cycles

(elongation) of 95 ^{o}C for 30 sec, 55 ^{o}C for 1 min, 68 ^{o}C for 8 min (1 min/Kb of plasmid DNA length).

2.5.8.3 *Dpn* I Digestion of the amplification product

Dpn I restriction enzyme (Stratagene) (1 µL) was added directly to each tube to digest the supercoiled DNA from the amplification product. The contents were mixed, transferred to 0.5 mL microfuge tubes and incubated for 1 h at 37 ^{o}C.

2.5.8.4 Transformation of XL1-Blue Supercompetent Cells

Transformation of XL1-Blue Supercompetent Cells (Stratagene) was performed according to the protocol provided and in a manner similar to 2.5.2. *Dpn* I-treated DNA (1 µL) was added to 50 µL thawed competent cells and incubated on ice for 30 min. Samples were heat-shocked for 45 sec at 42 ^{o}C, cooled on ice for 2 min and then incubated with S.O.C Medium (500 µL) for 90 min at 30 ^{o}C with shaking (225 rpm). Cells were subsequently spread onto L-agar plates (250 µL on each of two plates) as before (2.5.2).

2.5.8.5 Polymerase Chain Reaction using bacterial culture

Single colonies of competent cells from 2.5.8.4were picked as described before and grown in LB with appropriate antibiotic in a 96-well flat-bottomed plate for 2 hours at 37 ^{o}C (2.5.3). PCR amplication was conducted in thin-walled PCR tubes using Taq polymerase (Roche) and pcDNA3 primers (company). A 25 µL reaction was set up containing 0.25 µL Taq polymerase, 2.5 µL 10X Buffer, 1 µL forward primer 1 µL reverse primer, 0.75 µL dNTP mix (each dNTP at 10 mM), 18.5 µL injection water and finally 1 µL culture from the appropriate well of the 96-well flat-bottomed plate. The tubes were placed in a thermal cycler and the

following cycling parameters were applied: 1 cycle of 94 ^{o}C for 4 min; 37 cycles of 94 ^{o}C for 15 sec, 60 ^{o}C for 30 sec, 68 ^{o}C for 3 min followed by a final 10 min extension at 68 ^{o}C.

2.5.8.6 Purification of PCR product

Amplified DNA was purified using the PureLink™ PCR Purification Kit (Invitrogen) according to the protocol provided. This allowed primers, dNTPs, enzymes and salts to be removed from the PCR products before the DNA was used. Initially, 4 volumes of Binding Buffer was added to 1 volume of PCR product, mixed and transferred to a collection tube in a PureLink® Spin Column to allow the dsDNA to bind to the column. The mixture was centrifuged at 10,000 *x g* at room tempterature for 1 min after which, the flow-through was discarded. Wash Buffer with ethanol (650 µL) was added to the spin column and centrifuged at 10,000 *x g* at room temperature for 1 min to remove any impurities from the DNA. The flow-through was discarded and any remaining Wash Buffer was removed by further centrifugation at maximum speed. The collection tube was consequently discarded and the spin column placed into a fresh elution tube. Elution Buffer (50 µL) was added to the spin column and the contents were incubated for 1 min at room temperature before being centrifuged at maximum speed for 2 min. The purified DNA was stored at -20 ^{o}C until required.

2.5.8.7 Agarose gel electrophoresis

DNA was viewed on a 0.8% agarose gel. Agarose powder (0.8 g) (Sigma) was dissolved in 100 mL 1X TBE buffer (89 mM Tris, 89 mM Boric Acid, 2 mM EDTA) and heated to boiling point. The agarose was allowed to cool before 1 µL Sybr Safe DNA gel stain (Invitrogen) was added. PCR product (1 µL) or DNA from a miniprep (5 µL) was diluted with 6X loading

buffer (0.25% Bromophenol blue, 30% glycerol in a 10 mM Tris, 1 mM EDTA, [pH 8.0] solution). The electrophoresis tank was filled with 1X TBE and diluted samples were loaded onto the agarose gel alongside a DNA molecular weight marker (Roche). Electrophoresis was conducted at 120 V for 30 min – 1 h after which, the DNA was visualised using a Safe Image blue-light transilluminator.

2.5.8.8 DNA sequencing

Mutations in plasmid DNA were validated by sequencing using the Big Dye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). A 20 µL sequencing reaction was made up containing 7 µL 5X sequencing buffer, 1 µL BigDye® terminator v3.1 Ready Reaction mix and 10 µL water. The reaction was placed in a thermal cycler and the following cycling parameters were applied; 25 cycles of 95 ^{o}C for 10 sec, 55 ^{o}C for 5 sec and 60 ^{o}C for 4 min. Precipitation of the PCR product (20 µL) was conducted by adding 2 µL 3 M sodium acetate and 50 µL 100% ethanol and incubating for 5 min at room temperature. The sample was centrifuged at 13,000 rpm for 20 min after which, the supernatant was discarded. Ethanol (70%) (250 µL) was added and the sample was centrifuged at 13,000 rpm for 5 min at room temperature. The supernatant was discarded and the sample was air-dried for at least 10 min to remove any residual ethanol. Hi-Dye (10 µL) (Applied Biosystems) was added to the sample to dissolve the DNA pellet, which was then denatured for 5 min at 95 ^{o}C. The sample was consequently placed on ice to quench any re-duplexing of DNA. Sequencing was performed in a 96-well plate in a ABI Prism 337 Automated DNA Sequencer using the Applied Biosystems, 3130*xl* Genetic Analyser. Sequences were analysed using FinchTV version 1.4.0 (Geospiza Inc.) software programme. Once the mutations in the sequence had

been analysed, plasmid DNA was purified using the PureLink™ HiPure Plasmid Filter Maxiprep Kit (2.5.4.2).

2.5.9 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

2.5.9.1 RNA Extraction

Cell were pelleted as before (2.3.1) except for the final wash in cold PBS, which was omitted, and stored at -80 ^{o}C until total RNA was extracted from cell lysates using the RNeasy Mini kit (Qiagen) according to the protocol provided. A maximum of 1 x 10^7 cells were lysed in RNeasy Lysis Buffer (buffer RLT) provided by passing the lysate at least 5 times through a 20-gauge needle (0.9 mm diameter) fitted to an RNase-free 1 mL syringe. One volume of 70% ethanol was added to the homogenised lysate and mixed by pipetting. This was transferred to an RNeasy spin column and centrifuged for 15 sec at 8000 x *g*. RNeasy Wash buffer (buffer RW1) was added to the RNeasy spin column and centrifuged for 15 sec at 8000 x *g*. Concentrated Wash Buffer (buffer RPE) (500 μL) was added to the RNeasy spin column and centrifuged for 15 sec at 8000 x *g*. Buffer RPE (500 μL) was added again to the RNeasy spin column and subjected to a longer centrifugation for 2 min at 8000 x *g*. RNA was eluted using RNase-free water (30-50 μL), incubated with DNase (1 μL) for 20 min at 37 ^{o}C and stored at -80 ^{o}C until further required.

2.5.9.2 Determination of RNA purity and concentration

The extracted RNA was run on 0.8% agarose gel to determine its integrity. When visualised using the Safe Image blue-light transilluminator, two clear bands should have been seen; these correspond to the 28S and 18S rRNA bands of intact RNA where the 28S band should be

approximately twice as intense as the 18S band (Figure 2.2). RNA concentration was determined by spectrophometric analysis where the absorbance at OD_{260nm} m was measured and calculated against 1 OD_{260nm} unit being an equivalent of 40 μg of RNA in 1 mL. The RNA was considered to be pure and free from DNA and protein concentration if the ratio of $OD_{260nm/280nm}$ was more than 1.7.

2.5.9.3 Production of genomic cDNA

Total RNA (1 μg) was converted into single-strand cDNA using the Reverse Transcriptase System (Promega). RNA from 2.5.9.1 was incubated for 10 min at 70 ^{o}C, centrifuged and placed on ice until further required. The reverse transcription reaction was prepared as follows: 4 μL $MgCl_{2,}$ 2 μL 10X Reverse Transcriptase Buffer, 2 μL dNTP mix (each dNTP at 10 mM), 0.5 μLRecombinant RNasin® Ribonuclease Inhibitor, 0.7 μL AMV (reverse transcriptase), 1 μL Random primers and 1 μg RNA that had been prepared previously. The reaction was made up to a volume of 20 μL with RNase-free water. The reaction was mixed, incubated for 10 min at room temperature to allow primer extension and then for a further 15 min at 42 ^{o}C. To inactivate the reverse transcriptase and to degrade the RNA, the reaction was incubated for 5 min at 95 ^{o}C before incubating it on ice for 5 min. The resulting cDNA was used as a template for the PCR assay using primers for specific genes (Table 2.6). All primers for qRT-PCR were obtained from Invitrogen and designed to anneal at 60 ^{o}C and were at a stock concentration of 200 μM.

2.5.9.4 Real-time PCR: SYBR® green detection

The PCR was performed in the Applied Biosytem 7500 Fast Real-Time PCR System using Fast SYBR® green as recommended by the manufacturer (Applied Biosystems). Fast SYBR® green containing SYBR Green I dye, (which binds to double-stranded DNA and allows its detection), AmpliTaq® Fast DNA polymerase Ultra Pure (UP) (amplifies the target sequence), dNTPs, Uracil-DNA Glycosylase (UDG) (prevents the reamplification of carryover PCR products) and ROX™ dye (internal reference to which the reporter-dye signal can be normalised during data analysis) was supplied as a 2X concentration in an optimised buffer. Each reaction contained the following: 0.25 μL Forward primer (200 nM), 0.25 μL Reverse primer (200 nM), 5.5 μL H_2O, 10 μL Fast SYBR® green and 4 μL cDNA (20 ng), to make the reaction volume up to 20 μL. The reactions were performed in MicroAmp™ Fast Optical 96-Well Reaction Plates (Applied Biosystems) according to the following thermal cycling conditions: 1 cycle of 95 ^{o}C for 20 sec (AmpliTaq®Fast DNA polymerase UP activation); 40 cycles of 95 ^{o}C for 3 sec (denaturing) and 60 ^{o}C for 30 sec (annealing and extending). The PCR reactions were subjected to a heat dissociation protocol present in the Applied Biosytem 7500 Fast Real-Time PCR System software.

Primer Name	Sequence
DDB2 for	5’-TCAAGGACAAACCCACCTTC-3’
DDB2 rev	5’-GTGACCACCATTCGGCTACT-3’
p21 for	5’-GACACCACTGGAGGGTGACT-3’
p21 rev	5’-GGCGGTTGGAGTGGTAGAAA-3’
MDM2 for	5’-ATGAAAGCCTGGCTCTGTGT-3’
MDM2 rev	5’-TCCTGATCCAACCAATCACCT-3’
Bax for	5’-TCTGACGGCAACTTCAACTG-3’
Bax rev	5’-CTCAGCCCATCTTCTTCCAG-3’
Actin for	5’-GTCTTCCCCTCCATCGTG-3’
Actin rev	5’-TCGCCCACATAGGAATCCTTC-3’
pcDNA3.1 for	5’-ACTCACTATAGGGAGACCCAAGC-3’
pcDNA3.1 rev	5’-GCAACTAGAAGGCACAGTCGAGG-3’

Table 2.6 Sequences of the primers used in this study
‘For’ denotes forward primer and ‘rev’ denotes reverse primer.

CHAPTER 3

HNRNPUL-1 REPRESSES THE TRANSCRIPTIONAL ACTIVITY OF P53

CHAPTER 3 HNRNPUL-1 REPRESSES THE TRANSCRIPTIONAL ACTIVITY OF p53

3.1 INTRODUCTION

The DDR is of fundamental importance to preserve eukaryotic genome integrity and to maintain cell viability. In response to genotoxic stress, numerous key regulators function to initiate a cascade of protein recruitment that promotes DNA repair and cell cycle arrest. An important component of the DDR is the transcription factor p53, which is described as the 'guardian of the genome' (Lane, 1992). Upon its activation in response to genotoxic stress, p53 prevents the propagation of damaged cells by inducing a number of possible cellular responses including cell cycle arrest, apoptosis or DNA repair (Vogelstein *et al.*, 2000). One of the main mechanisms whereby p53 mediates cell cycle arrest is through the transactivation of the CKI, p21. CDKs control progression through the mammalian cell cycle and their activities, in turn, are regulated by CKIs (Sherr and Roberts, 1999). The small 165 amino acid protein p21 (also known as $p21^{WAF/Cip1}$), inhibits cell cycle progression following DNA damage primarily through its ability to inhibit the kinase activity of CDK2 and CDK4. This, in turn inhibits the phosphorylation of the retinoblastoma protein, Rb leading to the inactivation of E2F1 activity in late G1 (Delavaine and La Thangue, 1999, Dimri *et al.*, 1996). Consequently, E2F1-dependent transcription of genes that would normally promote proliferation is inhibited and growth arrest in G1 occurs (Dimri *et al.*, 1996). There is also evidence to support a role for p21 in the inhibition of PCNA. Indeed, it has been shown that different domains of p21 are responsible for binding to and inhibiting different substrates; its

amino terminal domain is responsible for inhibiting the kinase activity of the CDKs and its carboxy terminal domain inhibits PCNA (Chen *et al.*, 1995). The ability of p21 to bind to PCNA makes it unique among the CKIs (Cayrol *et al.*, 1998).

Since it has a central role in coordinating the DDR, p53 is regulated by numerous mechanisms including post-translational modifications such as phosphorylation, acetylation, ubiquitylation and methylation (Lavin and Guevan, 2006). Under normal conditions (i.e. in unstressed cells), p53 is regulated and kept at low levels by the action of MDM2. As the main E3 ubiquitin ligase for p53, MDM2 targets p53 for proteosomal degradation (Haupt *et al.*, 1997). It is not only post-translational modifications that are in place to regulate p53; many co-factors exist that interact with p53 to stimulate or inhibit its activity and, consequently, affects promoter specificity (Kruse and Gu, 2009a). Co-factors can act directly or indirectly to regulate p53 activity and many are involved in influencing the promoter-specific activity of p53 to result in different p53-dependent cellular outcomes (Vousden and Prives, 2009). Some examples of p53 co-factors include Hzf, p300/CBP and members of the ASPP family of proteins (Das *et al.*, 2007, Lill *et al.*, 1997, Samuels-Lev *et al.*, 2001).

The cellular protein, hnRNPUL-1, was first identified as an adenovirus 5 (Ad 5) E1B-55K interacting protein and was found to share high sequence homology with the hnRNPU/SAF-A protein (Gabler *et al.*, 1998). In keeping with its membership of the hnRNP family, hnRNPUL-1 is involved in pre-mRNA processing, nucleocytoplasmic mRNA transport and mRNA localisation, translation and stability (Krecic and Swanson, 1999). However, its functions are not limited to mRNA metabolism; for example there is evidence to suggest that hnRNPUL-1 plays an integral part in the ATR-dependent signalling pathway in response to cellular stress and more recently, in DNA-end resection (Blackford *et al.*, 2008, Polo *et al.*,

2012). In addition, the involvement of hnRNPUL-1 in transcriptional regulation has previously been reported with the finding that it inhibits basal transcription in a manner that is similar to hnRNPU/SAF-A (Kzhyshkowska *et al.*, 2003). Notably, an interaction between hnRNPU/SAF-A and the transcriptional co-factor, p300 has been reported previously, which further suggests a role of the hnRNP family and transcriptional regulation (Martens *et al.*, 2002). The link between the hnRNP family and the regulation of transcription can be further studied using p53, since the latter is one of the most widely-studied transcription factors. Indeed, it has already been established that another member of the hnRNP family, hnRNPK, acts as a transcriptional co-activator for p53 in response to DNA damage (Moumen *et al.*, 2005). Previous work from our laboratory has shown that hnRNPUL-1 can bind directly to p53 via the C-terminal region of p53 and over-expression studies revealed that increasing exogenous levels of hnRNPUL-1 resulted in the repression of p53-dependent transcriptional activity, although the exact mechanism by which this occurs remains unknown (Barral *et al.*, 2005). These data suggest that there is a relationship between transcriptional regulation and hnRNPUL-1.

This study aims to:

- Confirm, using an siRNA approach, whether hnRNPUL-1 is acting to repress p53 transcriptional activity
- Ascertain whether hnRNPUL-1 affects p53 target genes and consequent responses that are mediated by p53 transcriptional activation
- Determine the site of interaction on hnRNPUL-1for p53

3.2 RESULTS

3.2.1 hnRNPUL-1 interacts with p53 *in vivo* before and after DNA damage

To confirm that hnRNPUL-1 does indeed associate with p53, co-immunoprecipitation assays were conducted. These were also undertaken to examine whether the interaction between the two proteins would be affected following the induction of DNA damage. Endogenous hnRNPUL-1 was, therefore, immunoprecipitated from U2OS cells before and after treatment with 10 Gy IR and 50 J m^{-2} UV. Immunoblotting was used to detect any association between hnRNPUL-1 and p53 *in vivo*. The results from the co-immunoprecipitation assay revealed that p53 was found in hnRNPUL-1 immunocomplexes before and after both types of damage (Figure 3.1A). A reciprocal co-immunoprecipitation experiment was performed to confirm these results, where endogenous p53 was immunoprecipitated from similarly-treated U2OS cells and immunoblotting was used to detect the amount of complexed hnRNPUL-1. The results in Figure 3.1B indicate that hnRNPUL-1 associated with p53 before and after treatment with IR and UV. These data indicate that hnRNPUL-1 and p53 associate with each other before and after DNA damage.

3.2.2 Depletion of hnRNPUL-1 increases the transcriptional activity of p53

To investigate further the relationship between hnRNPUL-1 and p53 and because of previous over-expression work implicating hnRNPUL-1 in the regulation of the transcriptional activity of p53 (Barral *et al.*, 2005), the effect of hnRNPUL-1 depletion on p53 transcriptional activity

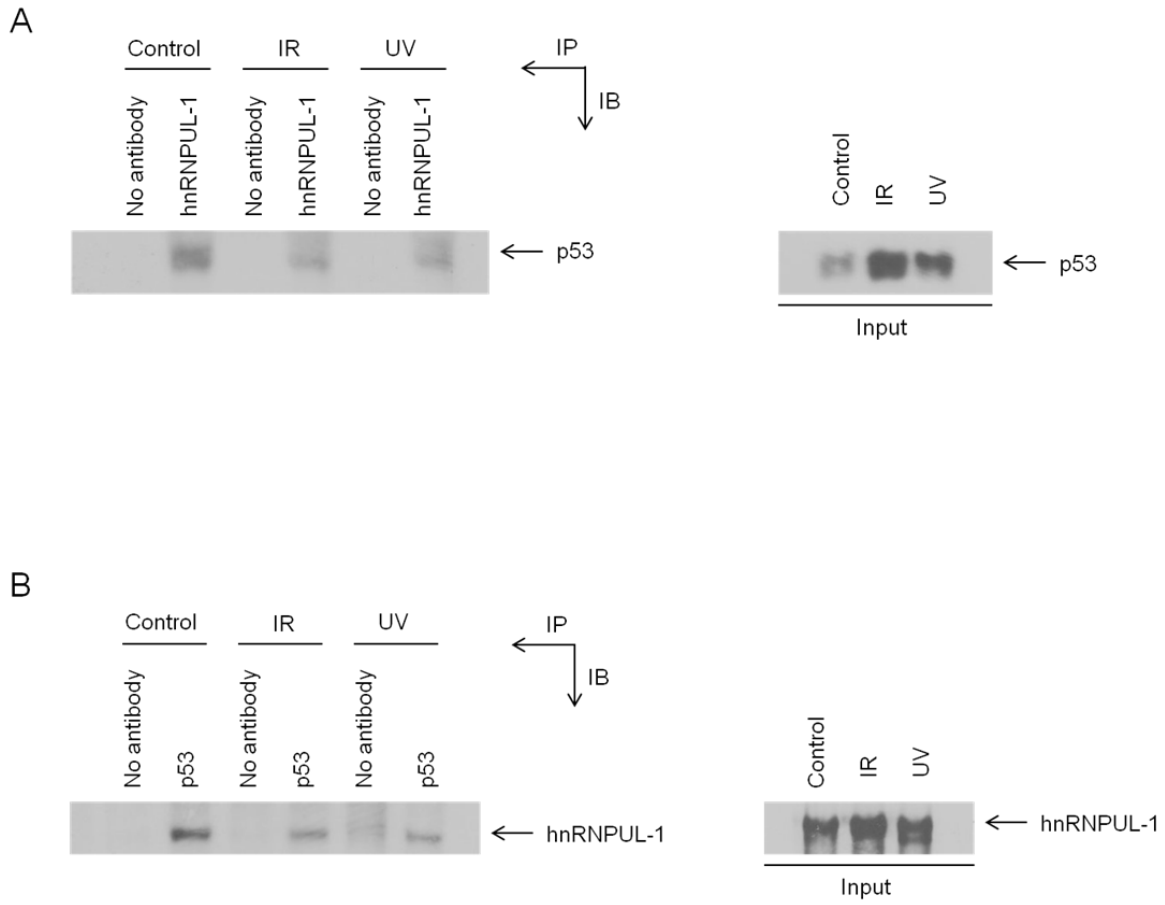

Figure 3.1 hnRNPUL-1 and p53 associate before and after DNA damage *in vivo*
U2OS cells were mock-treated or treated with 10 Gy IR or 50 J m^{-2} UV as indicated and harvested 1 h following irradiation. (A) hnRNPUL-1 was immunoprecipitated from whole cell extracts and western blotting was used to detect co-immunoprecipitating protein, p53; (B) Reciprocal co-immunoprecipitation experiment where p53 was immunoprecipitated and western blotting used to detect complexed hnRNPUL-1. Immunoprecipitates performed using no antibodies were used as controls. IP and IB denote immunoprecipitation and immunoblot respectively.

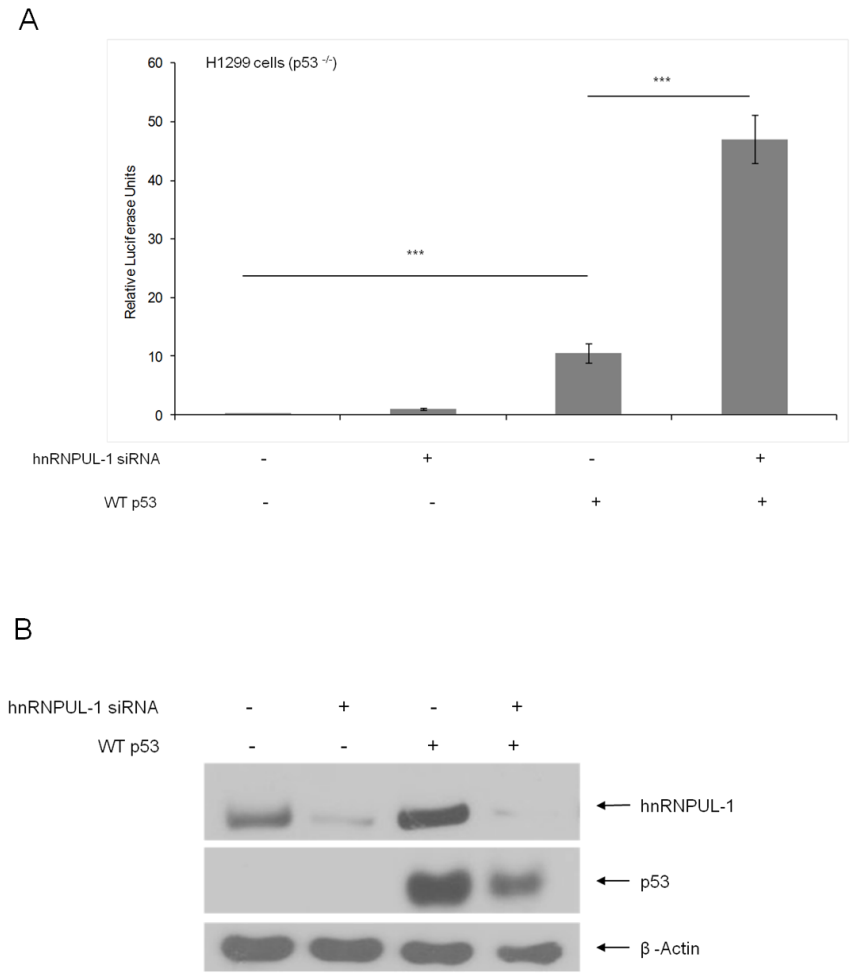

Figure 3.2 hnRNPUL-1 negatively regulates p53 transcriptional activity
(A) H1299 (p53 null) cells were transfected with siRNA, 150 ng p53 luciferase reporter construct, 50 ng *Renilla* and 5 ng WT p53 as indicated. Luciferase activity was determined after 24 h and normalised to *Renilla* activity. Standard deviations from three independent experiments are presented, with P-values depicting significant differences (*=P<0.05 significant, **=P<0.01 highly significant, ***=P<0.001 very highly significant); (B) H1299 cells were transfected as before and whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with antibodies against hnRNPUL-1 and p53. An antibody against β-actin was used as a loading control.

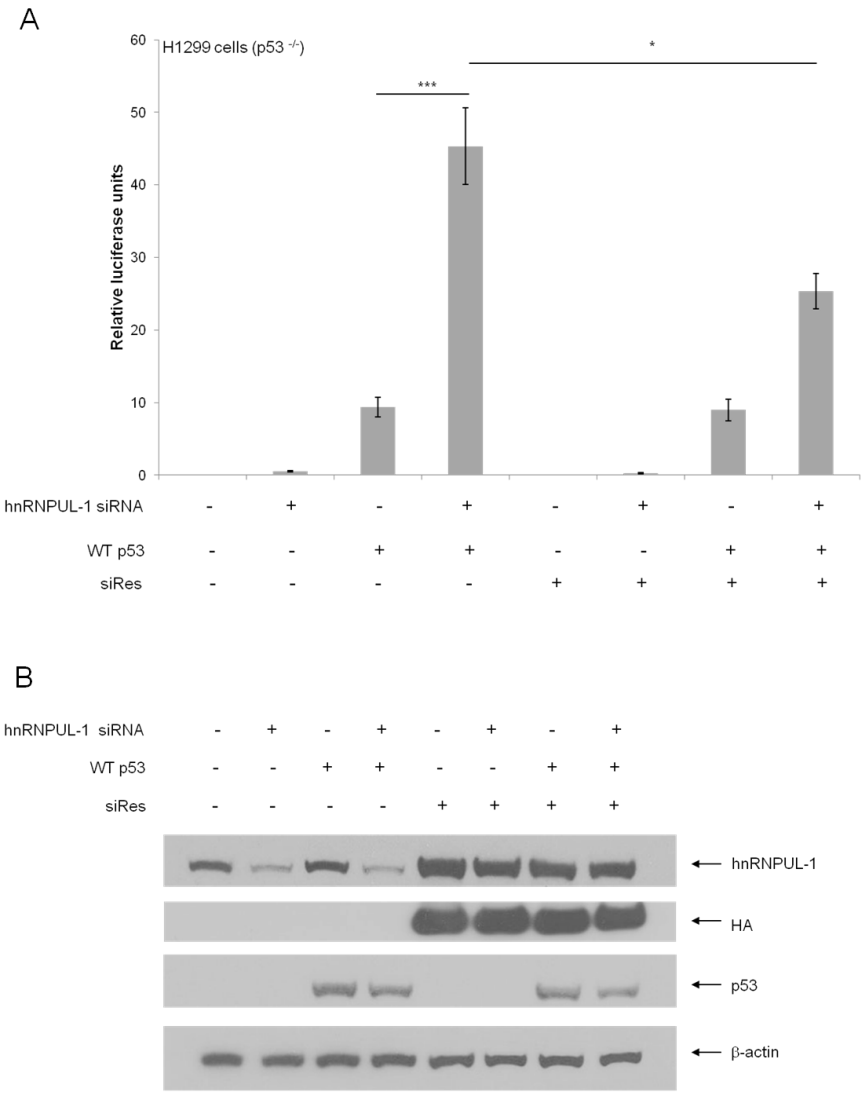

Figure 3.3 hnRNPUL-1-mediated effects are reversed by siRNA-resistant hnRNPUL-1 expression

(A) H1299 cells were transfected as before (Figure 3.2) with siRNA-resistant hnRNPUL-1 as indicated. Luciferase activity was determined and normalised to *Renilla* activity. Standard deviations from three independent experiments are presented, with P-values depicting significant differences (*=P<0.05 significant, **=P<0.01 highly significant, ***=P<0.001 very highly significant); (B) H1299 cells were transfected as before and whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with antibodies against hnRNPUL-1 and p53. An antibody against β-actin was used as a loading control. An antibody against HA was used to assess the expression of siRNA-resistant hnRNPUL-1.

was examined using a luciferase reporter assay. The p53-null H1299 cell line was transfected with control or hnRNPUL-1 siRNA, WT p53 as indicated and a reporter construct containing p53 responsive elements upstream of the luciferase gene. Following transfection of WT p53 into the control siRNA-treated cells, a 10-fold increase in luciferase activity was observed compared to cells without p53 (Figure 3.2A). This indicates that binding of p53 to the p53 binding site on the promoter construct resulted in the transcription of the luciferase gene and consequent luminescence. The difference between the relative luciferase units was reflected in the very highly significant P-value, which indicated that the response was p53-dependent. Interestingly, following transfection of p53 in cells depleted of hnRNPUL-1, the p53-dependent luciferase activity increased 50-fold compared to cells containing endogenous hnRNPUL-1, which demonstrated that hnRNPUL-1 negatively regulates p53-dependent transcription (Figure 3.2A). Again, a very highly significant P-value revealed the difference between the sample transfected with WT p53 containing endogenous hNRNPUL-1 and the sample transfected with WT p53 that was depleted of hnRNPUL-1.

The efficiency of the depletion of hnRNPUL-1 by siRNA was confirmed at the protein level by Western blotting (Figure 3.2B). Figure 3.2B also confirmed that the increase in p53 transcriptional activity after hnPNPUL-1 depletion was not due to an increase in p53 protein levels: indeed, there was reproducibly less p53 in samples treated with hnRNPUL-1 siRNA compared with samples treated with control siRNA. Reasons for this are not clear at the moment.

To confirm that the results obtained using the hnRNPUL-1 siRNA were not due to ‘off target’ effects, an expression vector encoding WT hnRNPUL-1 was designed with 3 point mutations within the siRNA-targeting regions to render it resistant to siRNA (2.5.8.1). Luciferase assays

were then used to examine whether the expression of siRNA-resistant hnRNPUL-1 could restore the phenotype observed in hnRNPUL-1-depleted H1299 cells i.e. whether the increase in p53 transcriptional activity observed in Figure 3.2A would be diminished upon transfection of siRNA resistant hnRNPUL-1. As expected, expression of siRNA-resistant hnRNPUL-1 decreased p53 transcriptional activity appreciably, though not entirely, indicating that the effect is due to depletion of hnRNPUL-1 (Figure 3.3A). The lack of total suppression may be due to an incomplete transfection of siRNA-resistant hnRNPUL-1. The transfection of siRNA-resistant hnRNPUL-1 with control or hnRNPUL-1 siRNA and WT p53 was seen at the protein level in Figure 3.3B. The expression of HA-tagged siRNA resistant hnRNPUL-1 was observed using an antibody against HA (Figure 3.3B). Taken together, these data confirm that hnRNPUL-1 is acting to negatively regulate p53 transcriptional activity.

3.2.3 p53-dependent transcription of specific target genes increases after hnRNPUL-1 depletion

Since it was observed that hnRNPUL-1 affected the transcriptional activity of p53, the effect of hnRNPUL-1 depletion on specific p53 transcriptional targets was assessed. One major transcriptional target of p53 is the CKI, p21, which plays an important role in p53-mediated G1 arrest (Deng *et al.*, 1995, El-Deiry *et al.*, 1993). Using the p53 specific response element in the p21 promoter in the reporter construct, luciferase assays were conducted again to examine whether hnRNPUL-1 affects p53 transactivation of p21. H1299 cells were transfected with control or hnRNPUL-1 siRNA, WT p53 and a reporter construct containing the gene coding for luciferase under the control of the p53-responsive *p21* promoter. Luciferase expression was strongly induced in cells that had been transfected with hnRNPUL-

1 siRNA and WT p53 compared to cells treated with control siRNA and WT p53, therefore indicating that hnRNPUL-1 negatively regulates the p53-mediated transactivation of p21 (Figure 3.4A). This was reflected in the P-value depicting a highly significant difference in relative luciferase units in samples containing endogenous hnRNPUL-1 compared to samples depleted of hnRNPUL-1 (Figure 3.4A). Surprisingly, Figure 3.4A also showed that when hnRNPUL-1 was depleted, there was a small increase in p21 transactivation in the *absence* of p53.

Evidence of hnRNPUL-1 depletion by siRNA was seen by Western blotting in Figure 3.4B. Again, it is of note that p53 protein levels are slightly reduced in samples treated with hnRNPUL-1 siRNA despite the very highly significant increase in p53-dependent transactivation of p21 in these samples compared to samples containing endogenous hnRNPUL-1 (Figure 3.4B).

A similar experiment was conducted using a reporter construct containing the luciferase gene under the control of the p53-responsive *MDM2* promoter. Notably, in H1299 cells depleted of hnRNPUL-1 and transfected with WT p53, there was an increase in luciferase expression compared to cells treated with control siRNA and transfected with WT p53 (Figure 3.5A). Again, the P-value represents the highly significant increase in relative luciferase units in samples depleted of hnRNPUL-1 compared to those containing endogenouse hnRNPUL-1 (Figure 3.5A).

The depletion of hnRNPUL-1 at the protein level was examined by Western blotting in Figure 3.5B. This also showed that the p53 protein levels were reduced in samples depleted of hnRNPUL-1, confirming that the increase in p53-dependent transactivation of MDM2 was

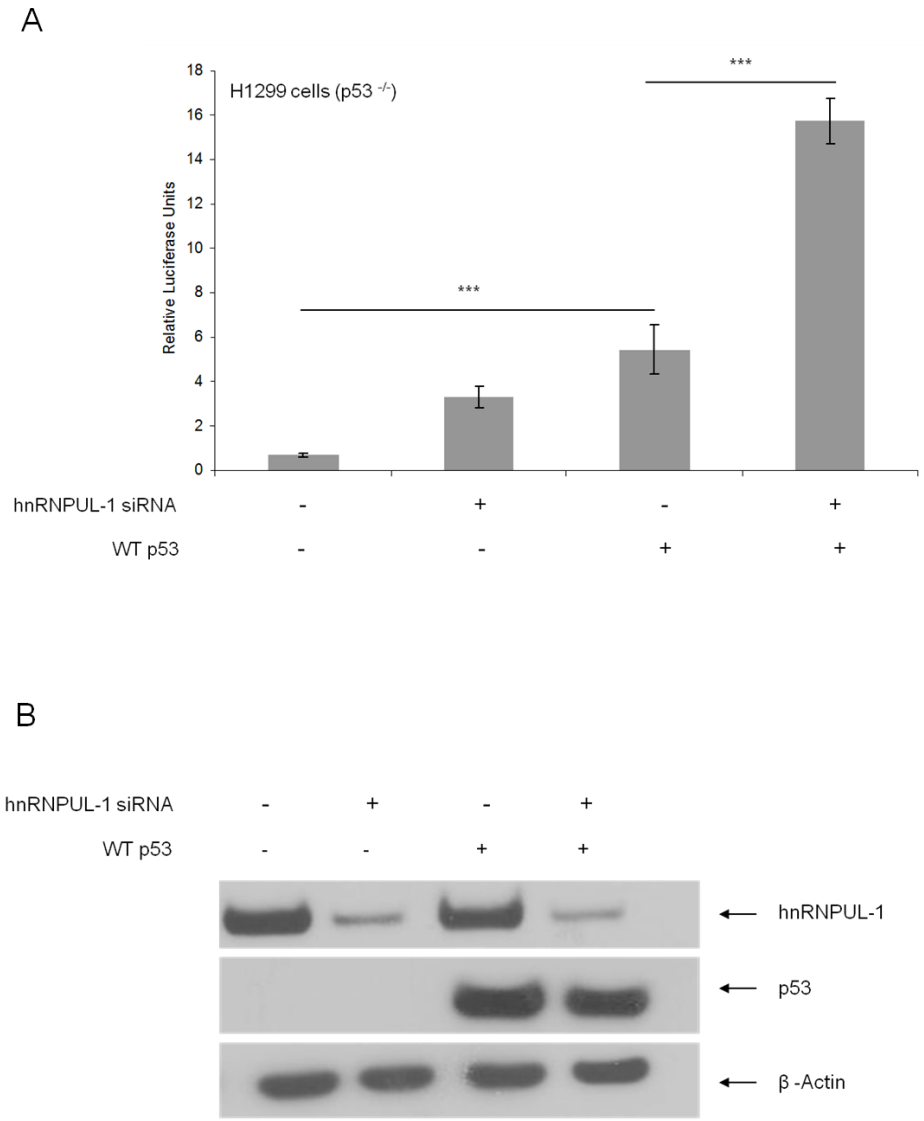

Figure 3.4 Depletion of hnRNPUL-1 results in an increase in p53-dependent transactivation of p21

(A) H1299 cells were transfected with siRNA, 150 ng *p21* promoter-specific reporter construct, 50 ng *Renilla* and 5 ng WT p53 as indicated. Luciferase activity was determined after 24 h and normalised to *Renilla* activity. Standard deviations from three independent experiments are presented, with P-values depicting significant differences (*=P<0.05 significant, **=P<0.01 highly significant, ***=P<0.001 very highly significant); (B) H1299 cells were transfected as before and whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with antibodies against hnRNPUL-1 and p53. An antibody against β-actin was used as a loading control.

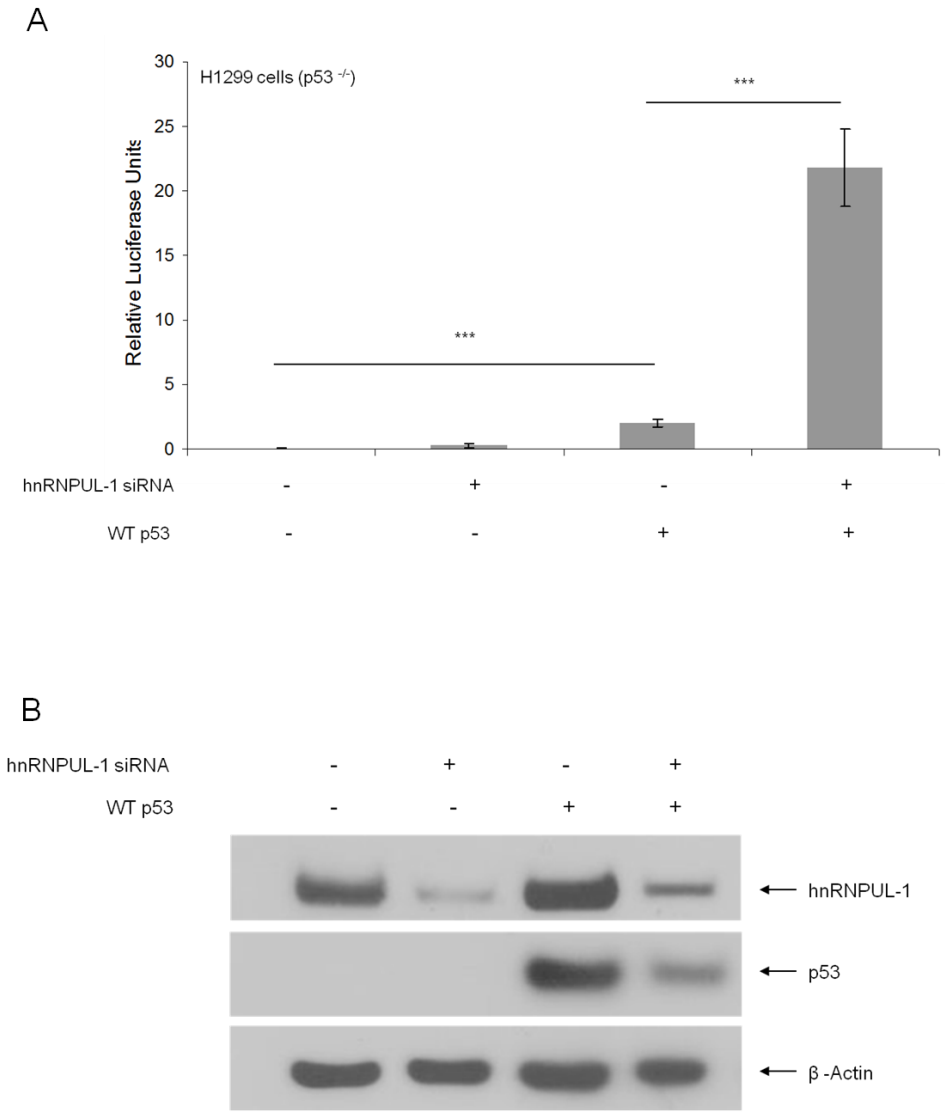

Figure 3.5 Depletion of hnRNPUL-1 results in an increase in p53-dependent transactivation of MDM2

(A) H1299 cells were transfected with siRNA, 150 ng *MDM2* promoter-specific reporter construct, 50 ng *Renilla* and 5 ng WT p53 as indicated. Luciferase activity was determined after 24 h and normalised to *Renilla* activity. Standard deviations from three independent experiments are presented, with P-values depicting significant differences (*=P<0.05 significant, **=P<0.01 highly significant, ***=P<0.001 very highly significant); (B) H1299 cells were transfected as before and whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with antibodies against hnRNPUL-1 and p53. An antibody against β-actin was used as a loading control.

not due to an increase in p53 protein levels. These data collectively indicate that the depletion of hnRNPUL-1 results in the activation of p53-dependent transactivation of p21 and MDM2. It is also possible that the down-regulation of hnRNPUL-1 may be mediating the transactivation of p21 in a p53-independent manner and in this regard, may be functioning to affect other proteins that are involved in the activation of p21. It is of note that the luciferase experiments were all conducted without the presence of a DNA damaging agent, therefore these results indicate that hnRNPUL-1 affects p53 transcriptional activity before DNA damage.

3.2.4 hnRNPUL-1 affects p53-dependent target genes in response to DNA damage

Since p53-dependent transactivation of target genes in response to damage is essential for cellular function and viability and that hnRNPUL-1 depletion appears to be affecting specific p53 transcriptional targets, the mechanism behind the observations described above was examined in a more physiological context and after genotoxic stress. It is known that p53 regulates the transcription of genes from different pathways (Helton and Chen, 2007). Therefore, the effect of hnRNPUL-1 depletion on a set of p53 target genes present in a range of pathways was assessed. To further address the role of hnRNPUL-1 in mediating the activation of p21 and MDM2, these two genes were included in the panel of p53 target genes that were used in the assay. In addition, DDB2 was included as a p53 target gene in qRT-PCR since it is involved in p53-mediated DNA repair pathways (Tan and Chu, 2002). Finally, the Bcl-2 family member, Bax, was chosen on the basis of its p53-dependent pro-apoptotic activity in response to DNA damage (Helton and Chen, 2007). Quantitative RT-PCR (qRT-PCR) was conducted on samples derived from U2OS cells that had been depleted of

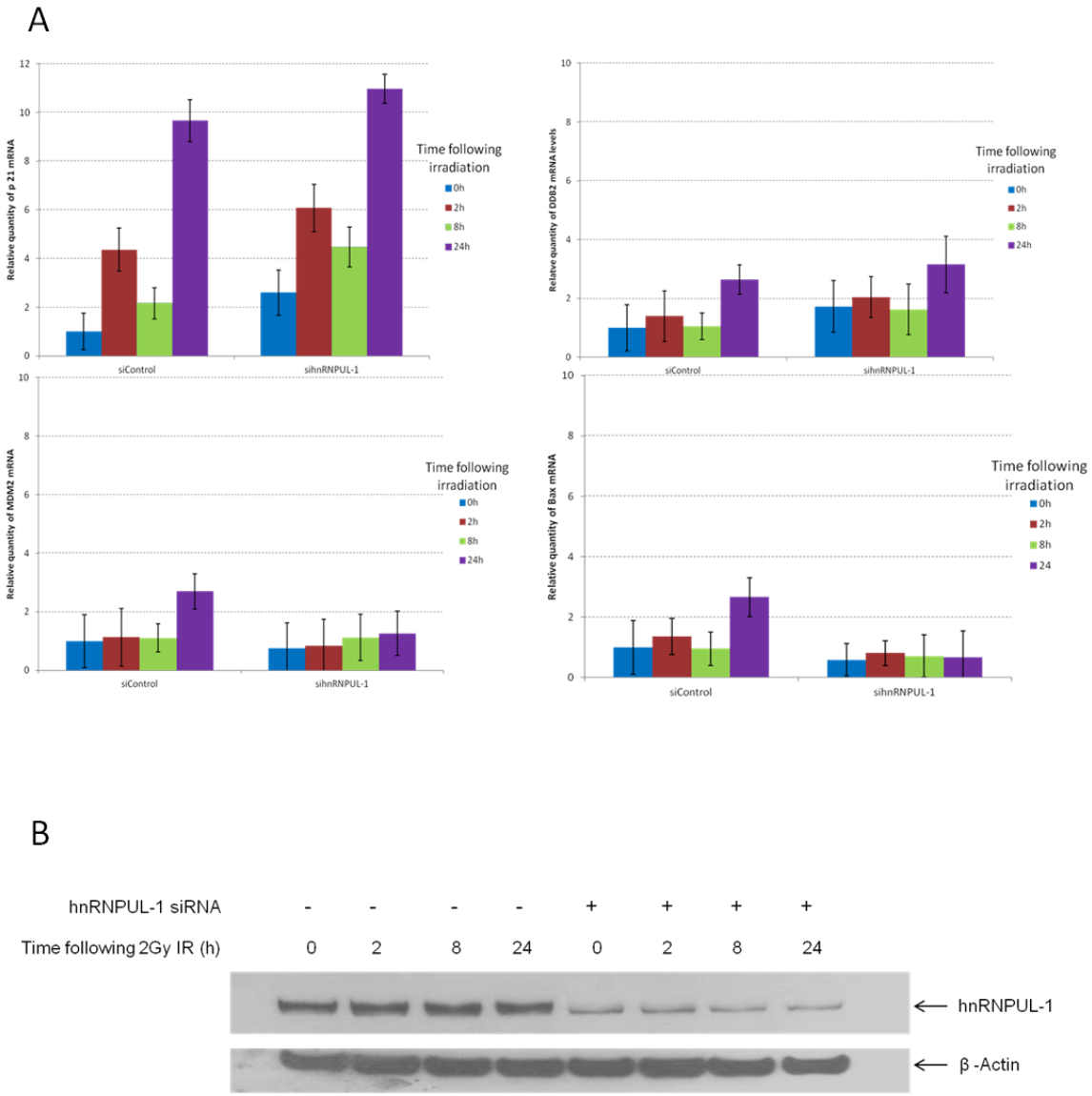

Figure 3.6 The depletion of hnRNPUL-1 affects mRNA levels of p53 target genes before and after IR

(A) U2OS cells were transfected with control or hnRNPUL-1 siRNA and treated with 2 Gy IR. qRT-PCR was performed in cDNA with primers recognising sequences within the mRNAs for p21,DDB2, MDM2 and Bax. CT values were normalised to β-actin. The results are presented as relative mRNA levels of each p53 target gene compared to control siRNA-treated and unirradiated cells at each time point and represent three independent experiments; (B) Whole cell extracts were prepared from U2OS cells treated in the same way, separated by SDS-PAGE and immunoblotted to assess hnRNPUL-1 depletion by siRNA. An antibody against β-actin was used as a loading control.

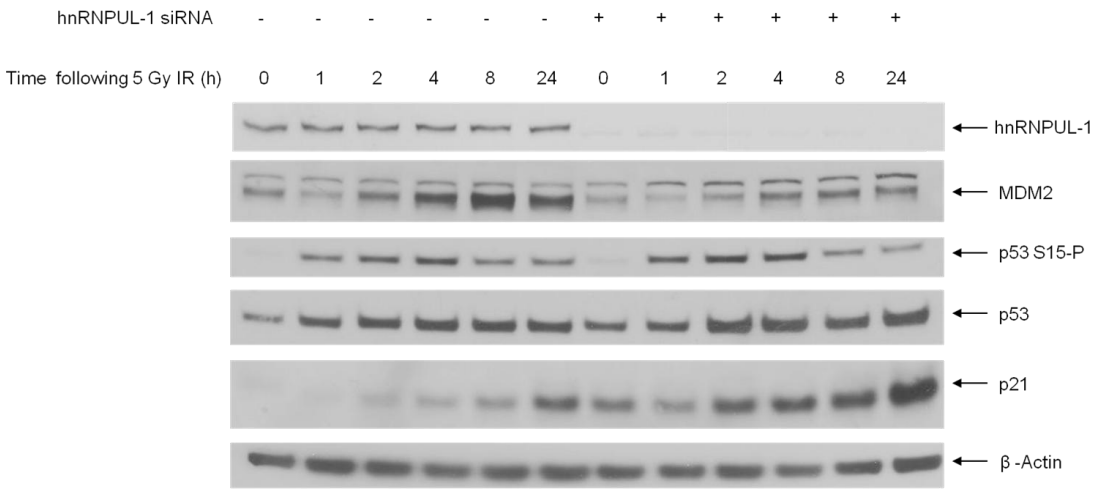

Figure 3.7 The depletion of hnRNPUL-1 affects protein expression levels of p53 target genes before and after IR

U2OS cells were transfected with control or hnRNPUL-1 siRNA and mock-treated or treated with 5 Gy IR. Whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with antibodies against p53 and its target genes, p21 and MDM2. An antibody against β-actin was used as a loading control. The Western blot is a representation of three individual experiments.

hnRNPUL-1 using siRNA and exposed to 2 Gy IR. The results were presented as relative mRNA levels of different p53 target genes at different times following irradiation compared to control siRNA-treated and unirradiated cells.

Interestingly, data from the qRT-PCR experiment showed that hnRNPUL-1 depletion increased IR induction of the mRNA for p21 (Figure 3.6A). This is consistent with the results shown in Figure 3.4A where luciferase expression from the p21 promoter was strongly induced in cells depleted of hnRNPUL-1. There was also an increase in fold change of mRNA for the p53 transcriptional target, DDB2, in cells treated with hnRNPUL-1 siRNA and IR (Figure 3.6A). Surprisingly, no induction of MDM2 mRNA levels were seen following hnRNPUL-1 depletion and damage with IR (Figure 3.6A) despite the increase in p53-dependent transactivation of MDM2 depicted in the luciferase data (Figure 3.5A). In addition, the down-regulation of hnRNPUL-1 did not cause an induction in the fold change in mRNA levels of Bax after damage and, in fact, resulted in the decrease of these levels (Figure 3.6A). The depletion of hnRNPUL at the protein level was confirmed by Western blotting in Figure 3.6B at each timepoint following IR.

To complement these results, protein expression levels of p53 and p53 target genes were examined in U2OS cells depleted of hnRNPUL-1 using siRNA and treated with 5 Gy IR. Western blot analysis was used to determine the protein expression levels of p53, MDM2 and p21. Significantly, following DNA damage by IR, p21 protein levels were increased in cells depleted of hnRNPUL-1 compared to cells containing endogenous levels of hnRNPUL-1 (Figure 3.7). In addition, even before damage with IR, the protein expression of p21 was higher in cells depleted of hnRNPUL-1 compared to cells treated with control siRNA (Figure 3.7). This observation also reflects the appreciable increase in fold change of p21 mRNA

levels observed after hnRNPUL-1 depletion and before DNA damage (Figure 3.6A). Notably, the down-regulation of hnRNPUL-1 appeared to cause a decrease in MDM2 protein levels following DNA damage (Figure 3.7). Although this is contrary to the *in vitro* luciferase data (Figure 3.5A) it does correlate with the lack of induction of this gene at the mRNA level in cells lacking hnRNPUL-1 (Figure 3.6A). In addition, hnRNPUL-1 depletion appeared not to affect IR-induced p53 stabilisation or indeed, phosphorylation on S15 of p53 following DNA damage (Figure 3.7). Taken together, these data suggest that hnRNPUL-1 contributes to the regulation of p53-mediated transcription of a subset of genes.

3.2.5 Cells depleted of hnRNPUL-1 exhibit an elevated G1/S checkpoint arrest

The depletion of hnRNPUL-1 results in the induction at both the mRNA and protein level of p21, which is the main mediator of p53-induced G1 arrest. Therefore, to address the possible role of hnRNPUL-1 in regulating cell cycle checkpoints, U2OS cells were transfected with control or hnRNPUL-1 siRNA, subjected to genotoxic stress by IR and analysed by flow cytometry. Notably, the down-regulation of hnRNPUL-1 resulted in an increase in the percentage of cells in G1 compared to cells containing endogenous hnRNPUL-1 (Figure 3.8). To confirm this cell cycle effect, the results from three independent experiments were collated and are presented using bar chart analysis. The data clearly show that the down-regulation of hnRNPUL-1 caused an increase in the percentage of cells that accumulated in G1 (Figure 3.9A). The P-values represent significant differences in the percentage of G1 cells in samples treated with hnRNPUL-1 siRNA compared with those treated with control siRNA at 0, 1, 2, 4

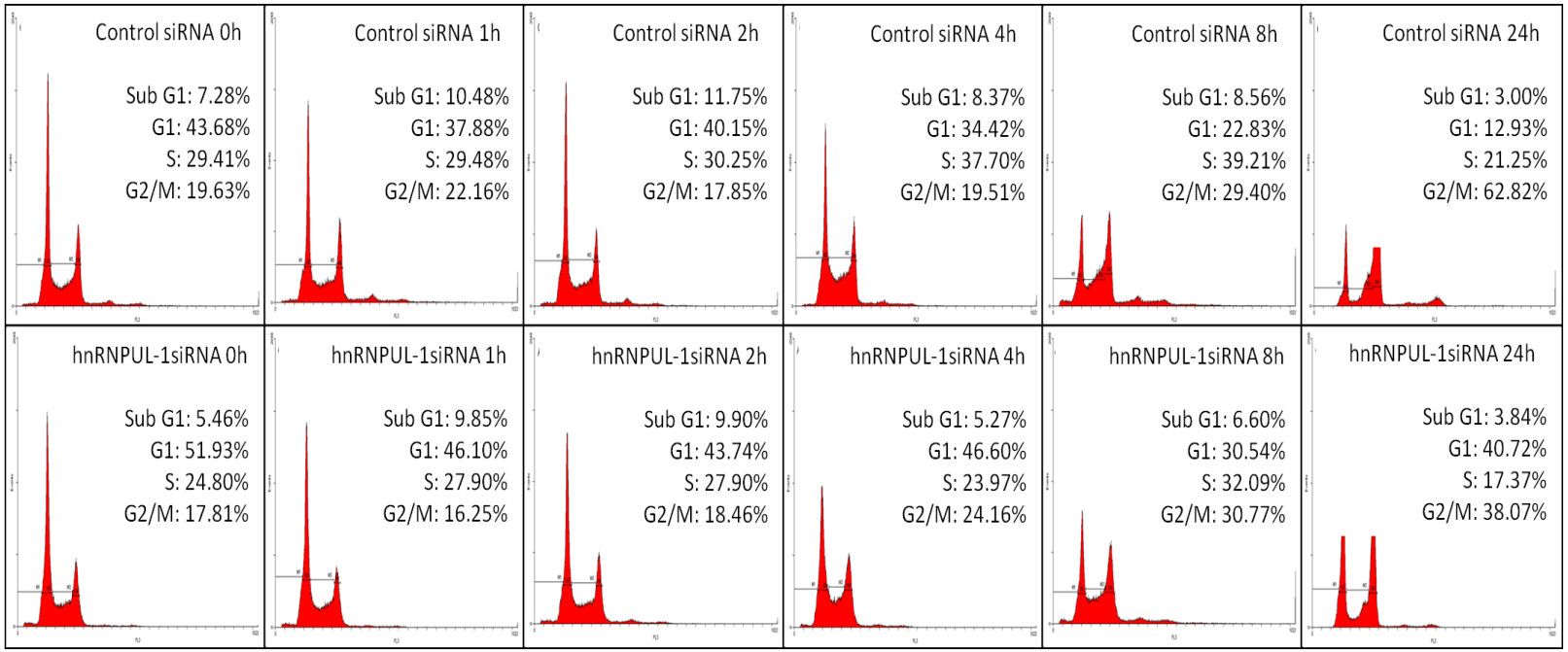

Figure 3.8 Cells depleted of hnRNPUL-1 exhibit an elevated G1/S checkpoint arrest before and after IR
U2OS cells treated with control or hnRNPUL-1 siRNA and 5 Gy IR were analysed by flow cytometry using PI dye to quantify DNA content during the cell cycle. The percentage of cells accumulating in sub G1, G1, S or G2/M is shown for control- and hnRNPUL-1-treated samples. The FACS profiles shown are representatives from three independent experiments

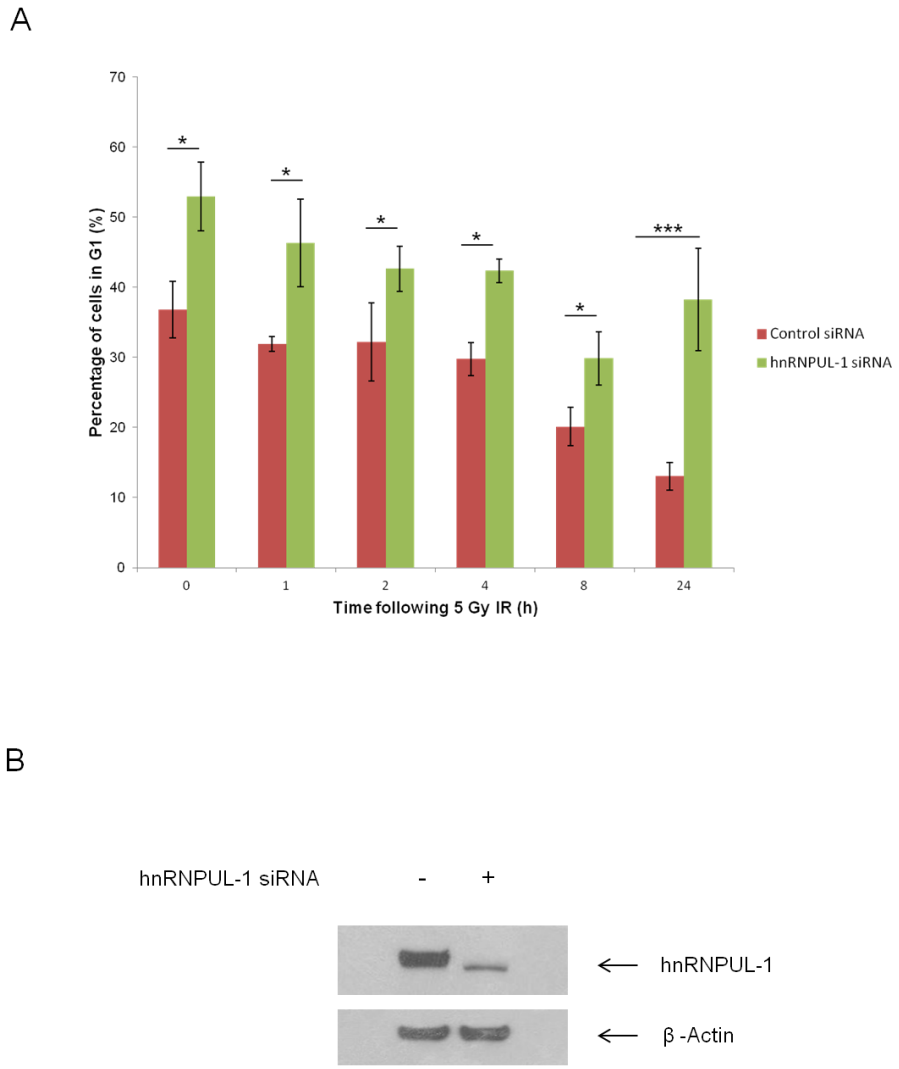

Figure 3.9 Depletion of hnRNPUL-1 results in an elevated G1/S checkpoint arrest before and after IR

U2OS cells treated with control or hnRNPUL-1 siRNA and 5 Gy IR were analysed by flow cytometry using PI dye to quantify DNA content during the cell cycle. **(A)** The percentage of cells accumulating in G1 is shown for irradiated cells following hnRNPUL-1 depletion. The plots represent the mean of four independent experiments with P-values depicting significant differences between control siRNA- and hnRNPUL-1 siRNA-treated samples (*=P<0.05 significant, **=P<0.01 highly significant, ***=P<0.001 very highly significant); **(B)** Whole cell extracts were prepared from U2OS cells in the same experiment, separated by SDS-PAGE and immunoblotted to assess hnRNPUL-1 depletion by siRNA. An antibody against β-actin was used as a loading control.

and 8 hours after treatment with IR (Figure 3.9A). The P-value at 24 h after IR reflects the highly significant difference between cells with reduced levels and those with endogenous levels of hnRNPUL-1 (Figure 3.9A). Figure 3.9B confirms the depletion of hnRNPUL-1 at the protein level by Western blotting. Taken together these data demonstrate that the depletion of hnRNPU-1 leads to an elevated G1/S checkpoint arrest compared to cells treated with control siRNA and may indicate that hnRNPUL-1 plays a role in p53-mediated cell cycle regulation, probably through its effect on p21 expression.

3.2.6 The interaction site on hnRNPUL-1 for p53 encompasses the BBS domain

The domain required on p53 for the interaction with hnRNPUL-1 has been shown to reside within the C-terminal region of p53 (Barral *et al.*, 2005). However, the interaction site on hnRNPUL-1 required for this association has not been mapped with any precision. Given that hnRNPUL-1 and p53 have been found to interact before and after DNA damage and that hnRNPUL-1 appears to negatively regulate p53 transcriptional activity, the domains required for the interaction on hnRNPUL-1 were mapped using various fragments encompassing the open reading frame of hnRNPUL-1 (Figure 3.10A).The hnRNPUL-1 'splice variant' commences at exon 2 and from this point it is identical to WT hnRNPUL-1 (Figure 3.10A) (Rusch, 2003). The hnRNPUL-1 fragments, together with full length hnRNPUL2, were *in vitro* translated, labelled with [^{35}S] methionine and incubated with GST-p53 or GST alone in a pull-down assay (Figure 3.10B). No binding of the hnRNPUL-1 fragments to GST was seen, whereas p53 bound to full length hnRNPUL-1, and to the C-terminal and central regions of hnRNPUL-1 (Figure 3.10B). Interestingly, p53 was shown to bind to full-length hnRNPUL-2, which is structurally and functionally similar to hnRNPUL-1 but does not

possess a PP region at its C-terminus (Figure 3.10A). This is the first demonstration that hnRNPUL-2 binds to p53 and also indicates that the PP region is not required for this interaction (Figure 3.10B). Virtually no binding of p53 to the N-terminal region of hnRNPUL-1 was observed (Figure 3.10B). This is consistent with the minimal binding observed between GST-p53 and the N-terminal region of hnRNPUL-1 reported in Barral *et al*., (2005). The fragment corresponding to the splice variant protein was also found to interact strongly with p53 (Figure 3.10B). The splice variant does not contain part of the N-terminal sequence of WT hnRNPUL-1 and the data is consistent with the fact that the N-terminal region of hnRNPUL-1 is not required by p53 to bind to this protein (Figure 3.10A) (Barral *et al.*, 2005). The deletion of the central BRD7-binding site (BBS) region of hnRNPUL-1 resulted a significantly decreased binding of p53 to hnRNPUL-1 (Figure 3.10B). To gain further insight into the nature of this interaction, hnRNPUL-1 deletion mutants with differing deletion of the BBS region were examined for their ability to bind to p53. Notably, the binding of p53 decreased with increasing deletion of the BBS region of hnRNPUL-1, indicating that it is necessary for the interaction between p53 and hnRNPUL-1 (Figure 3.10B).

To confirm that this region of hnRNPUL-1 does interact with p53, GST-fused p53 and GST alone were incubated with the RGG-BBS domain of hnRNPUL-1 that had been *in vitro* translated and labelled with [^{35}S] methionine (Figure 3.11A). GST-fused p53 was also incubated with [^{35}S] methionine-labelled hnRNPUL-1 and [^{35}S] methionine-labelled hnRNPUL-2 as controls and again as a confirmation that p53 does associate with the full length proteins (Figure 3.11A). The pull-down assay showed strong binding between p53 and the region corresponding to the BBR-RGG domain of hnRNPUL-1 (Figure 3.11A). A

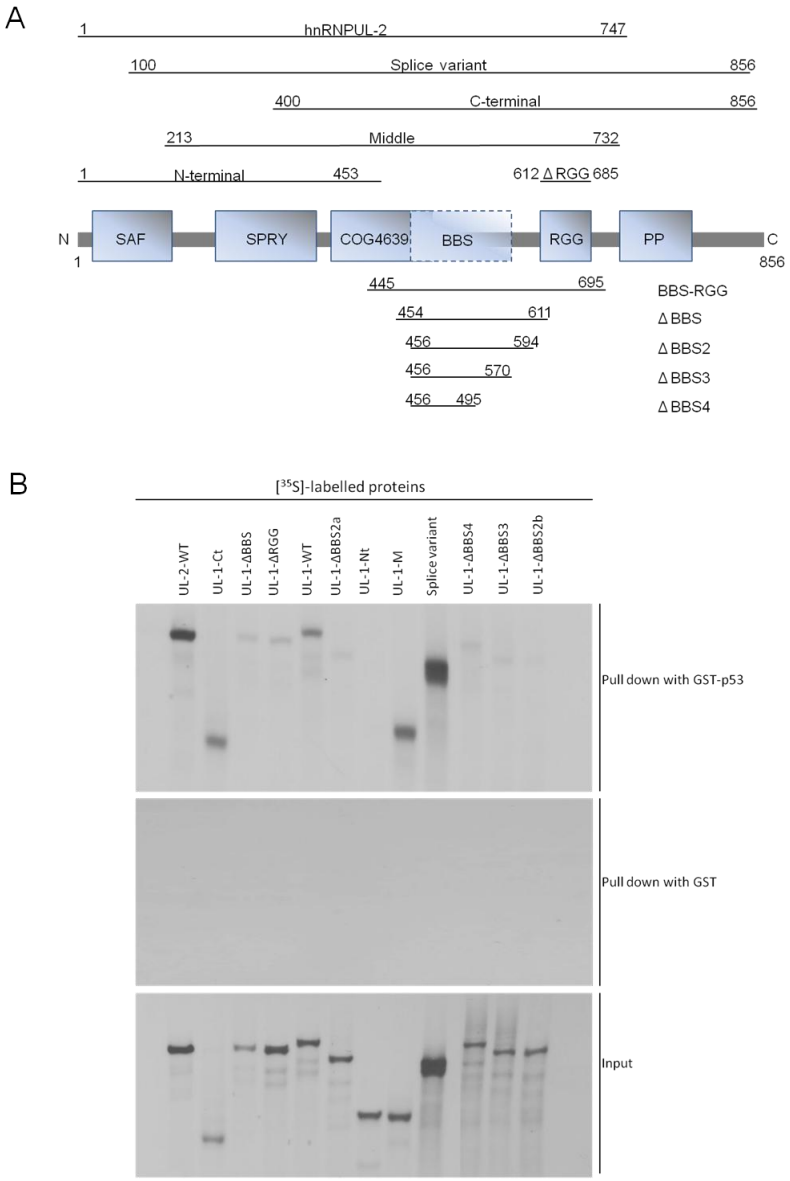

Figure 3.10 p53 requires the BBS domain of hnRNPUL-1 for binding
(A) Schematic of hnRNPUL-1 and the different [^{35}S] methionine-labelled fragments covering the open reading frame of hnRNPUL-1; (B) [^{35}S] methionine-labelled fragments were *in vitro* translated and incubated with GST-p53 or GST alone as a control. Δ denotes deleted sequences; UL-1 and Ul-2 denote full-length hnRNPUL-1 and hnRNPUL-2 respectively; WT, Nt, Ct and M denote wildtype, N-terminal, C-terminal and middle fragments respectively. UL-1 ΔBBS2a and 2b are different DNA preparations of the same construct. Input represents 5% of the radiolabeled protein or polypeptide used for the pull down assay.

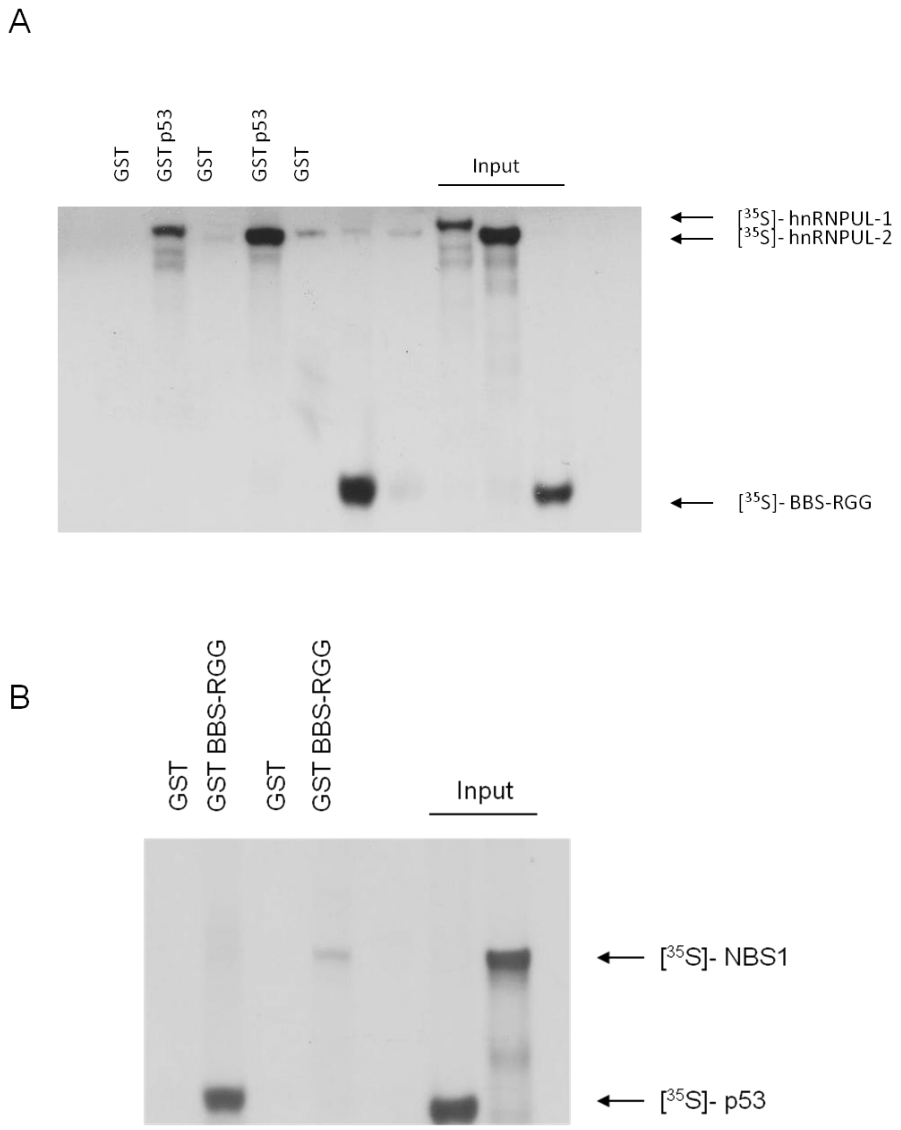

Figure 3.11 p53 binds to the BBS region of hnRNPUL-1
(A) GST pull-down using the indicated [^{35}S] methionine-labelled hnRNPUL-1, hnRNPUL-2 and RGG-BBS and GST-p53 or GST alone as a control; (B) Reciprocal pull-down using the indicated [^{35}S] methionine-labelled p53 and GST-BBS-RGG or GST alone as a control. Input represents 5% of the radiolabeled protein or polypeptide used for the pull down assay.

reciprocal pull-down assay was performed, where GST-BBS-RGG and GST alone were incubated with [^{35}S] methionine-labelled p53 (Figure 3.11B). This verified the association of the BBS-RGG domain of hnRNPUL-1 and p53 (Figure 3.11B). An interaction between the BBS-RGG domain and NBS1 was used as a control as reported by Polo *et al*. (2012).

3.3 DISCUSSION

p53 is a transcription factor that plays a major role in the DNA damage repair pathways and coordinates the cellular response to a range of genotoxic stresses. Many co-factors exist to regulate p53 activity during both normal stress-free conditions and following cellular stress, including those that influence p53-mediated cellular outcomes such as Hzf, which enhances p53 transactivation of cell cycle arrest genes, and ASPP1 and ASPP2 that promote p53 binding to promoters of pro-apoptotic genes (Das *et al.*, 2007, Samuels-Lev *et al.*, 2001). In addition to its role in mRNA metabolism, the hnRNP family member, hnRNPUL-1, has been shown to be involved in regulating p53 transcriptional activity following over-expression (Barral *et al.*, 2005). The data presented here confirm that hnRNPUL-1 functions as a transcriptional co-factor for p53 and is of particular importance in regulating p21 levels and, subsequently, of cell cycle arrest.

Consistent with previous GST pull-down and co-immunoprecipitation assays, studies here confirm that hnRNPUL-1 and p53 interact (Figure 3.1). This interaction could be seen before and after damage with two types of genotoxic stress; IR and UV. This suggests that at least a certain proportion of the cellular hnRNPUL-1 and p53 exists in a protein complex and that this complex is present before and after DNA damage. Presumably the reduction in the

amount of binding protein co-immunoprecipitated in both cases is due to the marked increase in p53 following DNA damage and the consequent 'dilution' of the complex.

Previous studies in which hnRNPUL-1 was over-expressed in human tumour cells had indicated that it could act as a repressor of p53 activity (Barral *et al.*, 2005). To confirm and to expand on these observations, an alternative approach was adopted here based on the reduction of hnRNPUL-1 levels using siRNA. Initially, luciferase reporter assays were performed in cells depleted of hnRNPUL-1. The data established that p53 transcriptional activity was enhanced when hnRNPUL-1 was down-regulated using siRNA (Figure 3.2). The effect on the promoters of specific p53 transcriptional targets, p21 and MDM2, was also examined using luciferase assays and these results showed that p53-mediated transcription of these genes was greatly increased upon hnRNPUL-1 depletion (Figures 3.4A and 3.5A). The findings provide significant evidence that hnRNPUL-1 may be acting to negatively regulate the transcriptional activity of p53.

A previous study has shown that hnRNPUL-1 interacts with p53 and requires C-terminal sequences of p53 to do so (Barral *et al.*, 2005). The C-terminal region of p53 is known as the regulatory domain and is the site of many post-translational modifications (Appella and Anderson, 2001). Many modifications at the C-terminus affect other regulatory events such as co-factor recruitment and the mediation of promoter-specific activity (Kruse and Gu, 2009a). In particular, 6 C-terminal lysine sites present on p53 are subject to modification by phosphorylation, ubiquitylation, methylation, sumoylation, neddylation and acetylation (Brooks and Gu, 2003). It is conceivable that the binding of hnRNPUL-1 may mask some of the modification sites on p53, therefore, affecting the level of post-translational modification that occurs on this tumour suppressor. It has been reported that, by mutating these lysine

residues to arginine, p53-dependent gene transcription was impaired (Feng *et al.*, 2005). Therefore, it is possible that masking of these sites at the C-terminus of p53 consequently affects its ability to transcriptionally activate target genes. The fact that hnRNPUL-1 and p53 have been shown to interact provides further evidence for the inhibition of p53 transcriptional activation in this way. Interestingly, the increase in p53 transcriptional activity when hnRNPUL-1 levels are reduced occurs in the absence of DNA damage, therefore suggesting that hnRNPUL-1 could be functioning to repress p53 transcriptional activity before genotoxic stress. Most noticeably, knockdown of hnRNPUL-1 expression results in a marked increase in p21 levels even in the absence of cellular stress (Figure 3.7).

There is evidence to suggest that p53 binds to DNA in unstressed cells and that it is even present at promoters of its target genes under normal homeostasis (Kaeser and Iggo, 2002). It is possible that hnRNPUL-1 and p53 may interact before assembly onto transcriptional promoters. This is supported by the observation that both proteins can be co-immunoprecipitated from cells that have been treated to genotoxic stresses and those that have not (Figure 3.1). However, further research would be needed to examine whether hnRNPUL-1 is recruited to the promoters of p53 target genes and whether the depletion of hnRNPUL-1 affects p53 recruitment to these promoters. Such a finding would be determined using Chromatin Immunoprecipitation (ChIP) analysis.

Using qRT-PCR, the effect of depletion of hnRNPUL-1 on a number of p53 target genes was assessed. Although p21 mRNA levels were appreciably increased, it is notable that Bax was not increased following hnRNPUL-1 depletion (Figure 3.6). Bax is a pro-apoptotic gene that is generally found to be upregulated in response to DNA damage in a p53-dependent manner. If hnRNPUL-1 is acting generally to negatively regulate p53-dependent transactivation it

would be expected that the levels of this gene would be increased following the down-regulation of hnRNPUL-1. However, there is evidence to suggest that specific mechanisms exist that activate different subsets of p53 target genes after genotoxic stress (Kruse and Gu, 2009a). For example, the activation of the subset of genes involved in apoptosis requires the recruitment of co-activators of p53 and modifications on p53 that are different to those required for the activation of the subset of genes involved in cell cycle regulation (Vousden and Prives, 2009). The acetylation of K120 in the DBD of p53 is indispensible for p53-mediated transcription of its apoptotic genes; however, the transcription of non apoptotic targets remained unaffected when this site was mutated (Sykes *et al.*, 2006). Indeed, no evidence of apoptosis was seen in any cells depleted of hnRNPUL1.

Redundancy exists amongst the lysine residues that are acetylated to enable the activation of p21 since it takes the simultaneous mutation of 8 lysine sites within p53 to inhibit p21 activation (Tang *et al.*, 2008). In contrast, more specific modification of p53 is required for the activation of pro-apoptotic genes. This emphasises the fact that the requirements for the full activation of apoptotic genes are more stringent, which is unsurprising since the apoptotic response is irreversible (Kruse and Gu, 2009a). In this manner, the lack of increase in Bax mRNA levels following hnRNPUL-1 depletion could possibly be because hnRNPUL-1 does not affect the more specific modifications on p53 that are needed for pro-apoptotic gene activation.

It is also possible that hnRNPUL-1 affects other co-factors that regulate p53 activity and alter their activity in a gene-specific manner. For example, a transcriptional target of p53, Hzf, binds to p53 after its induction and is preferentially recruited to promoters of cell cycle arrest genes (Das *et al.*, 2007). The opposite could also occur; co-factors exist to cause the

activation of the expression of pro-apoptotic genes alongside the inhibition of cell cycle genes. Co-factors of p53 belonging to the latter category include ASPP1 and 2, which aid p53 binding to promoters of apoptotic genes such as *Bax, PUMA* and *NOXA* (Samuels-Lev *et al*., 2001). Therefore, hnRNPUL-1 may repress or activate particular co-factors of p53 and in this regard, hnRNPUL-1 may be acting to interfere with the recruitment of such proteins and thus affect preferential transactivation by p53. Further experiments will have to be conducted to determine whether hnRNPUL-1 affects the expression of other p53-regulated genes and to identify any patterns that emerge indicating the transcriptional activation of a subset of p53 target genes.

Promoters of cell cycle genes such as *p21* possess high levels of bound RNA polymerase II before activation whereas lower levels exist at the promoters of pro-apoptotic targets (Espinosa *et al.*, 2003). This phenomenon has been further supported by the examination of core promoter structures of p53 targets, which are required to direct the initiation of transcription (Morachis *et al.*, 2010). It was found that, while the pro-apoptotic *Fas* promoter undergoes slow initiation component formation, the *p21* core promoter rapidly assembles these initiation components (Morachis *et al.*, 2010). Evidence points to the fact that promoters of cell cycle genes are highly responsive and it is more than likely that mechanisms exist to exercise some caution in activating these genes. It can be hypothesised that the inhibition of p53 transcriptional activity by hnRNPUL-1 is one factor contributing to repression of the activation of certain p53 target genes, in particular p21, and perhaps others involved in cell cycle regulation.

A similar argument could apply to the effects on MDM2. Although loss of hnRNPUL-1 results in hyperactivation of the p53-dependent DDR, the expression of MDM2 is reduced

compared to control cells (Figure 3.7). Taken at face value, this observation would suggest that, although hnRNPUL-1 functions to negatively regulate the p53 response, this does not apply to all known p53 target genes. However, MDM2 is known to be a negative regulator of p53 through its ability to ubiquitylate p53 and target it for proteolytic destruction by the proteasome (Haupt *et al.*, 1997). Therefore, if the function of hnRNPUL-1 is to repress p53 function, it would seem logical for hnRNPUL-1 to potentiate the expression of negative regulators, such as MDM2. The defective induction of MDM2 in cells devoid of hnRNPUL-1 following exposure to DNA damaging agents would lend support to this proposition.

Another possibility may be that since the phosphorylation of S15 of p53 remains unaffected by the depletion of hnRNPUL-1, this implies that hnRNPUL-1 does not affect the degradation of p53. It is known that phosphorylation at the N-terminal region of p53 causes the inhibition of the p53-MDM2 interaction (Kruse and Gu, 2009a). Therefore, if the absence of hnRNPUL-1 results in unchanged levels of S15 phosphorylation of p53 the inhibition of the interaction between p53 and MDM2 would remain and MDM2 protein levels would be reduced. Phosphorylation of T18 of p53 is also known to be important in the regulation of the p53-MDM2 interaction but unfortunately this has not been examined here (Schon *et al.*, 2002).

The discrepancy between the MDM2 *in vitro* luciferase data and the *in vivo* qRT-PCR and Western blot analysis may be explained by the fact that the luciferase assay is an artificial system, acting as a surrogate for measuring promoter activity. Therefore, this system may not reflect the true interactions that exist and may not fully recapitulate mRNA regulation *in vivo*. The mRNA levels and proteins levels of MDM2 following hnRNPUL-1 depletion depict a more accurate representation of what is actually happening in the cell in a more physiologically-relevant manner. In this regard, it is of interest that MDM2 levels at the

mRNA level are in agreement with those at the protein level in that they are decreased following hnRNPUL-1 depletion (Figures 3.6A and 3.7).

The heightened p53-dependent DDR after hnRNPUL-1 depletion is again seen in the cell cycle distribution where, interestingly, cells depleted of hnRPUL-1 exhibit an elevated G1/S checkpoint arrest compared to cells treated with control siRNA (Figures 3.8 and 3.9A). This presumably reflects the ability of hnRNPUL-1-depleted cells to induce p21, which is the main mediator of p53-dependent G1 arrest in response to DNA damage (Deng *et al.*, 1995). p21 usually mediates G1 cell cycle arrest by inhibiting CDKs and by inhibiting the role of PCNA in DNA replication (Abbas and Dutta, 2009). It is possible that by negatively regulating the expression of p21 levels, hnRNPUL-1 causes a functional effect by negatively regulating cell cycle progression after DNA damage.

The binding site on p53 for hnRNPUL-1 has been reported to be within the C-terminal domain of p53 (Barral *et al.*, 2005). However, the precise binding site on hnRNPUL-1 that is required for this interaction had not been determined. To map the hnRNPUL-1 region that mediates the interaction with p53, various hnRNPUL-1 fragments and deletion mutants, including those with deletions in the BBS were generated (Kzhyshkowska *et al.*, 2003, Kzhyshkowska *et al.*, 2001, Polo *et al.*, 2012) and used in GST pull-down assays. The BBS domain of hnRNPUL-1 was shown to be required for p53 binding to hnRNPUL-1(Figures 3.10B and 3.11). Incidentally, this region of hnRNPUL-1 was recently found to be partially responsible for its interaction with NBS1 (Polo *et al.*, 2012).

BRD7 is a bromodomain-containing gene and has been implicated in transcriptional regulation (Peng *et al.*, 2006). This is not surprising since the bromodomain is found in proteins that are involved in transcriptional regulation and there is evidence to suggest that

mutations in bromodomain genes contribute to the development of cancer (Jeanmougin *et al.*, 1997). Significantly, BRD7 has recently been found to bind to p53 and that this interaction requires the C-terminal region of p53 (Drost *et al.*, 2010). It is possible that the binding of BRD7 to p53 at the C-terminus of p53 may be inhibited when hnRNPUL-1 binds to the same region on p53. Since BRD7 has been found to function as a transcriptional co-factor of p53 (Drost *et al.*, 2010), the binding of hnRNPUL-1 to p53 may abrogate the ability of BRD7 to function in this manner. This will have to await future investigation.

Interestingly, BRD7 was shown to be required to activate a subset of p53 target genes, namely cell cycle genes such as *p21* (Drost *et al.*, 2010). The results in that study are consistent with the findings here, which show that the depletion of hnRNPUL-1 results in the activation of the cell cycle inhibitor, p21, but does not seem to cause an increase in levels of the pro-apoptotic protein, Bax. Further to this, it is known that BRD7 is found at promoters of non-apoptotic targets of p53 and that it induces histone acetylation around the p53-binding site of the *p21* promoter (Drost *et al.*, 2010). BRD7 is also able to interact with p300, a critical co-activator of p53 that is required for the acetylation of several p53 C-terminal lysine residues (Drost *et al.*, 2010, Gu and Roeder, 1997). In addition, there are also studies that support a role of BRD7 in BRCA1-dependent transcription such that BRD7 recruits BRCA1 to specific promoters (Harte *et al.*, 2010). Taken together, it is conceivable that BRD7 may be acting to regulate the activation of p53, an action that may be inhibited by the binding of hnRNPUL-1 to p53. In this manner it can be surmised that hnRNPUL-1 may be competing with BRD7 for the binding site on the C-terminal region of p53.

It has been previously shown that BRD7 is a binding partner of hnRNPUL-1 and that disruption of the BRD7-hnRNPUL-1 complex increased the ability of hnRNPUL-1 to inhibit

basic transcription (Kzhyshkowska *et al.*, 2003). Therefore, the fact that BRD7 competes for the same binding site on hnRNPUL-1 as p53 may indicate that the binding of p53 to hnRNPUL-1 displaces BRD7 and causes hnRNPUL-1 to act as a transcriptional co-repressor.

This study has revealed an interaction between p53 and hnRNPUL-2 that has not been demonstrated previously. It would be interesting to examine the nature of this interaction in the future since a lack of reagents such as antibodies and hnRNPUL-2 constructs hindered further such investigations.

Taken together, these findings confirm that hnRNPUL-1 is functioning to negatively regulate p53 transcriptional activity and may be acting at the transcriptional level. Given the ability of hnRNPUL-1 to bind to p53 and the fact that it can regulate p53 transcriptional activity, it can be hypothesised that hnRNPUL-1 plays a role in limiting the extent of p53 activity following DNA damage as well as shutting off the p53-dependent response once the damage has been repaired. It is also apparent that hnRNPUL-1 seems to be acting to regulate the p53-dependent transactivation of some genes, not others. Whether this relationship is dependent on interactions with, or the activity of, BRD7 is not clear at present. In summary, in the absence of hnRNPUL-1, the transcriptional activity of p53 significantly increases and these findings, observed in an artificial system, can also be reflected *in vivo* by an increase in cell cycle arrest. It can be speculated that hnRNPUL-1 is functioning as a co-repressor of p53 transcriptional activity.

CHAPTER 4

CHARACTERISATION OF THE DNA DAMAGE RESPONSE INDUCED BY THE NOVEL CHEMOTHERAPEUTIC AGENT, ALCHEMIX

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4.1 INTRODUCTION

The plethora of cellular pathways that detect DNA damage and replication stress to orchestrate repair processes and the resolution of DNA replication are collectively known as the DDR. The key proteins that mediate the DDR belong to the family of PIKKs – ATM, ATR and DNA-PK. ATM and DNA-PK primarily respond to DSBs, whereas ATR is activated mainly in response to ssDNA regions that are generated at stalled replication forks (Lovejoy and Cortez, 2009). These components of the DDR activate cellular responses to DNA damage to preserve the integrity of the genome. This is through the activation of cell cycle arrest and the repair of DNA lesions or through the promotion of apoptosis if the damage cannot be repaired (Khanna and Jackson, 2001).

Anti-cancer drugs usually exert their cytotoxic action through their ability to generate DNA damage in tumour cells, such that they are forced to undergo cell death whilst normal cells are spared (Al-Ejeh *et al.*, 2010). The DNA lesions that arise from the use of DNA damaging agents are diverse and, therefore, activate a variety of DDR pathways, which function to stimulate numerous downstream processes that include DNA repair pathways. Therefore, an understanding of the DDR pathways that become activated in response to a particular DNA damaging anti-cancer drug is of fundamental importance since specific abrogation of

components in these pathways may increase the effectiveness of the chemotherapeutic drug in question.

Alkylating agents are some of the most commonly prescribed anti-cancer drugs and are often used in combination therapy. It is important that particular components of DNA repair pathways that recognise alkyl lesions are inhibited alongside treatment with an alkylating agent to allow the conversion of the damage into fatal replication lesions (Helleday *et al.*, 2008). Another class of chemotherapeutic drug of interest is the topoisomerase inhibitors, which can be classified into TOP1 or TOP2 inhibitors. The TOP2 inhibitors can be divided further into the following: TOP2 poisons or TOP2 catalytic inhibitors. TOP2 poisons, such as etoposide, doxorubicin and mitoxantrone, function by trapping the TOP2cc so that they reach lethal levels. DSBs are subsequently formed, which activate apoptosis. The catalytic inhibitors of TOP2 disrupt the formation of the TOP2cc by reducing the activity of TOP2 (Larsen *et al.*, 2003). Chromosome decatenation is consequently inhibited and cell death ensues through mitotic catastrophe (Giménez-Abián *et al.*, 2000, Wang, 2002).

A common challenge in the use of standard chemotherapeutic agents is the increasing prevalence of drug resistance, which can occur through a number of mechanisms. Mutations in known tumour suppressors such as *ATM* and *TP53* are often the primary cause of resistance in tumours after prolonged exposure to chemotherapeutic DNA damaging agents (Austen *et al.*, 2007, Lowe *et al.*, 1993). Interestingly, another resistance mechanism is the reinstatement of repair of drug-induced DNA damage due to acquired secondary mutations in components of the DDR that allow once defective components of the repair machinery to regain some function (Lord and Ashworth, 2012). For example, secondary mutations in *BRCA2* result in a partially functional protein that leads to the resistance to PARP inhibitors

that would otherwise rely on inactive BRCA2 (Bryant *et al.*, 2005, Edwards *et al.*, 2008). Furthermore, the MMR pathway is actually required to mediate the toxicity of some anti-cancer agents e.g. 6-thioguanine; therefore, defects in this repair pathway are often associated with an increased resistance to some chemotherapeutic drugs (Fink *et al.*, 1998, Helleday *et al.*, 2008). The over-expression of drug efflux transporters such as P-glycoprotein, poses another problem with chemotherapeutic resistance (Longley and Johnston, 2005). This is of particular concern since substrates of these efflux transporters are vast; therefore, the design and development of compounds that are not recognised by such pumps is of fundamental importance.

In view of these resistance mechanisms, the use of single agents as the choice of anti-cancer therapy has been largely superseded by a range of combination therapies or by the use of agents that cause DNA damage through two or more independent mechanisms. This is in the hope that the tumour cell would be sensitised to the damaging effect of the drug whilst reducing the ability of the tumour cell to select for genetic events that allow its survival (Aziz *et al.*, 2012). In addition, the response to multi-functional chemotherapeutic agents should be sufficiently lethal so as not to be affected by mutations in proteins that act downstream of damage.

ALX belongs to the anthraquinone family of anti-cancer drugs. The anthraquinones have long been used as anti-cancer drugs and they act by specifically inhibiting TOP2 (Sparreboom *et al.*, 2005). Anthraquinone-based drugs are centred on a tricyclic pharmacophore and several natural derivatives of the anthraquinones have been identified, although it was clear that these were too toxic to enter clinical trials (Lown, 1993). This prompted the use of these natural products as leads for the design of new drugs by attaching functional groups such as the

hydroxyethyl moiety to the tricyclic structure. Mitoxantrone and several other drugs emerged as clinically effective but since many are substrates for multi-drug resistance mechanisms, tumours rapidly develop a tolerance to these compounds (Pors *et al.*, 2004). The generation of a hybrid class of agents consisting of both intercalating and alkylating capabilities has been undertaken: of these compounds, ALX demonstrated the most potent anti-tumour activity, most notably in a cisplatin-resistant cell line (A2780/cp70) and a doxorubicin-resistant tumour cell line (2780AD) (Pors *et al.*, 2004, Pors *et al.*, 2003). Incidentally, the latter cell line also contained elevated levels of the P-glycoprotein efflux pump, indicating that ALX is not a substrate of this drug transporter (Pors *et al.*, 2003). ALX itself is a prototype of the non-symmetrical 1,4 disubstituted anthraquinone hybrid structure, specifically called a chloroaminoanthraquinone and, therefore, functions as both a TOP2 inhibitor as well as possessing alkylating abilities (Figure 4.1) (Pors *et al.*, 2003). Interestingly, the symmetrical 1,4-disubstituted anthraquinones containing alkylating groups on both side chains lose their effectiveness in drug-resistance cancer cells while their non-symmetrical counterparts such as ALX remain cytotoxic (Pors *et al.*, 2003). In addition, recent studies have shown that the length of the alkylating side-chain of ALX (ethyl) is more effective than longer butyl and pentyl side-chains (Abdallah *et al.*, 2012). Despite these promising findings, little is known about the mode of action of this novel agent.

This study aims to:

- Determine which DDR pathways become activated in response to ALX
- Identify which of its two functions induce a greater DDR
- Examine whether the inactivation of a number of key DDR components results in a reduced signalling response to ALX
- Identify the mode of cell death that ALX initiates.

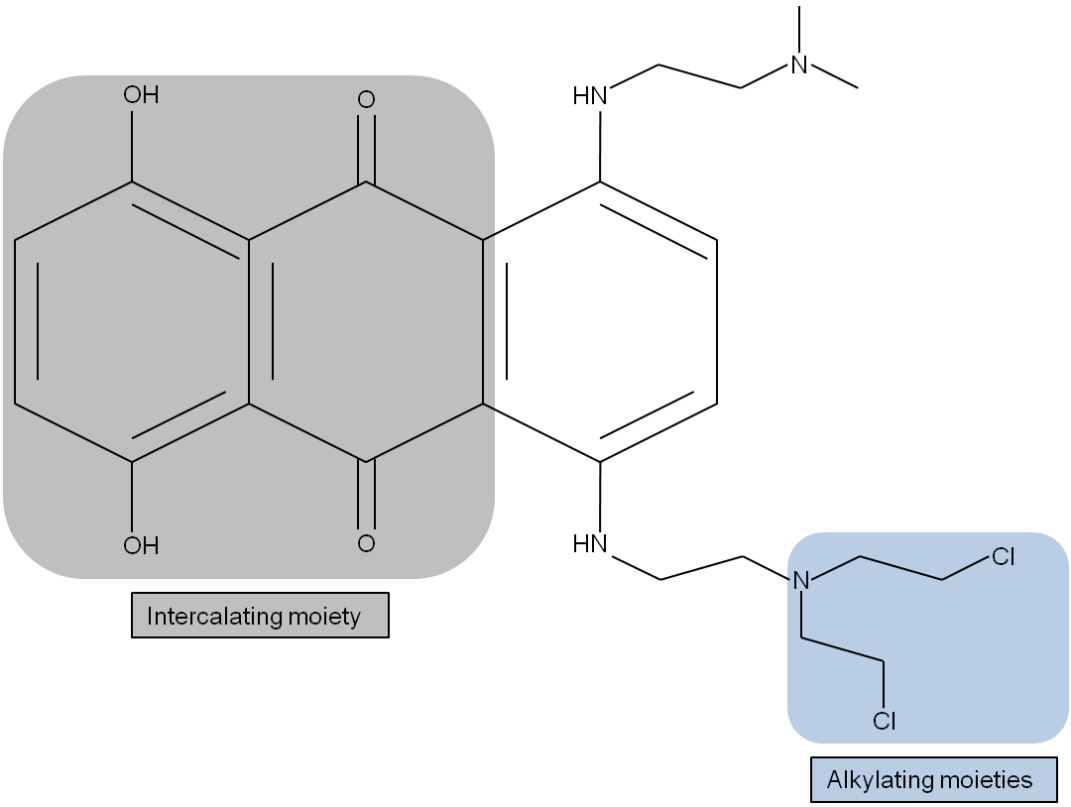

Figure 4.1 Schematic representation of the structure of Alchemix
The alkylating moieties of ALX are highlighted in blue, including the bis-chloroethyl moieties. It is clear that ALX contains non-symmetrical side-chains. The intercalating or topoisomerase-inhibiting moiety of ALX is highlighted in grey.

4.2 RESULTS

4.2.1 Treatment with ALX induces a DDR in multiple cancer cell lines

Dose response experiments were conducted before any other experiments to determine the sensitivity of two different cancer cell lines, H1299 and U2OS, to serial dilutions of ALX and also to identify the dose of ALX that stimulated a cellular response. This preliminary analysis indicated that ALX was capable of inducing a DDR even at nano-molar concentrations after both 5 h and 24 h of treatment in H1299 and U2OS cells (Figures 4.2 and 4.3 respectively). The results also indicated that 50 nM ALX would be sufficient to induce a DDR.

To ascertain whether this response could be recapitulated in cancer cell lines that differ in both their DNA repair and apoptotic capacities, U2OS (WT *TP53*), H1299 (*TP53* null), DLD1 (*MSH6* mutant), and HCT116 (*MLH1* and *MRE11* mutant) cells were treated with ALX (50 nM). Western blotting was used to examine the activation of the DDR using phospho-specific antibodies for DDR proteins. Interestingly, ALX induced a DDR in the p53 proficient U2OS cell line, the p53 proficient but *MLH1* and *MRE11* mutant HCT116 cell line, the *TP53* and *MSH6* mutant DLD1 cell line, and the *TP53* null H1299 cell line, indicating that there is a widespread response to ALX regardless of the p53 status and despite the difference in their DNA repair and apoptotic capabilities (Figures 4.4, 4.5, 4.6 and 4.7 respectively). More robust phosphorylation of RPA2 was observed in U2OS cells in response to ALX compared to other cell lines where extremely low levels of phosphorylated RPA2 were detected (Figure 4.4). The levels of phosphorylated DNA damage proteins after ALX treatment were lower in HCT116 cells in comparison to more robust phosphorylation observed in the other cell lines (Figure 4.5). These differences in the kinetics of the signalling response to ALX could be

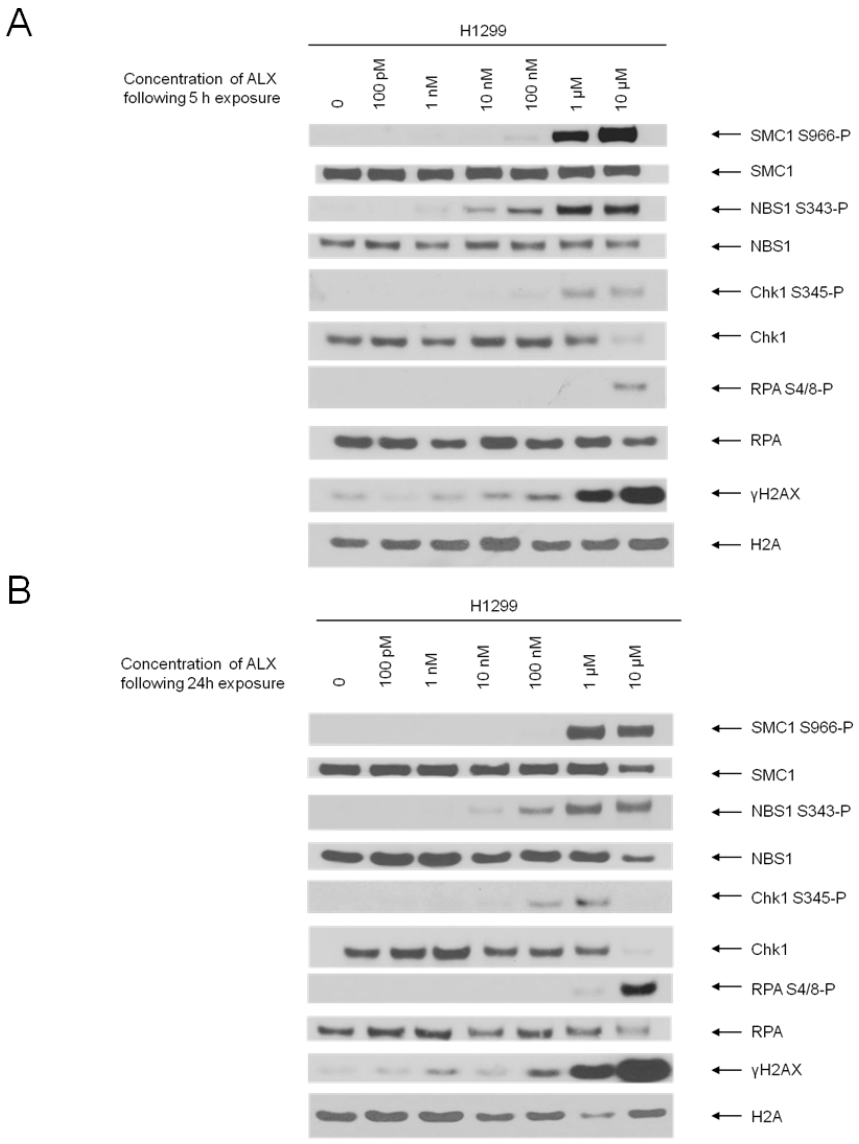

Figure 4.2 Treatment with serial dilutions of ALX for 5 and 24h induces a DDR in H1299 cells

(A) H1299 cells were treated to 0, 100 pM, 1 nM, 10 nM, 100 nM, 1 µM and 10 µM ALX and harvested after 5h and; (B) 24h. Whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with the indicated antibodies against DNA damage proteins. Antibodies against total proteins were used as loading controls.

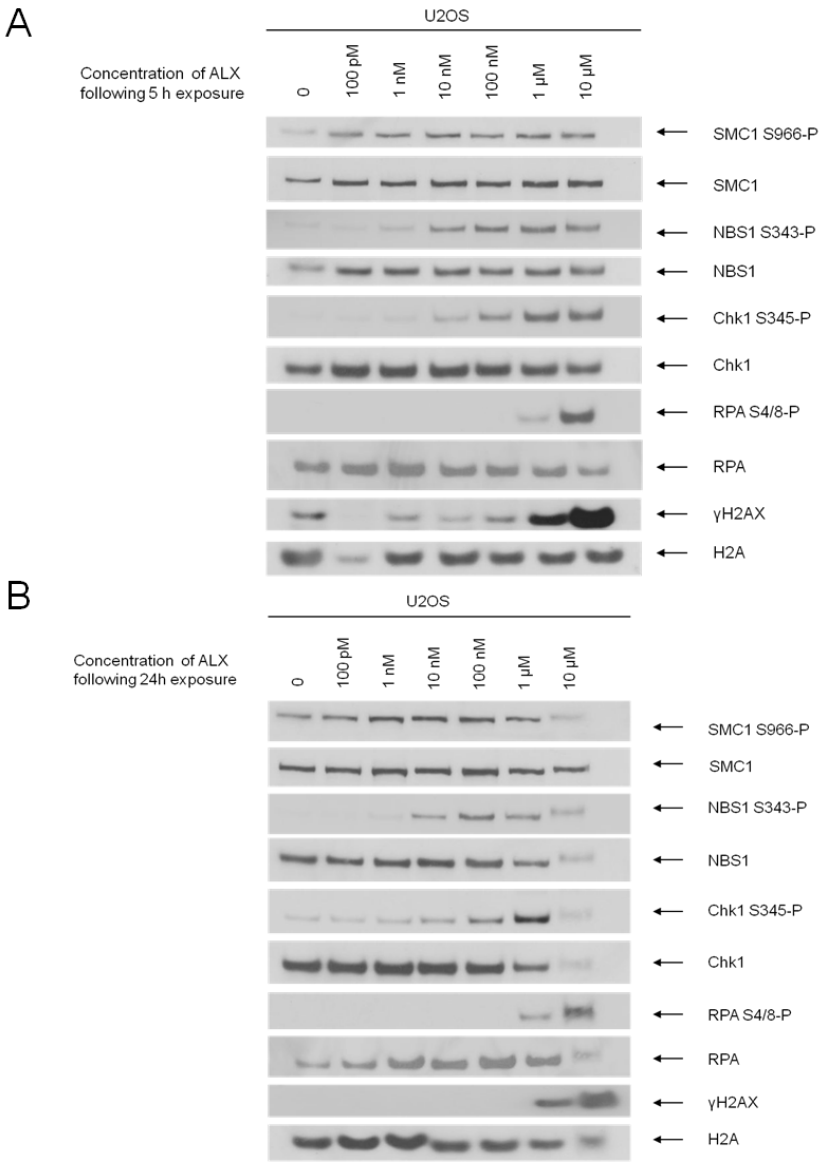

Figure 4.3 Treatment with serial dilutions of ALX for 5 and 24h induces a DDR in U2OS cells

(A) U2OS cells were treated to 0, 100 pM, 1 nM, 10 nM, 100 nM, 1 µM and 10 µM ALX and harvested after 5h and; (B) 24h. Whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with the indicated antibodies against DNA damage proteins. Antibodies against total proteins were used as loading controls.

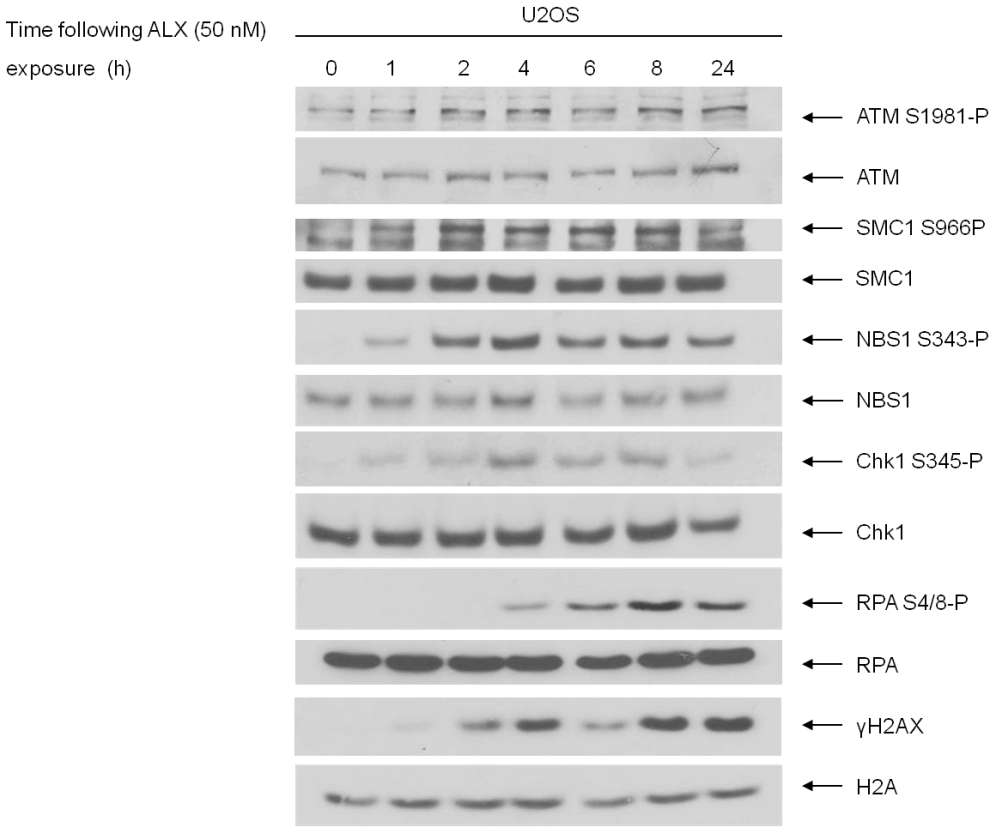

Figure 4.4 ALX induces a DDR in the U2OS cancer cell line
p53 proficient U2OS cells were treated to 50 nM ALX and harvested at the times indicated after treatment. Whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with the indicated antibodies against DNA damage proteins. Antibodies against total proteins were used as loading controls.

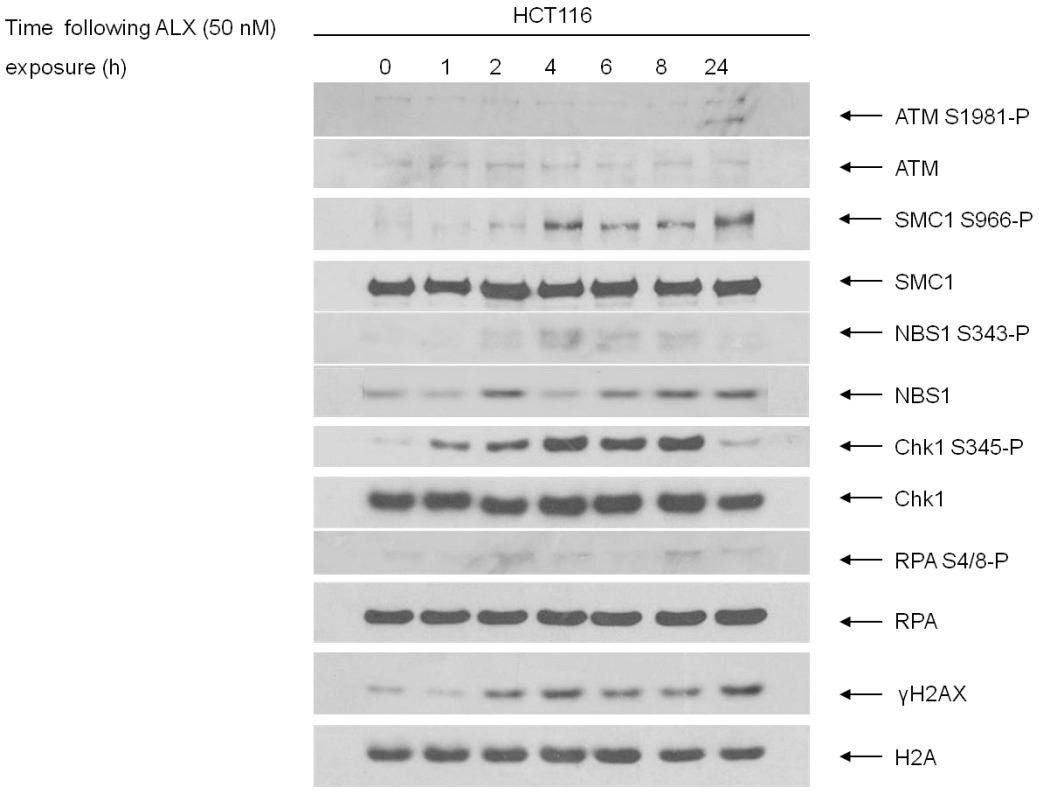

Figure 4.5 ALX induces a DDR in the HCT116 cancer cell line
p53 proficient HCT116 cells that also express mutant MLH1 and mutant MRE11 were treated to 50 nM ALX and harvested at the times indicated after treatment. Whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with the indicated antibodies against DNA damage proteins. Antibodies against total proteins were used as loading controls.

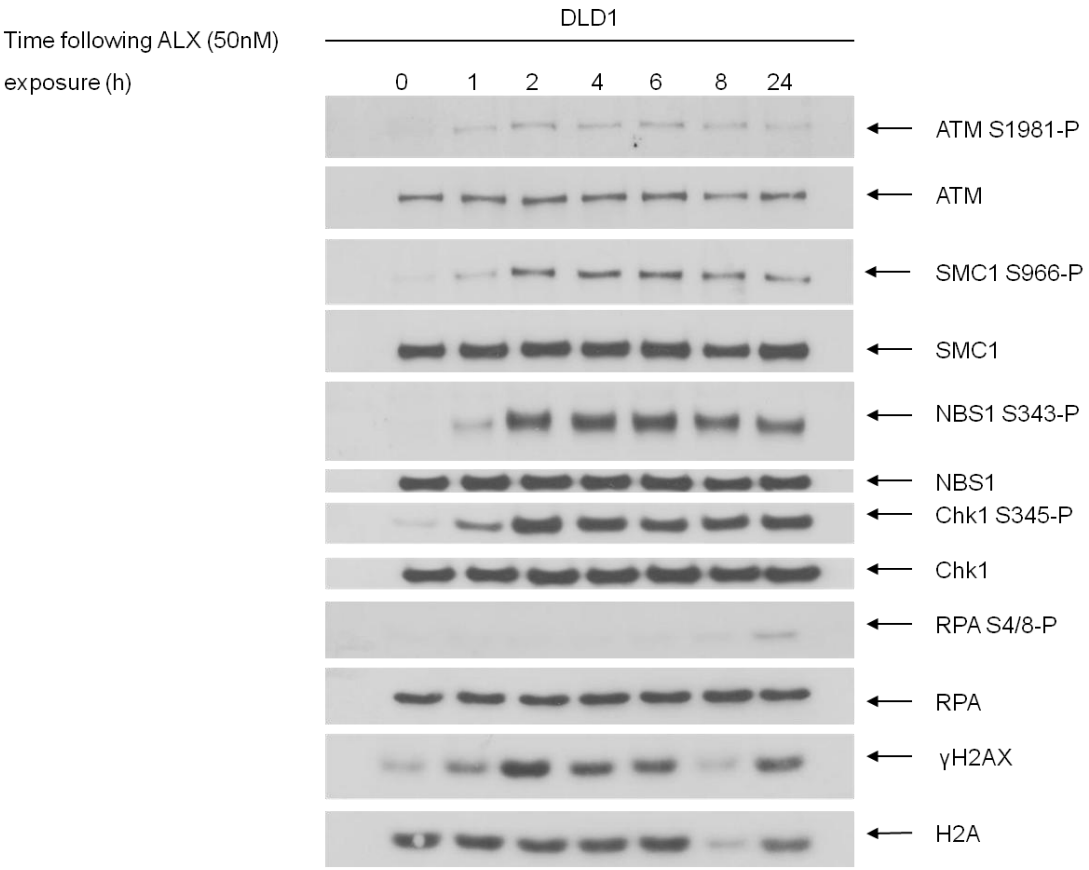

Figure 4.6 ALX induces a DDR in the DLD1 cancer cell line
DLD1 cells that express mutant p53 and mutant MSH6 were treated to 50 nM ALX and harvested at the times indicated after treatment. Whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with the indicated antibodies against DNA damage proteins. Antibodies against total proteins were used as loading controls.

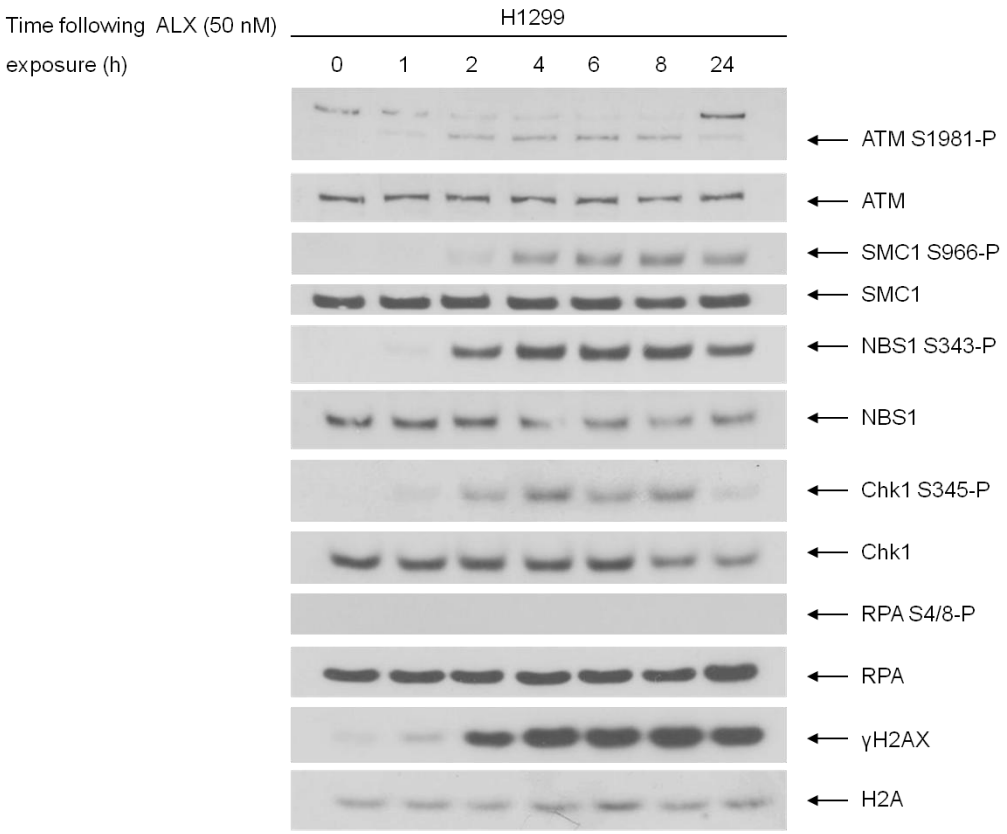

Figure 4.7 ALX induces a DDR in the H1299 cancer cell line
p53 null H1299 cells were treated to 50 nM ALX and harvested at the times indicated after treatment. Whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with the indicated antibodies against DNA damage proteins. Antibodies against total proteins were used as loading controls.

due to the differences in the genetic background of the cells.

To ascertain whether ALX could induce DSBs, HeLa cells were treated with ALX and fixed at certain time points over a 24 h time period. DSB formation was measured using fluorescence microscopy coupled with antibodies recognising phosphorylated H2AX (γH2AX) and 53BP1. Although previous studies showed that γH2AX foci were a reliable marker for the quantification of DSBs, evidence now suggests that the numbers may not reflect true DSBs induced by different damaging agents (Bouquet *et al.*, 2006, Sedelnikova *et al.*, 2002). Therefore, 53BP1foci were used alongside γH2AX for an accurate measurement of DSB formation (Schultz *et al.*, 2000). Although Western blot analysis had shown phosphorylation of H2AX relatively early in response to ALX (Figures 4.4-4.7), visible γH2AX/53BP1 foci were only detected at later time points of 8h-24 h (Figure 4.8A). This result indicates that DSBs are induced only after prolonged exposure to ALX. In addition, the level of damage after 24 h ALX treatment was assessed by microscopy and by looking at the different types of staining pattern. The results indicated that, after 24 h of ALX exposure, 50% of cells contain DSBs, as shown by the cumulative percentage of cells showing types III and IV staining, again suggesting that it is only after the prolonged treatment times that ALX induces DNA DSBs (Figure 4.8B). Taken together, these data suggest that the anti-tumour activity of ALX is likely to be, in part, mediated by its ability to induce DNA damage.

4.2.2 The activation of the DDR by ALX is ATM independent

The onset of DNA damage triggers the activation of ‘sensors’ such as the PIKK family, which signal different types of DNA damage. To discern which pathway the cell utilises to induce

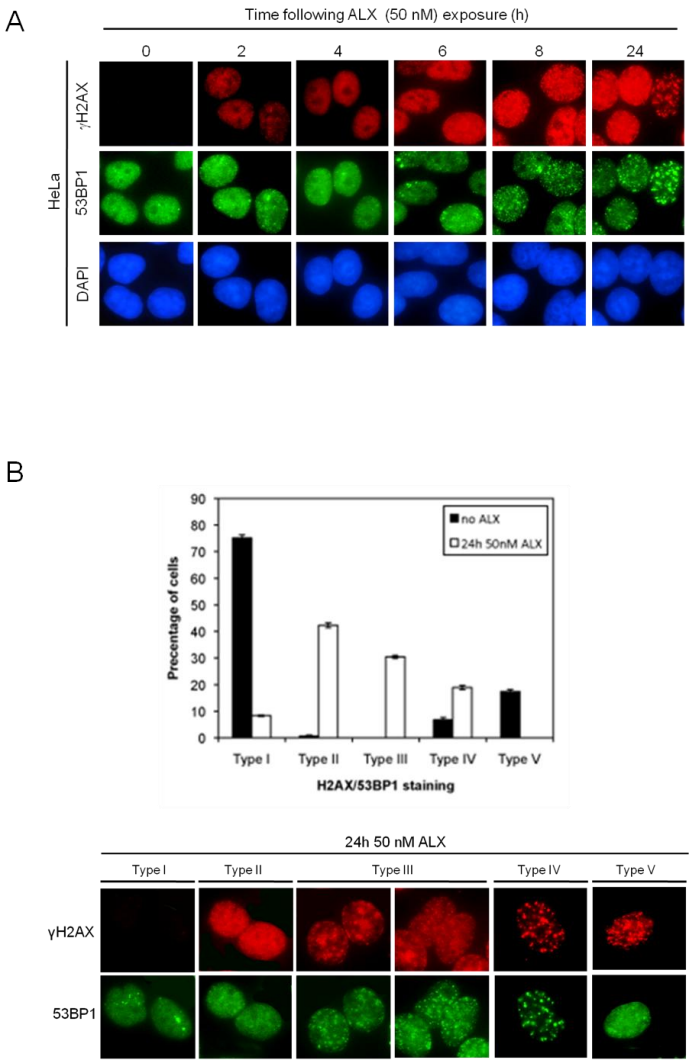

Figure 4.8 Treatment with ALX induces DSBs at later timepoints
(A) HeLa cells were treated with 50 nM ALX and fixed at the times indicated after treatment. Cells were processed for immunofluoresence using antibodies recognising γH2AX and 53BP1; (B) The level of damage in HeLa cells that had been fixed 24 h after ALX treatment was assessed by looking at different staining patterns: type I represents no damage; type II depicts the increased retention of protein on the chromatin and not DSBs; types III and IV show foci that represents DSBs and type V represents DSBs that may have merged into one.

the observed DDR following ALX treatment, the effects of inhibiting key kinases was examined. Since ALX is a putative TOP2 inhibitor, it was conceivable that the signalling response after treatment with ALX was due to the activation of the ATM kinase pathway, which responds primarily to DSBs; this seemed likely due to the fact that ALX was shown to induce DSBs at later time points (Figure 4.8). To determine whether this was the case, H1299 cells were pre-treated with the ATM inhibitor, KU-55933, before being treated with ALX. Western blotting was used to assess the level of phosphorylation of different DDR proteins over a time course of treatment with the drug in combination with the ATM inhibitor. Interestingly, the inhibition of ATM kinase did not affect the signalling response compared to cells that had not been treated with the ATM inhibitor (Figure 4.9). The combination of treatment with ionising radiation and the ATM inhibitor was used as a positive control (Figure 4.9).

This was repeated in different cell lines and using a greater range of time points to ascertain whether the observed response was cell-type specific. Figures 4.10A and 4.10B show that the response did not change in U2OS and HeLa cells respectively. The results indicate that the inhibition of ATM kinase does not affect the DDR after treatment with ALX and correlates with the very weak auto-phosphorylation of ATM observed in the four different cancer cell lines after ALX treatment (Figures 4.4-4.7). Taken together, this indicates that ATM is not strongly activated following treatment with this drug. HeLa cells were further treated with ALX and Western blotting was used to assess the levels of phosphorylation of ATM-specific substrates, KAP-1 and Chk2. ALX did not induce the activation of either protein as shown by the lack of phosphorylation (Figure 4.11). Therefore, despite the potential of ALX to generate DSBs (Figure 4.8), ATM does not appear to be activated and combining the ATM inhibitor

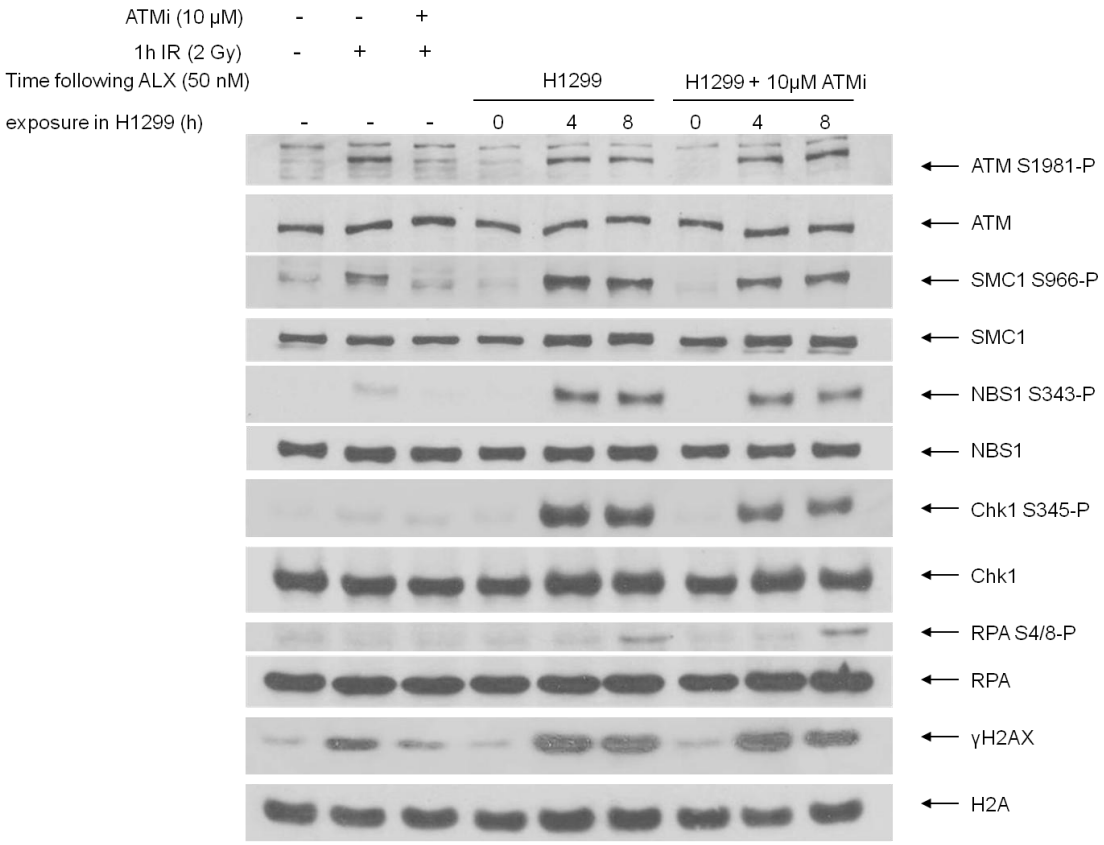

Figure 4.9 ALX acts independently of ATM kinase in an initial timecourse experiment
H1299 cells were pre-treated with 10 μM ATM inhibitor, treated with 50 nM ALX after 1h and harvested at the times indicated. Whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with the indicated antibodies against DNA damage proteins. Irradiated cells were used as controls alongside cells pre-treated with 10 μM ATM inhibitor and irradiated with 2 Gy. Antibodies against total proteins were used as loading controls. ATMi denotes ATM inhibitor.

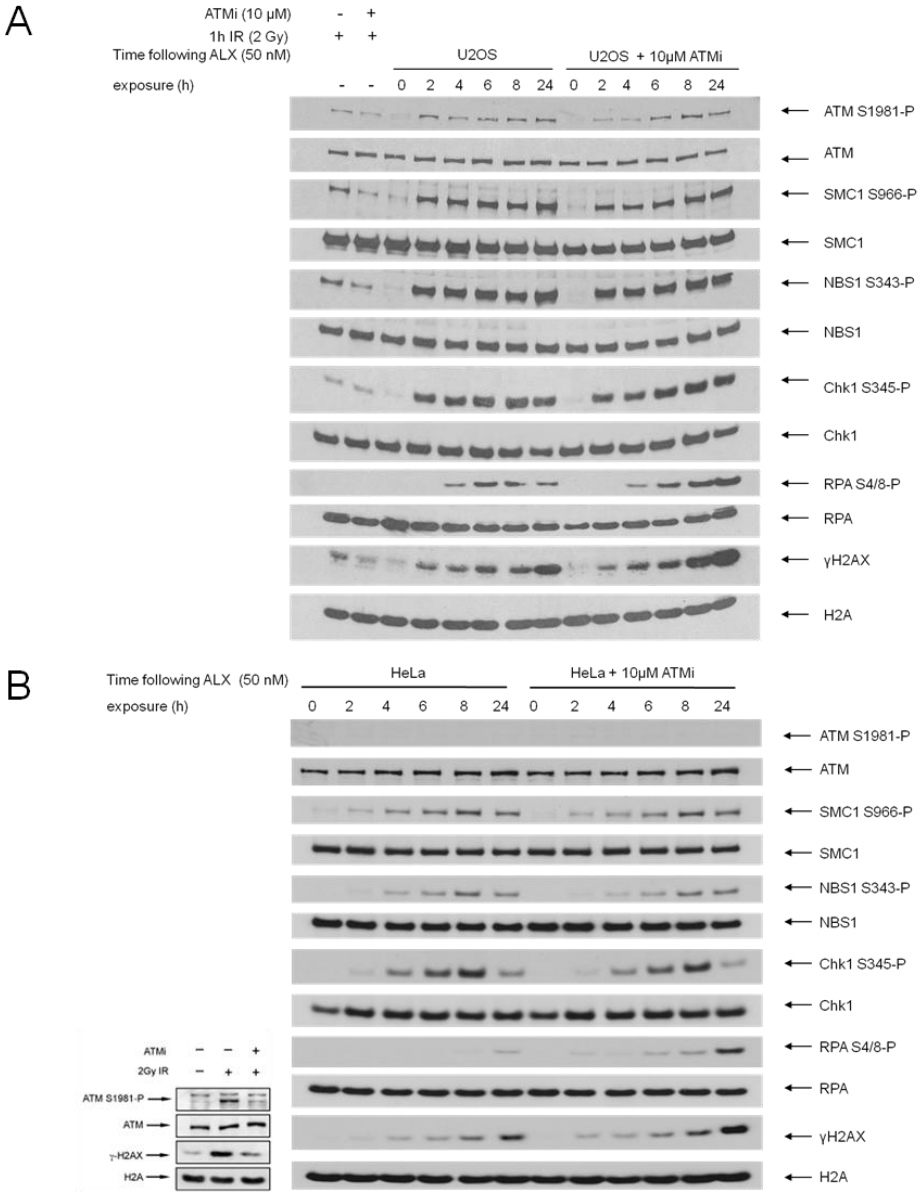

Figure 4.10 ALX acts independently of ATM kinase
(A) U2OS cells were pre-treated with 10 μM ATM inhibitor, treated with 50 nM ALX after 1h and harvested at the times indicated. This was repeated in; (B) HeLa cells. Whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with the indicated antibodies against DNA damage proteins. Irradiated cells were used as controls alongside cells pre-treated with 10 μM ATM inhibitor and irradiated with 2 Gy. Antibodies against total proteins were used as loading controls. ATMi denotes ATM inhibitor.

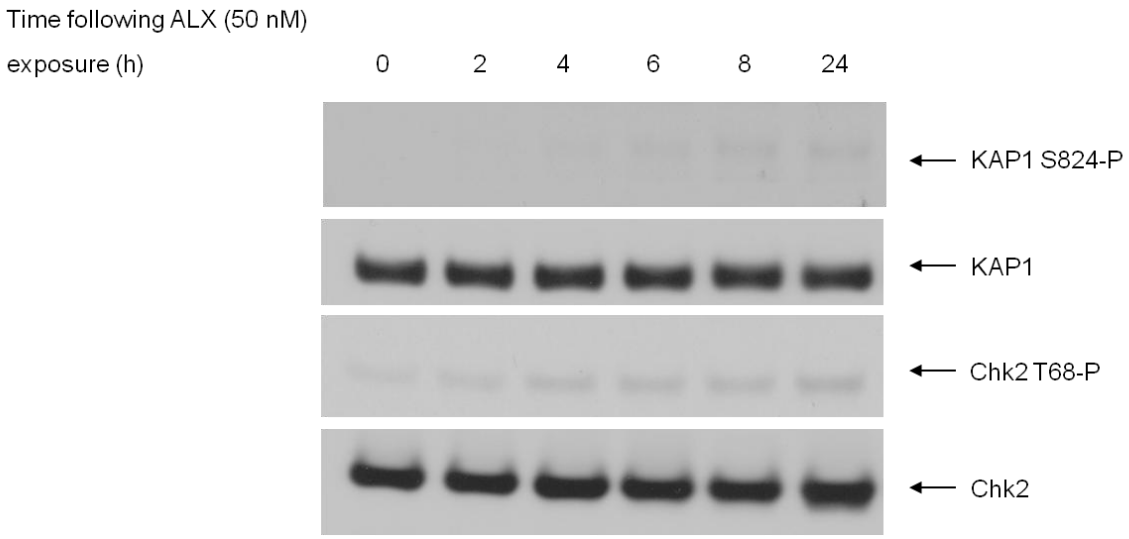

Figure 4.11 ALX does not activate ATM-specific substrates
HeLa cells were treated with 50 nM ALX and cells were harvested at the times indicated after treatment. Whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with antibodies recognising the phosphorylated forms of KAP-1 and Chk2. Antibodies against total proteins were used as loading controls.

with ALX does not affect the DDR.

The MRN mediator complex senses DSBs in the cell and recruits ATM to these sites following damage (Lee and Paull, 2005). Any disruption to the MRN complex results in both ATM localisation and activation defects. Since ALX-induced phosphorylation was lower in the *MRE11* mutant HCT116 cells compared to the other cell lines, the function of this component of the MRN complex was inhibited. Two methods were used to investigate the effect this would have on the response to ALX. Firstly, the MRE11 inhibitor, Mirin, was used to pre-treat cells before they were exposed to ALX. Mirin inhibits MRE11-associated exonuclease activity. The results indicate that, despite a slight decrease in both NBS1- and Chk1- phosphorylation and a concomitant increase in RPA2- and H2AX-phosphorylation, treatment with Mirin did not affect the signalling response to ALX (Figure 4.12A). Camptothecin-treated cells were used as a positive control (Figure 4.12A).

Secondly, MRE11 protein levels were depleted in U2OS cells using MRE11 siRNA prior to treatment with ALX. The reduced levels of MRE11 were confirmed by Western blotting (Figure 4.12B). The levels of NBS1 were also reduced in cells depleted of MRE11; this is unsurprising since it is known that depletion of MRE11 affects levels of all three MRN components (Figure 4.12B) (Stewart *et al.*, 1999). The results in Figure 4.12B show that the down-regulation of MRE11 protein levels did not affect the response to ALX and re-enforces the lack of involvement of the ATM-MRN pathway in the ALX-dependent DDR.

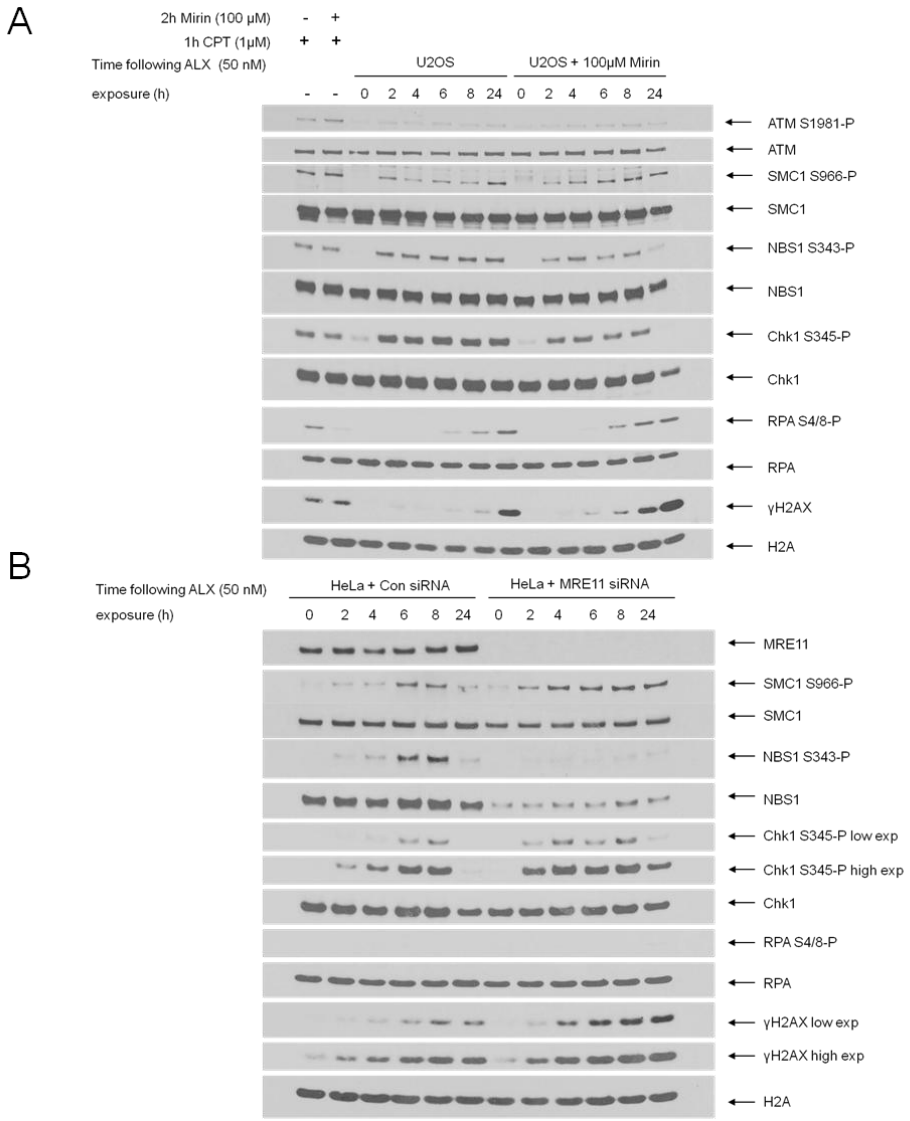

Figure 4.12 The MRN complex does not play a role in the cellular response to ALX
(A) U2OS cells were pre-treated with 100 μM Mirin, treated with 50 nM ALX and harvested at the times indicated. Cells treated with 1 μM CPT were used as controls, alongside cells pre-treated with 100 μM Mirin and treated with 1 μM camptothecin; (B) HeLa cells depleted of MRE11 using siRNA were treated as in (A). Whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with the indicated antibodies against DNA damage proteins. Antibodies against total proteins were used as loading controls. Experiments were performed twice to ensure reproducibility. CPT denotes camptothecin. Low exp and high exp denote low exposure and high exposure respectively.

4.2.3 Exposure to ALX induces a DDR that is mediated by ATR and, partly, by DNA-PK

Both TOP2 inhibitors and alkylators can induce replication-associated DNA damage, which suggests that ATR may play a role in activating the damage-induced signalling pathways in response to ALX. To assess whether the ATR kinase pathway was involved in the response to ALX, DLD1-Seckel cells were treated with ALX. These cells contain a hypomorphic mutation at the *ATR* locus that, at the cellular level, significantly decreases ATR expression due to aberrant splicing of the *ATR* mRNA transcript (O'Driscoll *et al.*, 2003). Cells that were engineered to re-express ATR were treated with ALX alongside the DLD1-Seckel cells. The phosphorylation response observed in cells with low levels of ATR was markedly different to that seen in the ATR complemented cell line, indicating that ALX is acting in an ATR-dependent manner (Figure 4.13A). Reducing the level of ATR caused a dramatic reduction in the phosphorylation of a number of DDR proteins such as NBS1 and Chk1; this was rescued by the re-expression of ATR (Figure 4.13A). Interestingly, the phosphorylation of H2AX and RPA2 in DLD1-Seckel cells was highly elevated following ALX treatment compared to the complemented cells (Figure 4.13A). This observation may be due to an increase in cellular replication stress since ATR-deficient cells contain high levels of fragile site breakage where stalled forks collapse and DSBs accumulate (Cimprich and Cortez, 2008). Consistent with this, the observed enhanced H2AX response in the DLD1-Seckel cells is indicative of an increase in DSB formation (Figure 4.13A).

The activation of Chk1, a checkpoint kinase regulated by ATR, after DNA damage is known to require the phosphorylation of sites within its C-terminal region, which includes the highly conserved serine 317 residue (S317). ATR is able to phosphorylate Chk1 at this site, which is

essential to the activation of downstream DNA damage signalling as well as to enhance efficiency of phosphorylation on neighbouring serine residues such as S345 (Zhao and Piwnica-Worms, 2001). Since ALX treatment induces an ATR-dependent cellular DDR, the DNA damage signalling function of Chk1 in response to ALX was examined using DLD1 cells that have a point mutation in the *Chk1* locus, which abrogates the S317 residue (S317A) (Wilsker *et al.*, 2008). These cells and parental DLD1 cells were treated with ALX and Western blotting was used to assess the phosphorylation levels of DDR proteins. As expected, levels of S345 phosphorylated Chk1 were reduced in the Chk1 S317A mutant cells (Figure 4.13B). Interestingly, cells harbouring the mutant Chk1 phosphorylation site appeared to exhibit a similar exacerbated signalling response after ALX treatment as the DLD1-Seckel cells (Figure 4.13B). The levels of phosphorylated RPA and H2AX were increased in Chk1S317A mutant cells compared to levels in the DLD1 parental cells after treatment with ALX (Figure 4.13B). These data indicate that mutations in the ATR-Chk1 pathway affect the cellular response to ALX induced DNA damage.

Notably, when DLD1-Seckel cells were treated with ALX in combination with the ATM inhibitor, the induced replication stress was not alleviated (Figure 4.14). Although ATM signalling can occur at collapsed replication forks, the data suggest that ATM does not mediate the signalling response to ALX even in the absence of ATR.

A level of redundancy does exist amongst PIKK signalling and, although it appears that ATM does not play a role in the DDR following treatment with ALX, it may be possible that DNA-PK does. There is evidence that DNA-PK becomes activated in response to stalled replication forks that have generated DSBs upon Chk1 inhibition, implying that DNA-PK does not only function in NHEJ (McNeely *et al.*, 2010). The effect on the levels of phosphorylation of

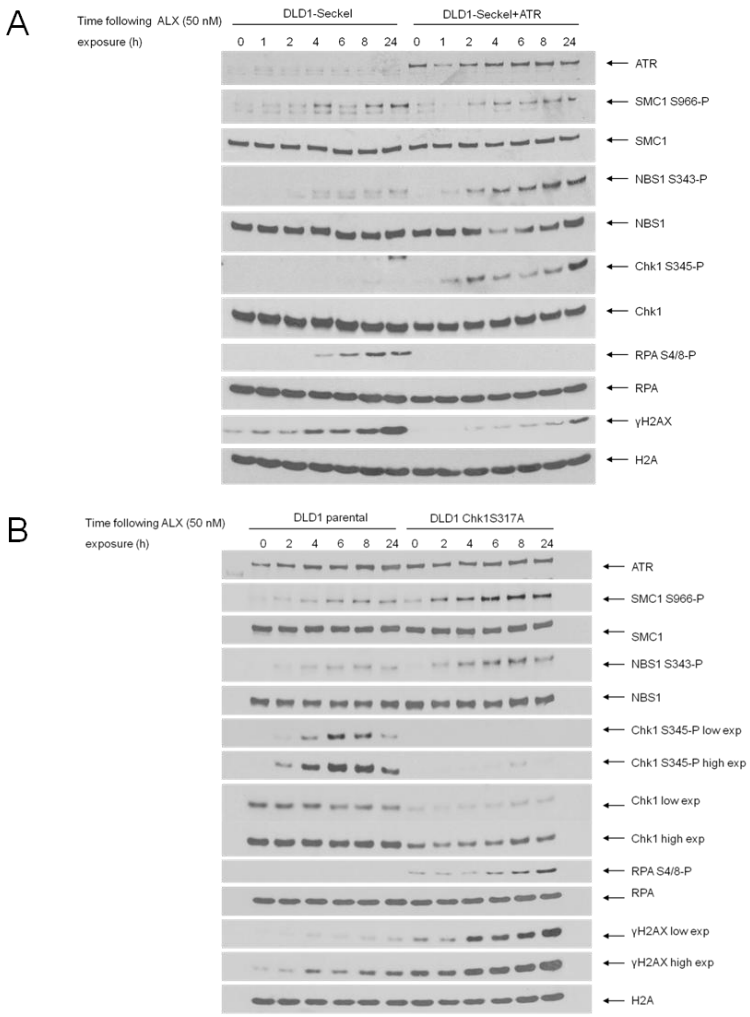

Figure 4.13 The ATR-Chk1 pathway is involved in the signalling response to ALX
(A) DLD1 Seckel and ATR complemented cells were treated with 50 nM ALX and harvested at the times indicated; (B) DLD1 parental and Chk1S317A mutant cells were treated as in (A). Whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with the indicated antibodies against DNA damage proteins. Antibodies against total proteins were used as loading controls. Low exp and high exp denote low exposure and high exposure respectively.

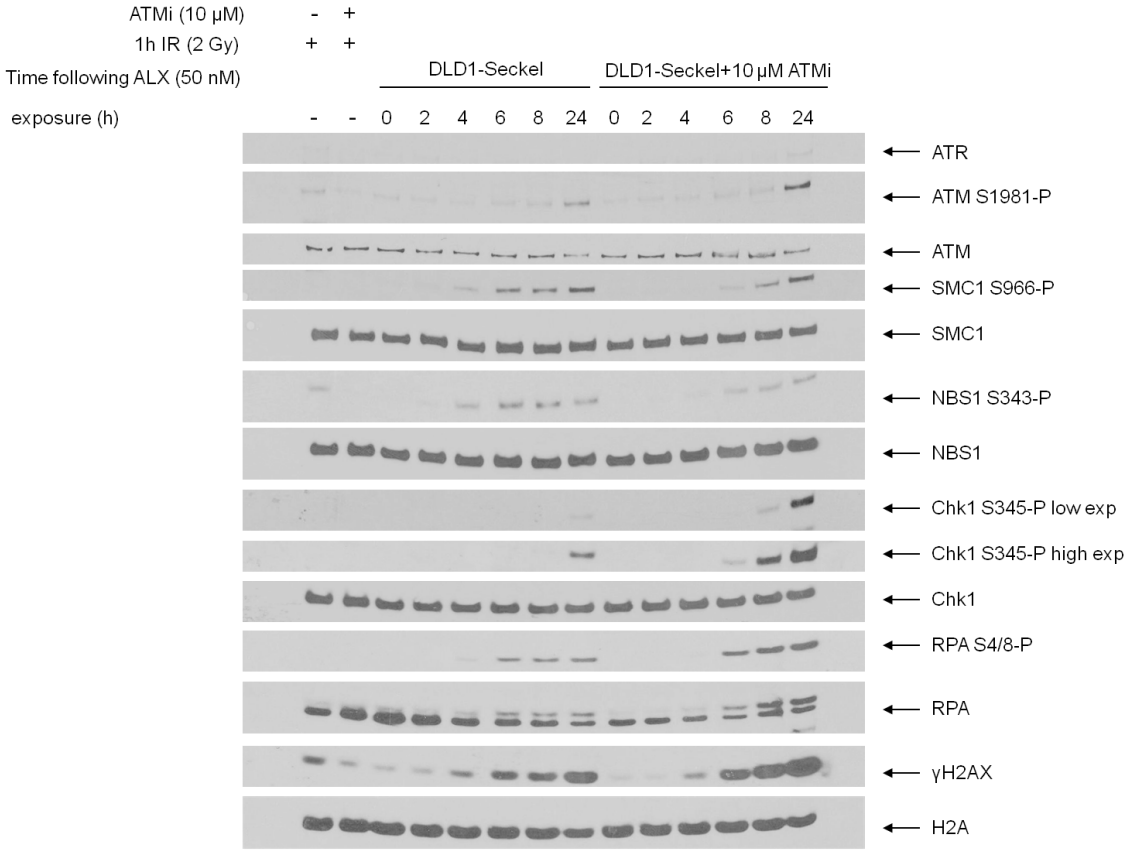

Figure 4.14 ATM does not mediate the signalling response to ALX if ATR is depleted
DLD1 Seckel cells were pre-treated with DMSO or 10 µM ATM inhibitor, then treated with 50 nM ALX after 1h and harvested at the times indicated. Whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with the indicated antibodies against DNA damage proteins. Irradiated cells were used as controls alongside cells pre-treated with 10 µM ATM inhibitor and irradiated with 2 Gy. Antibodies against total proteins were used as loading controls. ATMi denotes ATM inhibitor. Low exp and high exp denote low exposure and high exposure respectively.

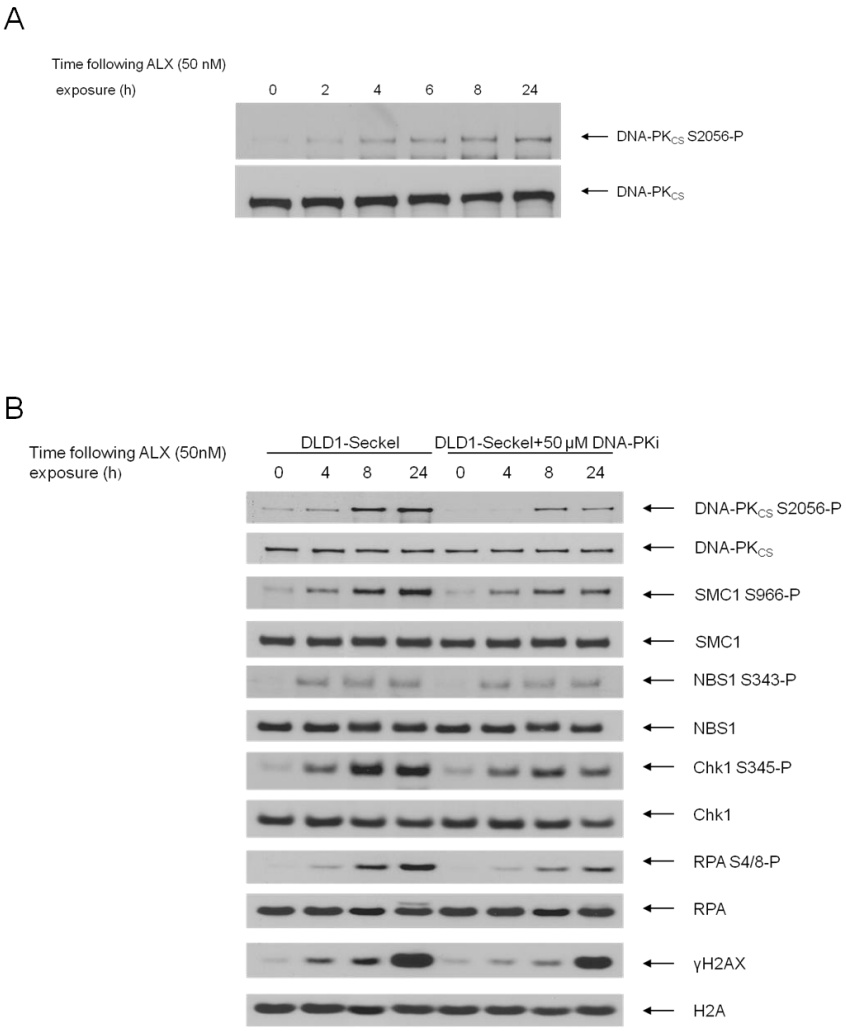

Figure 4.15 DNA-PK is involved in mediating the DDR in response to ALX
(A) HeLa cells were treated to 50 nM ALX and cells were harvested at the times indicated; (B) DLD1-Seckel cells were pre-treated with 50 µM DNA-PK inhibitor, treated with 50 nM ALX after 1h and harvested at the times indicated. Whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with the indicated antibodies against DNA damage proteins. Antibodies against total proteins were used as loading controls. DNA-PKi denotes DNA-PK inhibitor.

DNA-PK after treatment with ALX was, therefore, assessed in HeLa cells treated with the drug over a 24 h time period. It was observed that there were increasing levels of phosphorylated DNA-PK with increasing time of exposure to ALX (Figure 4.15A). These results indicate that DNA-PK becomes activated in response to ALX treatment.

To further address this, DLD1-Seckel cells were treated with ALX in the presence or absence of the DNA-PK inhibitor, DMNB. Western blot analysis showed a significant reduction in H2AX and RPA2 phosphorylation in DLD1-Seckel cells that had been pre-treated with DMNB, despite only partial inhibition of DNA-PK kinase activity upon treatment with this inhibitor (Figure 4.15B). In addition, levels of phosphorylated Chk1 and SMC1 were decreased in cells that had been treated with the DNA-PK inhibitor compared to cells that had not, giving rise to the suggestion that, in response to ALX, DNA-PK may be functioning to activate cell cycle checkpoints as well as in the response to replication-associated DSBs (Figure 4.15B). Together, these data suggest that the DDR, upon exposure to ALX, is primarily mediated by ATR and, to a lesser extent, DNA-PK. In contrast, ATM appears to play no significant role in the signalling response to this novel anthraquinone.

4.2.4 ALX requires both TOP2α and TOP2β and its alkylating function to elicit a DDR

Although ALX is a TOP2 inhibitor, it also contains an alkylating moiety, potentially capable of inducing DNA crosslinks and causing DNA base damage. To determine which function of ALX activates the observed DDR, HeLa cells were exposed to ALX or a derivative of ALX, ZP275, which lacks the alkyating side chain whilst retaining its ability to inhibit TOP2. Interestingly, both compounds elicited the phosphorylation of known PIKK downstream

targets, including SMC1, NBS1, Chk1, RPA2 and H2AX (Figure 4.16). However, the loss of the alkylating side chain severely attenuated this response, implying that both functions of ALX are required for its ability to activate the DDR (Figure 4.16). Interestingly, the phosphorylation of Chk1 was severely compromised in cells treated with ZP275, indicating that the alkylating ability of ALX is required for the ATR-dependent DDR in response to the drug (Figure 4.16). However, the fact that the signalling response is not completely abolished in response to ZP275 does indicate that both functions of ALX contribute to its ability to induce a DDR (Figure 4.16).

It is known that alkylation damage is a potent inducer of the TLS damage tolerance pathway that functions to bypass bulky DNA lesions allowing the replication fork to progress. Activation of this pathway is primarily controlled by the mono-ubiquitylation of PCNA by the E3 ubiquitin ligase Rad18, which is potently induced by the treatment of cells with agents that induce replication-associated DNA damage, e.g. UV, H_2O_2 and alkylating agents. To determine whether the replication-associated DNA damage observed in cells treated with ALX coincided with activation of the TLS pathway, HeLa cells were treated with ALX, etoposide (a known TOP2 inhibitor) or UV. It was observed that, unlike UV, ALX or etoposide treatment of HeLa cells failed to induce the mono-ubiquitylation of PCNA (Figure 4.17). This result indicates that the DNA alkylating ability of ALX does not conform to the mechanism that is typical to the class of alkylating agents as a whole. It is also possible that ALX might be functioning via its alkylating ability only at sites of DNA that contain bound TOP2, i.e. at regions of DNA where TOP2 has been inhibited.

Since ALX, as an anthraquinone derivative, has previously been found to inhibit TOP2α, this suggests that DNA damage, particularly the DSBs, generated from ALX exposure, would

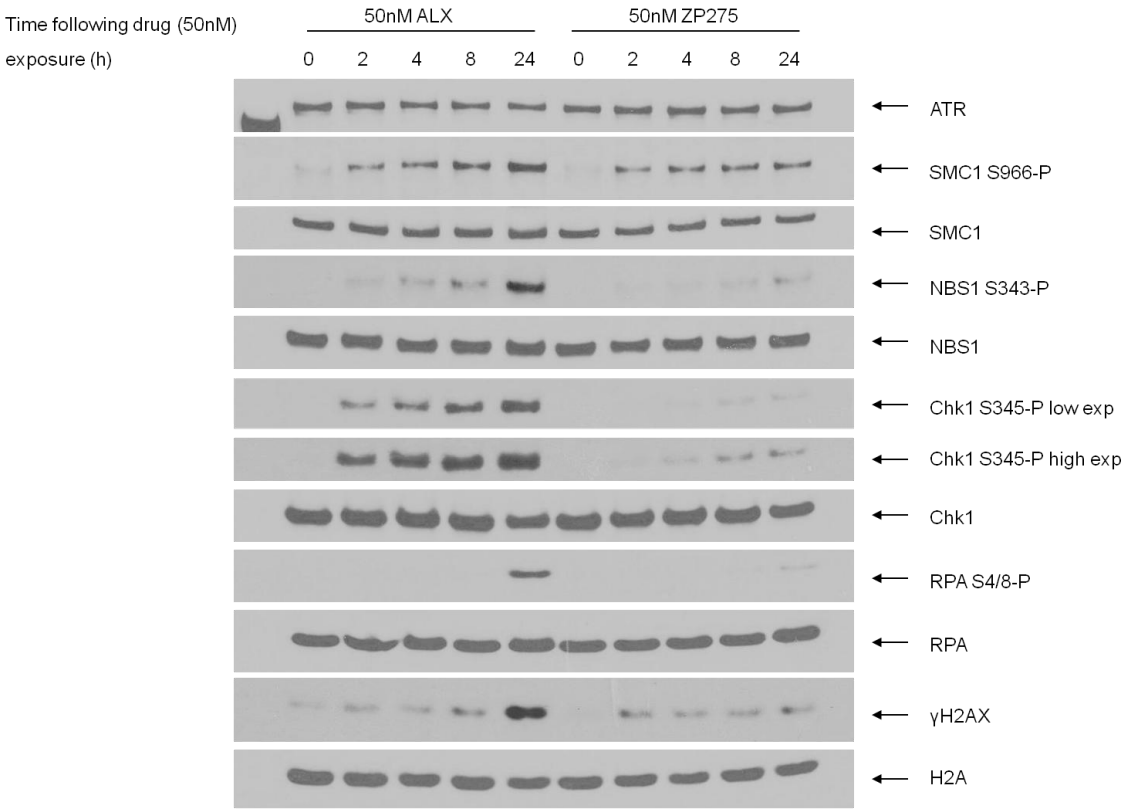

Figure 4.16 ALX requires a combination of its ability to inhibit TOP2 and its capacity to alkylate DNA to induce a DDR

HeLa cells were treated to 50 nM ALX or 50 nM ZP275 (a derivative of ALX that lacks the alkylating side chain). Cells were harvested at the times indicated. Whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with the indicated antibodies against DNA damage proteins. Antibodies against total proteins were used as loading controls. Low exp and high exp denote low exposure and high exposure respectively.

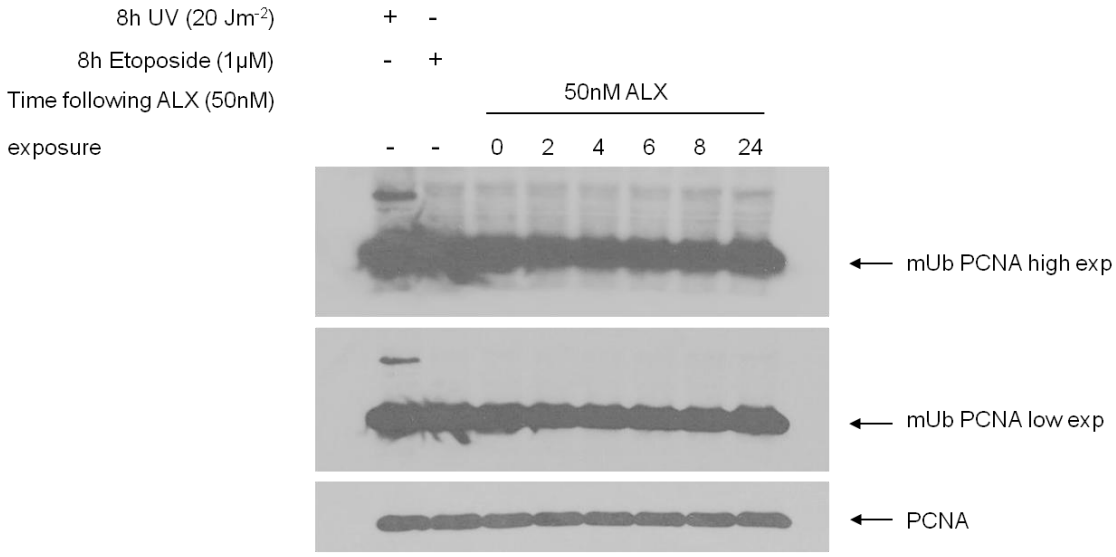

Figure 4.17 ALX does not induce PCNA mono-ubiquitylation
HeLa cells were treated with 50 nM ALX and harvested at the times indicated. Whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with the indicated antibodies against PCNA. UV-irradiated cells and etoposide-treated cells were used as controls after 8 h exposure to each respective damaging agent. mUb denotes mono-ubiquitylation. Low exp and high exp denote low exposure and high exposure respectively.

depend on the presence of TOP2. To determine whether this was the case, HeLa cells were transfected with either TOP2α or TOP2β siRNA and treated with ALX over a 24 h time period. The DDR was subsequently determined using Western blotting. Surprisingly, the depletion of either TOP2α or TOP2β alone did not alleviate the cytotoxicity of ALX since many of the downstream targets in the DDR such as SMC1, NBS1, Chk1, RPA2 and H2AX were still phosphorylated (Figure 4.18A). This indicates that ALX does not solely depend on the presence of either TOP2α or TOP2β, but may be capable of inhibiting multiple class II-type topoisomerase enzymes.

To investigate the possibility of redundancy between the two isoforms of TOP2, HeLa cells were treated with siRNA to both TOP2α and TOP2β prior to exposure to ALX. Interestingly, the siRNA-mediated depletion of both topoisomerases resulted in decreased levels of phosphorylated RPA2 and H2AX, implying that TOP2α and TOP2β can both be targeted by ALX, not just TOP2α (Figure 4.18B). Despite this depletion of both TOP2α and TOP2β, the checkpoint response, as measured by Chk1 phosphorylation induced by ALX, was not abrogated, supporting a role for the alkylating function of ALX being important for ATR-dependent cell cycle regulation (Figure 4.18B).

Since etoposide is a known TOP2α inhibitor, it was used as a positive control to compare the response to ALX. The reduced response to etoposide in cells treated with TOP2α siRNA compared to those cells treated with control is expected since etoposide requires the presence of TOP2α to induce a DDR (Figure 4.19) (Baldwin and Osheroff, 2005). This is in contrast the response of these cells to ALX where the depletion of TOP2α did not alleviate the response to ALX (Figure 4.18A). The lack of a checkpoint signalling response observed in

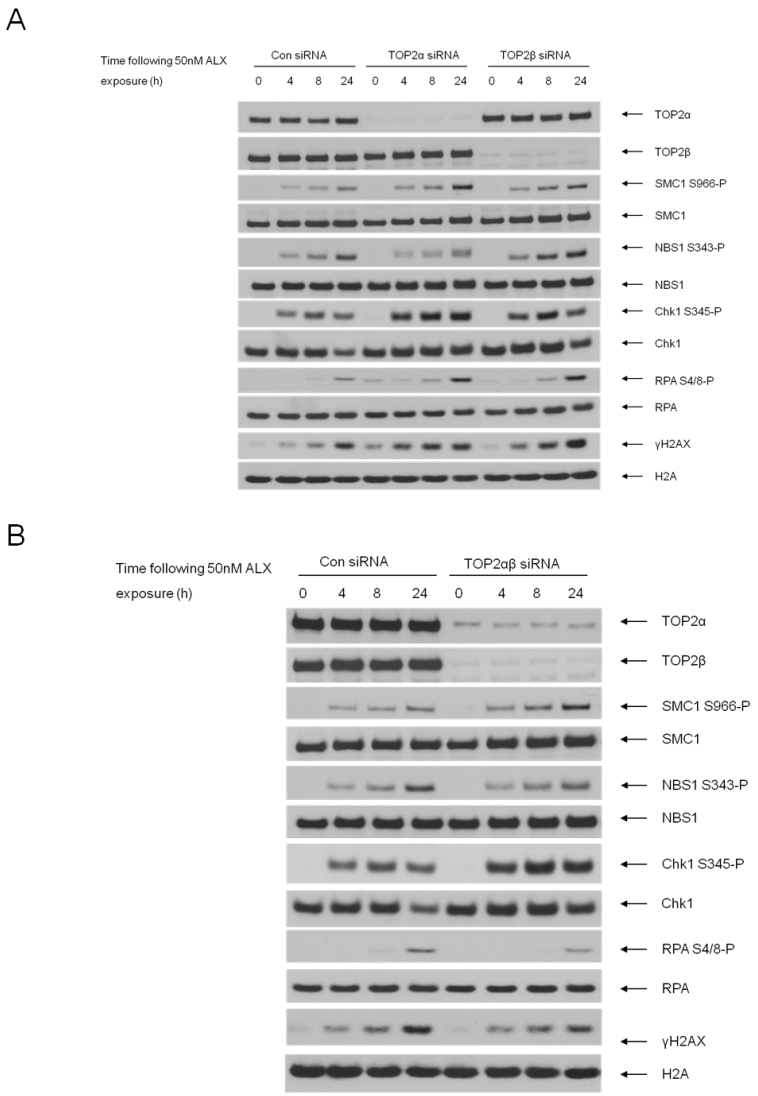

Figure 4.18 The ALX-induced DDR requires both TOP2α and TOP2β but is not dependent on either enzyme

(A) HeLa cells depleted of either TOP2α or TOP2β using siRNA were treated with 50 nM ALX; (B) HeLa cells depleted of both TOP2 enzymes simultaneously using siRNA were treated as in (A). Cells were harvested at the times indicated and whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with the indicated antibodies against DNA damage proteins. Antibodies against total proteins were used as loading controls. Experiments were performed twice to ensure reproducibility.

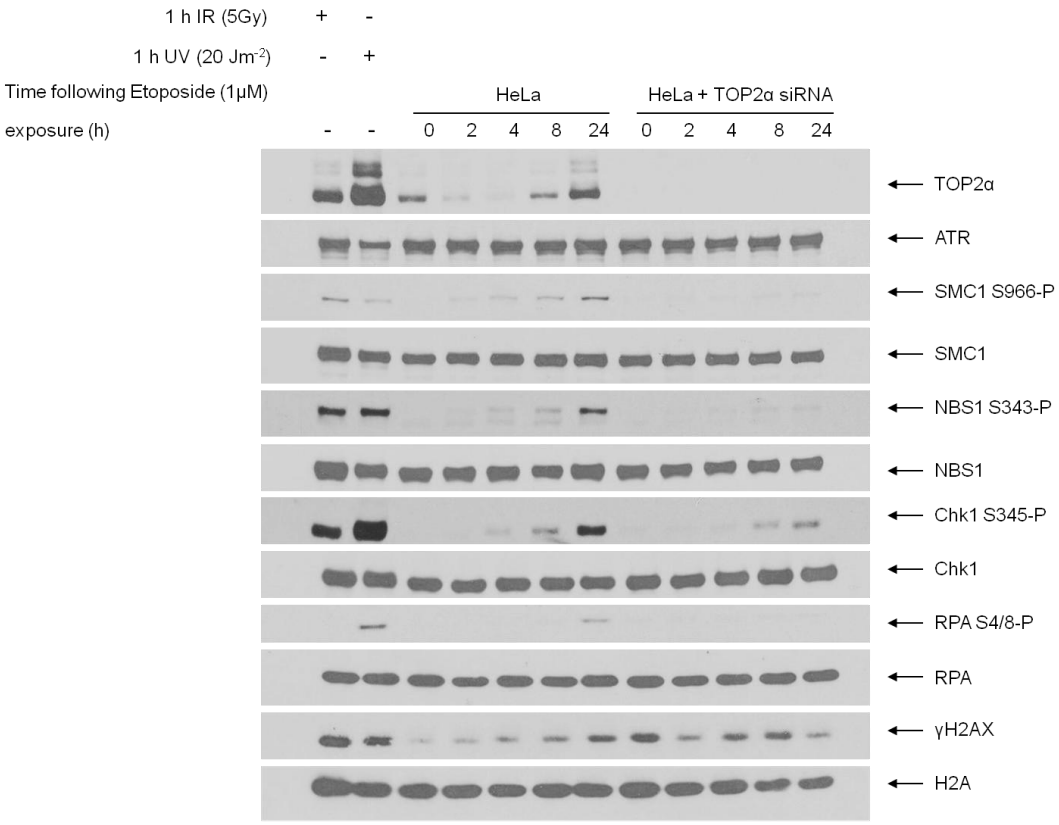

Figure 4.19 The DDR induced by etoposide solely requires TOP2α
HeLa cells were depleted of TOP2α and treated with 1 μM etoposide. Cells were harvested at the times indicated and whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with the indicated antibodies against DNA damage proteins. Antibodies against total proteins were used as loading controls. IR- and UV-treated cells were used as controls after 1 h exposure to each respective damaging agent.

cells treated with etoposide further supports the idea that ALX uses its alkylating function to activate ATR. IR- and UV-treated cells were used as controls (Figure 4.19).

Collectively, these data suggest that the DDR that is induced by ALX requires its ability to inhibit both isoforms of TOP2. Furthermore, the ALX-induced ATR-dependent checkpoint response appears to be reliant on the alkylating properties of this chemotherapeutic drug.

4.2.5 ALX does not require the MMR pathway or the FA repair pathway

Whilst it is clear that ALX activates the ATR-Chk1 replication stress pathway, the components of cellular repair pathways that are involved in the response to ALX are not known. The MMR pathway is known to recognise and remove a number of alkyl lesions and MSH2, in particular, is required for the activation of ATR following treatment with the methylating agent, MNNG (Wang and Qin, 2003). Since it has been shown that the alkylating ability of ALX is required for the activation of the ATR-dependent DDR, it is conceivable that elements of the MMR pathway are required for the response to ALX. To determine whether the MMR pathway becomes activated upon exposure to ALX, siRNA targeting MSH2 was transfected into HeLa cells that were then treated with ALX over a 24 h time period. Rather than alleviating the ATR-dependent DDR after ALX treatment, the depletion of MSH2 resulted in heightened activation of Chk1, thus indicating that MSH2 is not required for the activation of ATR in response to ALX (Figure 4.20). The levels of phosphorylated SMC1, RPA2 and H2AX were also increased in cells depleted of MSH2, which suggests that the signalling response to ALX is not recognised by components of the MMR pathway (Figure 4.20). This is important since tumour cells with deficiencies in the MMR pathway may prove to be sensitive to the cytotoxic effects of ALX.

The FA pathway is also involved in the repair of lesions that are encountered during DNA replication and may, therefore, become activated in response to ALX. The 2008 ovarian carcinoma cell line is deficient in the FA pathway due to methylation of the *FANCF* promoter region, and this is restored by the functional complementation with *FANCF* (Taniguchi *et al.*, 2003). The loss of the mono-ubiquitylation of FANCD2 in the FANCF deficient cells is a marker of function of this pathway and is, therefore, restored in the complemented cells since FANCF is a component of the E3 ubiquitin ligase that targets FANCD2 in the FA pathway (Taniguchi *et al.*, 2003). These cells were treated with ALX over a 24 h time period and the activation of the DDR was determined using Western blotting. The deficiency in FANCF in the 2008 cell line was shown by the lack of FANCD2 ubiquitylation in these cells compared to the levels of ubiquitylated FANCD2 in the complemented cells (Figure 4.21). The lack of any difference in the signalling response to ALX between cells with and without FANCF showed that the FA pathway is dispensable for the ALX-induced DDR (Figure 4.21). This suggests that any perturbations within the FA pathway will not affect the response after ALX treatment and will, therefore, be unlikely to contribute to any resistance to ALX. It also suggests that ALX is unlikely to induce DNA crosslinks that are recognised by the FA pathway.

4.2.6 Exposure to ALX leads to a G2/M checkpoint arrest, mitotic abnormalities and death by mitotic catastrophe

TOP2 inhibitors and alkylating agents induce DNA damage that occurs during replication; therefore, it is likely that the progression of cells through S phase will be affected by such agents. Since ALX possesses both of these properties, and given the fact that that TOP2 is

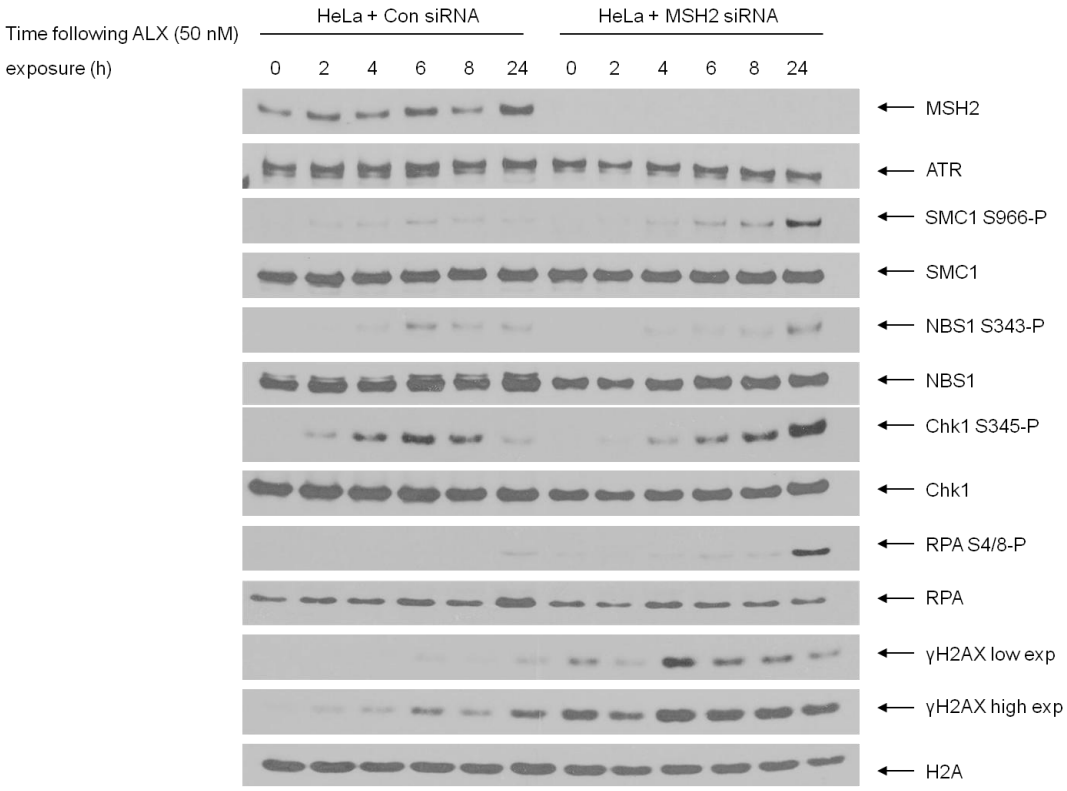

Figure 4.20 ALX-induced alkylated damage is not recognised by the MMR pathway
HeLa cells depleted of MSH2 using siRNA were treated with 50 nM ALX and harvested at the times indicated. Whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with the indicated antibodies against DNA damage proteins. Antibodies against total proteins were used as loading controls. Low exp and high exp denote low exposure and high exposure respectively.

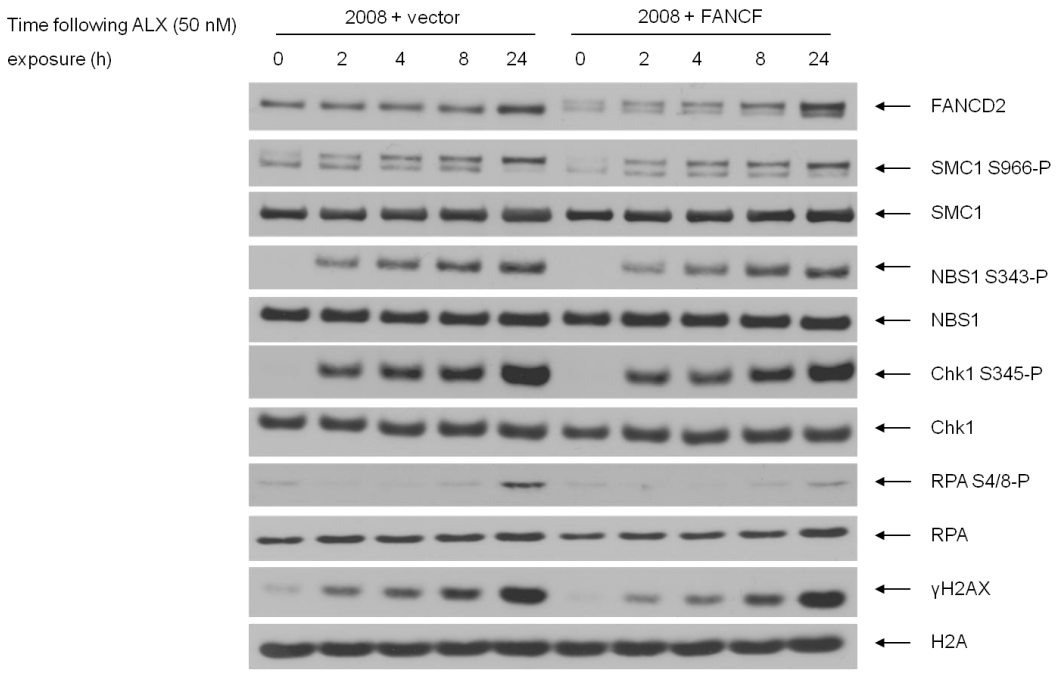

Figure 4.21 ALX-induced alkylated damage is not recognised by the FA pathway
2008+vector (FANCF deficient) and 2008+FANCF (FANCF complemented) cells were treated to 50 nM ALX and harvested at the times indicated. Whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with the indicated antibodies against DNA damage proteins. Antibodies against total proteins were used as loading controls.

also involved in chromosome segregation, the possibility that cells would be disrupted in both S phase and mitosis in response to ALX is likely.

To investigate the effect of ALX on the cell cycle, flow cytometric analysis was performed in HeLa cells treated with ALX over a 24 h time period. The results indicate that treatment with ALX induced an S phase arrest of the cell cycle between 4 h and 8 h, which is consistent with the early activation of the ATR-mediated checkpoint response observed in Figures 4.4-4.7 (Figure 4.22A). In addition, at the later time of 24 h, cells appeared to arrest in late S phase/G2-phase of the cell cycle, an observation that is consistent with the late induction of DSBs and the activation of a DNA-PK-mediated DDR upon exposure to ALX depicted in Figures 4.8 and 4.15 respectively (Figure 4.22A).

To further support the fact that ALX-treated cells accumulate in late S/G2, HeLa cells that had been treated with ALX for 24 h were analysed by Western blotting using antibodies recognising specific markers of mitosis. These markers included phosphorylated histone H3 (H3 S10-P), which is a marker of mitotic cells, and cyclin A and cyclin B1, which are normally degraded as cells progress through mitosis. H3 S10-P levels were greatly reduced in ALX-treated cells compared to cells that had not been treated with ALX, indicating that cells exposed to ALX for 24 h have undergone activation of a G2 checkpoint (Figure 4.22B). Consistent with this are the elevated levels of cyclin A and cyclin B1 in these cells, showing that cells have not progressed through mitosis (Figure 4.22B).

Despite this, mitotic cells could still be observed by immunofluorescence microscopy after treatment with ALX for 24 h, indicating that some cells had bypassed the G2/M checkpoint observed in Figure 4.22A (Figure 4.23). Interestingly, these cells were found to contain mitotic abnormalities, including chromosome segregation and DNA decatenation defects such

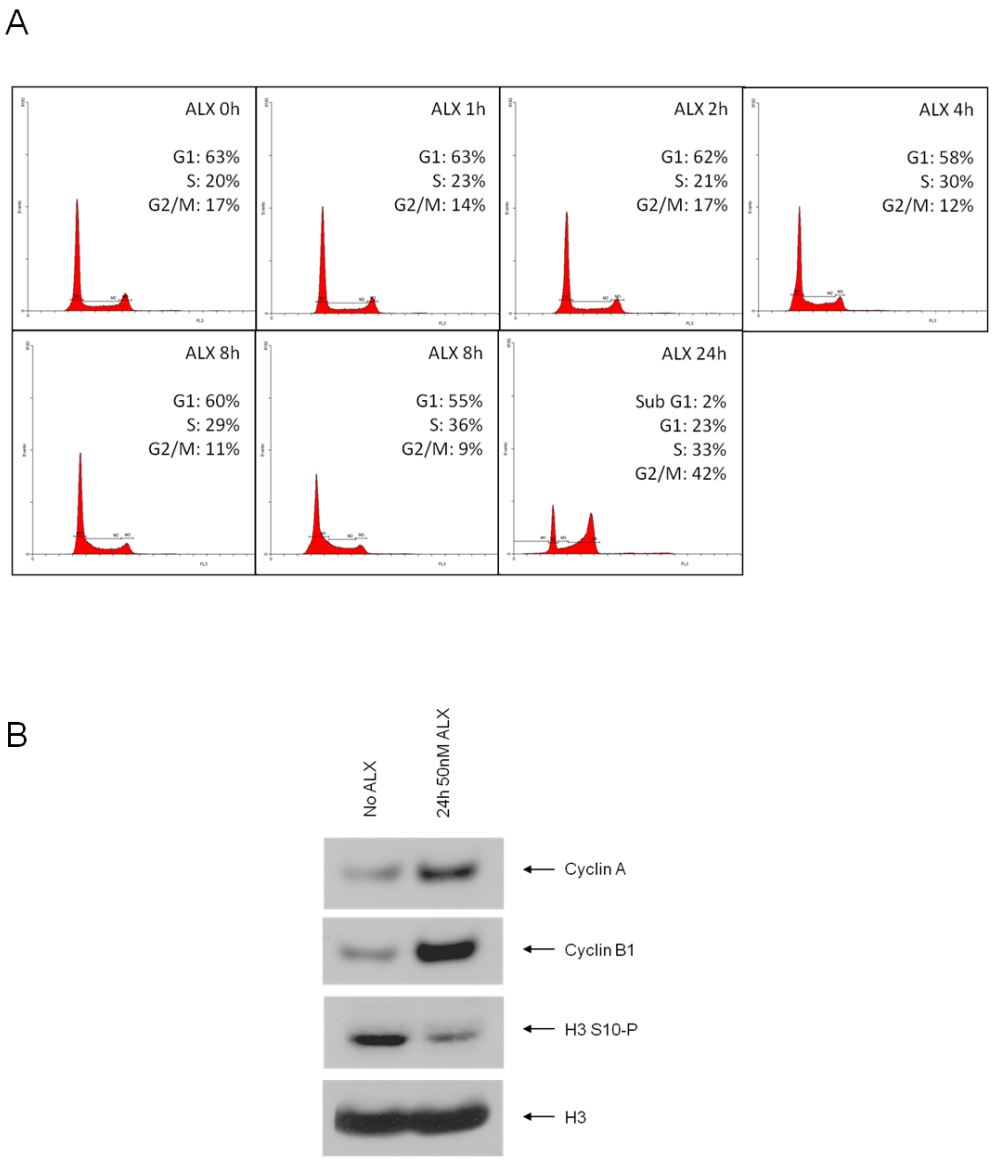

Figure 4.22 ALX induces a cell cycle arrest in S phase and, after 24 h, induces a G2/M arrest

(A) HeLa cells treated with 50 nM ALX were analysed by flow cytometry using PI dye to quantify DNA content during the cell cycle. The percentage of cells accumulating in sub G1, G1, S or G2/M is shown for DMSO- (0h) and ALX-treated samples at the times indicated. The FACS profiles shown are representatives from two independent experiments; (B) HeLa cells treated with 50 nM ALX were harvested after 24 h. Whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with the antibodies recognising cyclin A, cyclin B and H3 S10-P. The antibody against total histone H3 was used as a loading control.

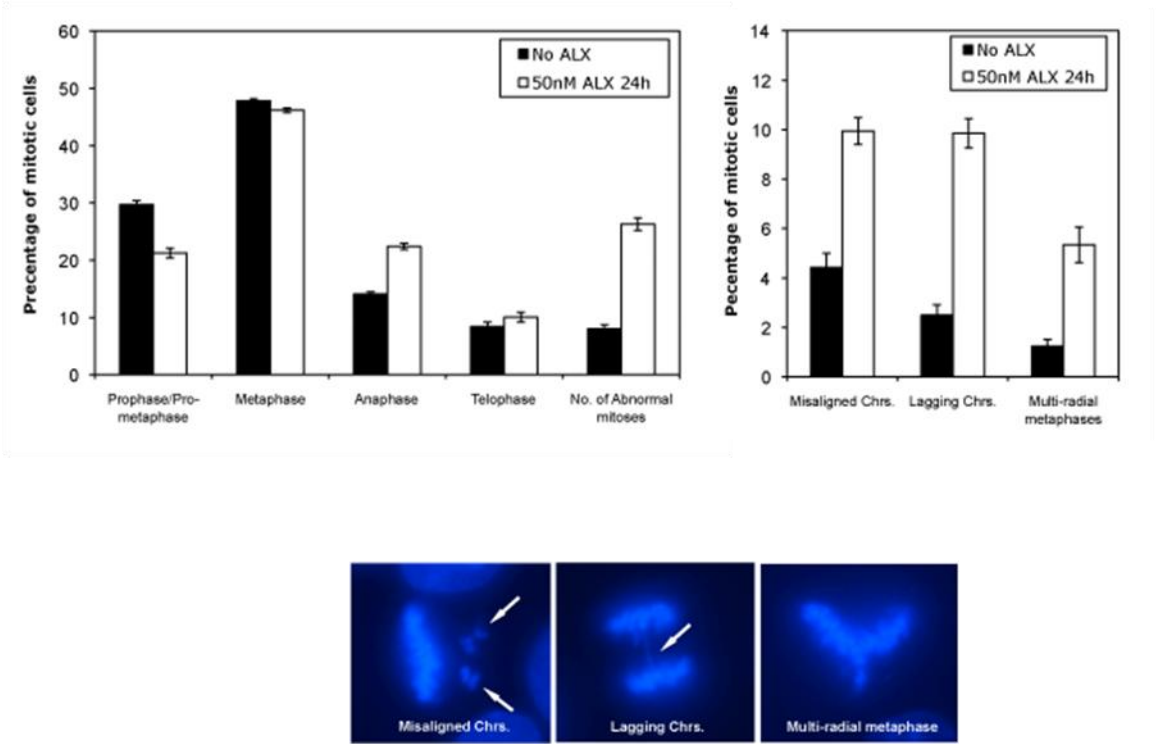

Figure 4.23 A small number of cells bypass the ALX-induced G2/M checkpoint and contain mitotic abnormalities

HeLa cells were treated with 50 nM ALX and fixed after 24 h treatment. Cells were washed and mounted in Vectashield containing DAPI before being visualised using a Nikon Eclipse E600 Microscope. A graphical representation of the mitotic abnormalities is depicted alongside the images.

as misaligned chromosomes, lagging chromosomes depicted by anaphase bridging and multi-polar metaphase spindles (Figure 4.23). Together, these data indicate that, although cells accumulate in late S/G2 after treatment with ALX, some cells bypass this checkpoint and enter mitosis with unrepaired breaks and mitotic abnormalities.

To establish whether the unrepaired DNA damage induced by ALX in cells entering mitosis contributed to cell death, HeLa cells were treated with two doses of ALX and harvested for FACS analysis every 24 h for 5 days. It was observed that both concentrations of ALX could induce a significant amount of cell death, as depicted by the increase in the sub G1 population of these cells compared to untreated cells (Figure 4.24). Cisplatin-treated cells were used as a control to induce cell death through the apoptotic pathway and these samples showed extremely high levels of cells that had accumulated in sub G1 (Figure 4.24).

The sub G1 peak is not specific for apoptosis: it is an indicator of dead cells with less than a 2n DNA content, which includes apoptotic cells, necrotic cells, cells that have undergone mitotic catastrophe and cells that have undergone autophagy. Therefore, to establish the mode of cell death that is induced by ALX treatment, HeLa cells were exposed to ALX and analysed by Western blotting using antibodies recognising specific markers of apoptosis. These markers included the reduction of procaspase 3 levels, signifying its cleavage/activation and consequent induction of apoptosis, reduced lamin B levels to represent the loss of nuclear membrane integrity during apoptosis, and the cleavage of PARP1 that again, signifies apoptosis. Interestingly, the levels of procaspase 3 in cells treated with the two high concentrations of ALX were no different to the levels in untreated cells, implying that ALX-induced cell death is not associated with this marker of apoptosis (Figure 4.25A). In contrast, cisplatin-treated cells exhibited significantly reduced levels of procaspase 3,

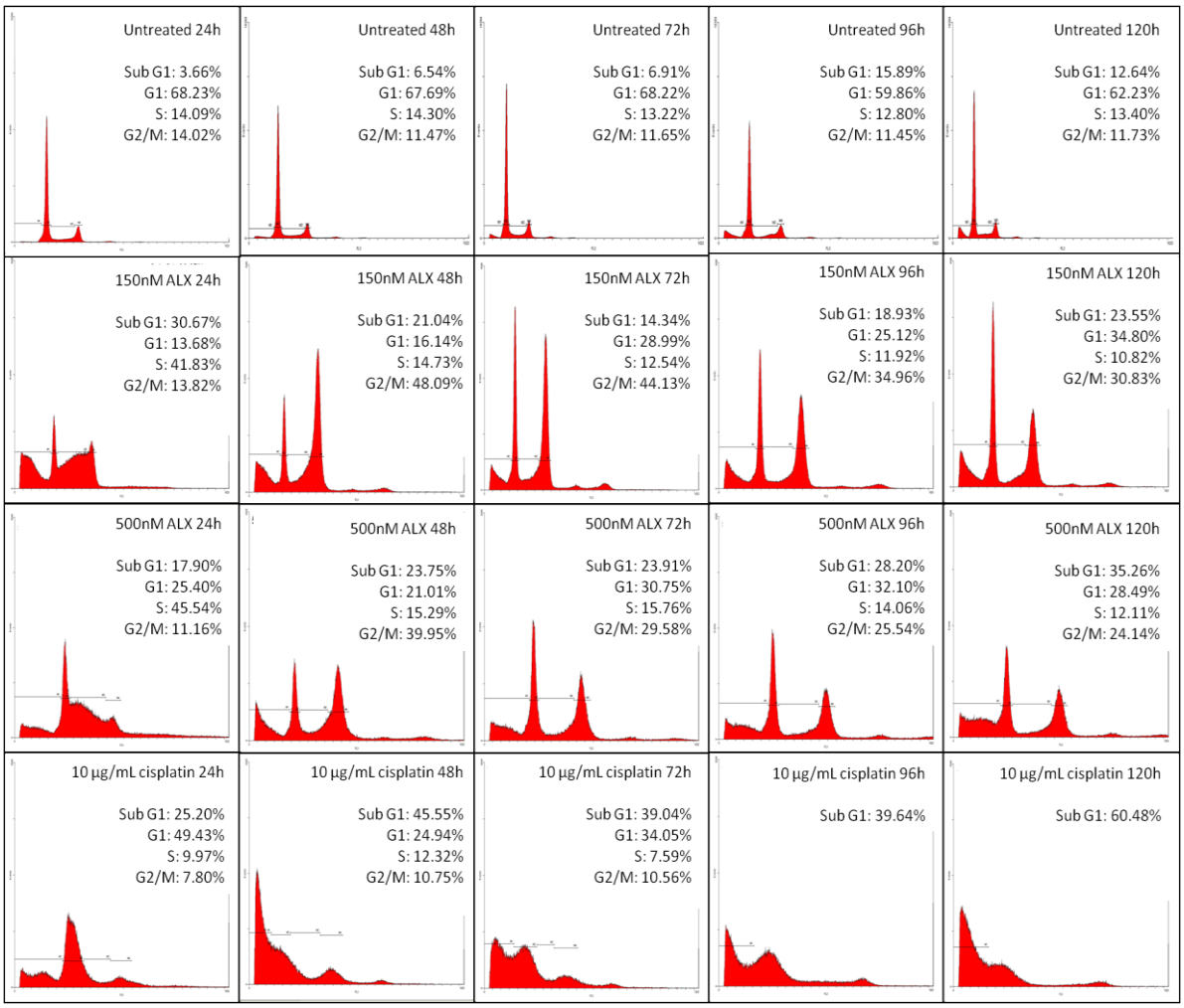

Figure 4.24 Damaged mitotic cells from ALX treatment cause cell death in HeLa cells
HeLa cells were treated with 150 nM and 500 nM ALX and harvested every 24 h for 5 days. Samples were analysed by flow cytometry using PI dye to quantify DNA content during the cell cycle. The percentage of cells accumulating in sub G1, G1, S or G2/M is shown for untreated and ALX-treated samples at the times indicated. Cisplatin-treated cells were used as controls.

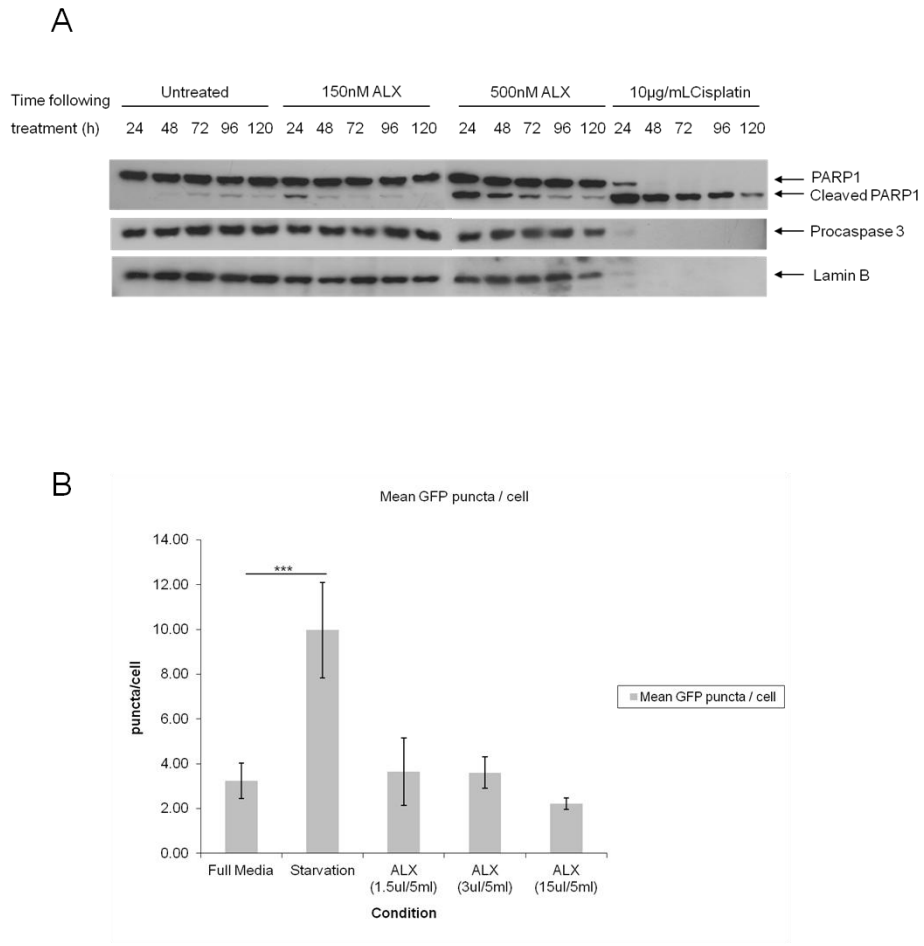

Figure 4.25 The mode of cell death induced by ALX is not through apoptosis or autophagy

(A) HeLa cells were treated with 150 nM and 500 nM ALX and harvested every 24 h for 5 days. Whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with the antibodies recognising PARP1, procaspase 3 and lamin B. Cisplatin-treated cells were used as controls; (B) 293 cells stably expressing the autophagy GFP-LC3 fusion protein were treated with three doses of ALX and then serum-starved for 4 h. Cells were fixed and mounted on glass slides. GFP-LC3-labelled puncta were visualised on a Nikon Eclipse E600 Microscope. Media-replenished cells and serum-starved cells were used as controls. Standard deviations from three independent experiments are presented, with P-values depicting significant differences (*=P<0.05 significant, **=P<0.01 highly significant, ***=P<0.001 very highly significant).

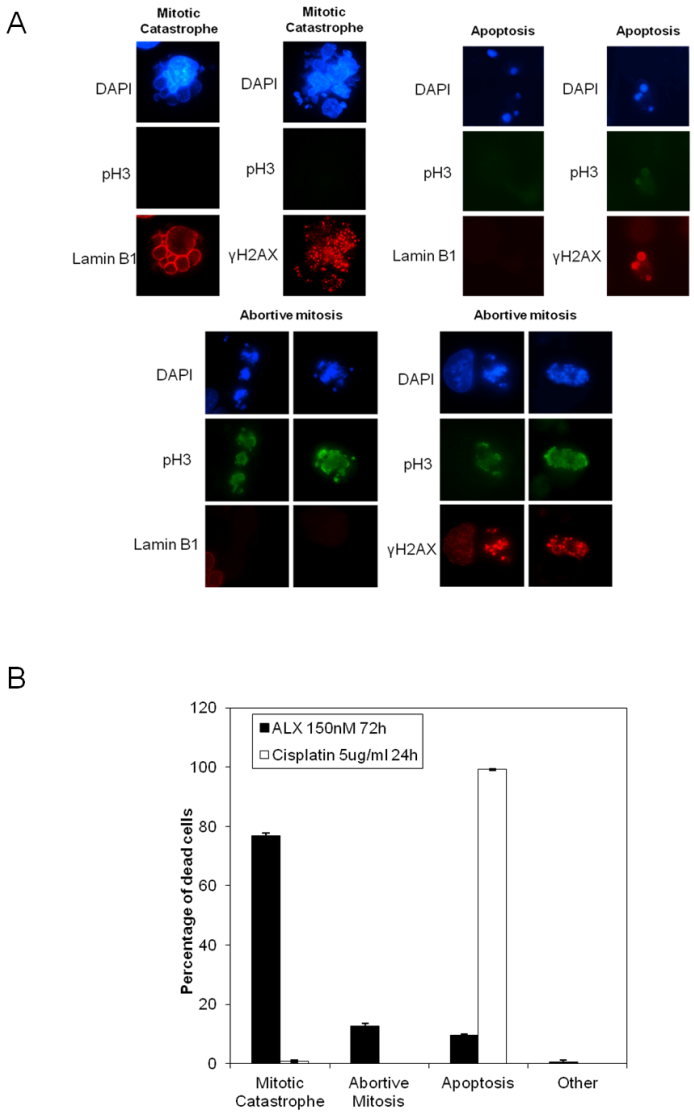

Figure 4.26 Exposure to ALX leads to cell death primarily by mitotic catastrophe
HeLa cells were treated with 150 nM ALX and fixed 72 h after treatment. Cells were processed for immunofluoresence using antibodies recognising γH2AX, lamin B and H3 S10-P. (A) Visualisation of cells using a Nikon Eclipse E600 Microscope; (B) Graphical representation of the percentage of dead cells undergoing each mode of cell death. For quantification, a minimum of 500 cells were counted per experiment. pH3 denotes H3 S10-P.

signifying the induction of apoptosis (Figure 4.25A). In addition, the unchanged lamin B1 levels in cells exposed to ALX signified no change to the nuclear membrane of these cells compared to the loss of nuclear membrane integrity in cisplatin-treated cells (Figure 4.25A). Notably, a small amount of PARP1 cleavage could be observed, more significantly at early times, following ALX treatment, implying that a small proportion of ALX-treated cells may be undergoing apoptosis (Figure 4.25A). However, at late times no major PARP1 cleavage product could be seen, as is the case with the cisplatin-treated cells (Figure 4.25A).

The possibility that ALX may be inducing cell death through autophagy was investigated by using an autophagosomal marker LC3 (that had been fused to GFP) in cells treated with ALX. During autophagy, cytoplasmic constituents are sequestered into autophagosomes, of which LC3 is a membrane component. Treatment with ALX did not induce LC3 puncta in contrast to serum starvation, which is known to induce autophagy (Figure 4.25B). The lack of an increase in LC3 puncta in ALX-treated cells indicates that the mode of cell death after ALX treatment is not through autophagy (Figure 4.25B).

Immunofluorescence microscopy was conducted on ALX-treated HeLa cells for further analysis of the mode of ALX-mediated cell death. Apoptotic cells were distinguished from cells undergoing mitotic catastrophe by the presence of a blebbed nuclear morphology, intense γH2AX staining and a lack of an intact nuclear membrane as depicted by reduced lamin B1 staining (Figure 4.26A). Cells undergoing mitotic catastrophe could be differentiated from those undergoing other forms of cell death by the presence of multiple micronuclei, an intact nuclear matrix and no H3 S10-P staining. The γH2AX staining in cells undergoing mitotic catastrophe is indicative of the large number of unrepaired DSBs (Figure 4.26A). Cells that were going through abortive mitosis exhibited fragmented chromosomes, a

loss of lamin B1 staining but retained H3 S10-P staining and γH2AX foci (Figure 4.26A). At the microscopic level, ALX-treated cells were found to primarily undergo cell death through mitotic catastrophe compared to cisplatin-treated cells that went through apoptosis (Figure 4.26B).

It can be seen that a small percentage of cells do undergo apoptosis after treatment with ALX (Figure 4.26B). This is consistent with the small amount of PARP1 cleavage depicted in Figure 4.25A. Recently, it has been found that cells that are subjected to a prolonged arrest in mitosis can undergo a p53-dependent apoptotic response (Orth *et al.*, 2012). Consistent with this, ALX is able to induce the stabilisation of p53 in the p53-proficient U2OS cell line (Figure 4.27). The exposure to ALX in these cells also led to the up-regulation of transcriptional targets of p53, including MDM2 and p21, although the levels of these proteins were only increased at later times of between 8-24 h following ALX treatment (Figure 4.27). The activation of p53 at later times after exposure to ALX coincides with the formation of DSBs at later times during the ALX-induced DDR (Figure 4.8) and the cells that undergo mitotic abnormalities (Figure 4.23).

To investigate whether ALX could induce cell death in other cell lines, p53 null H1299 cells and p53 WT U2OS cells were treated with varying doses of ALX and harvested every 24 h for FACS analysis over 4 or 5 days respectively. Similar to the response in HeLa cells, ALX induced a significant amount of cell death in both H1299 and U2OS cells as shown by the higher percentage of cells in sub G1 compared to untreated cells (Figures 4.28 and 4.29 respectively). Cisplatin treatment was again used as a positive control.

Together, the results indicate that, despite ALX treatment causing an arrest in late S and G2 phases of the cell cycle, some cells do enter mitosis with abnormalities, although a small

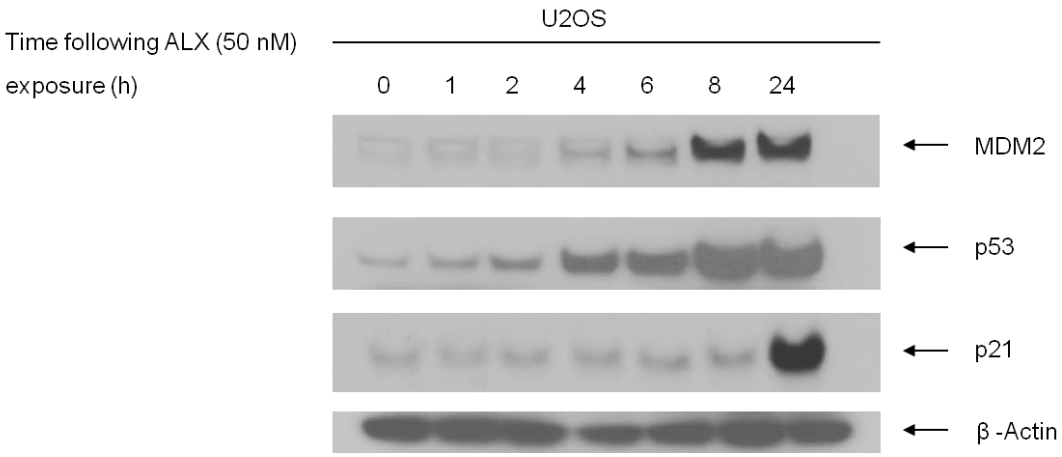

Figure 4.27 ALX stabilises p53 and causes its transcriptional activation at later times
U2OS cells were treated with 50 nM ALX and harvested at the times indicated. Whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with the indicated antibodies recognising p53, MDM2 and p21. An antibody against β-actin was used as a loading control.

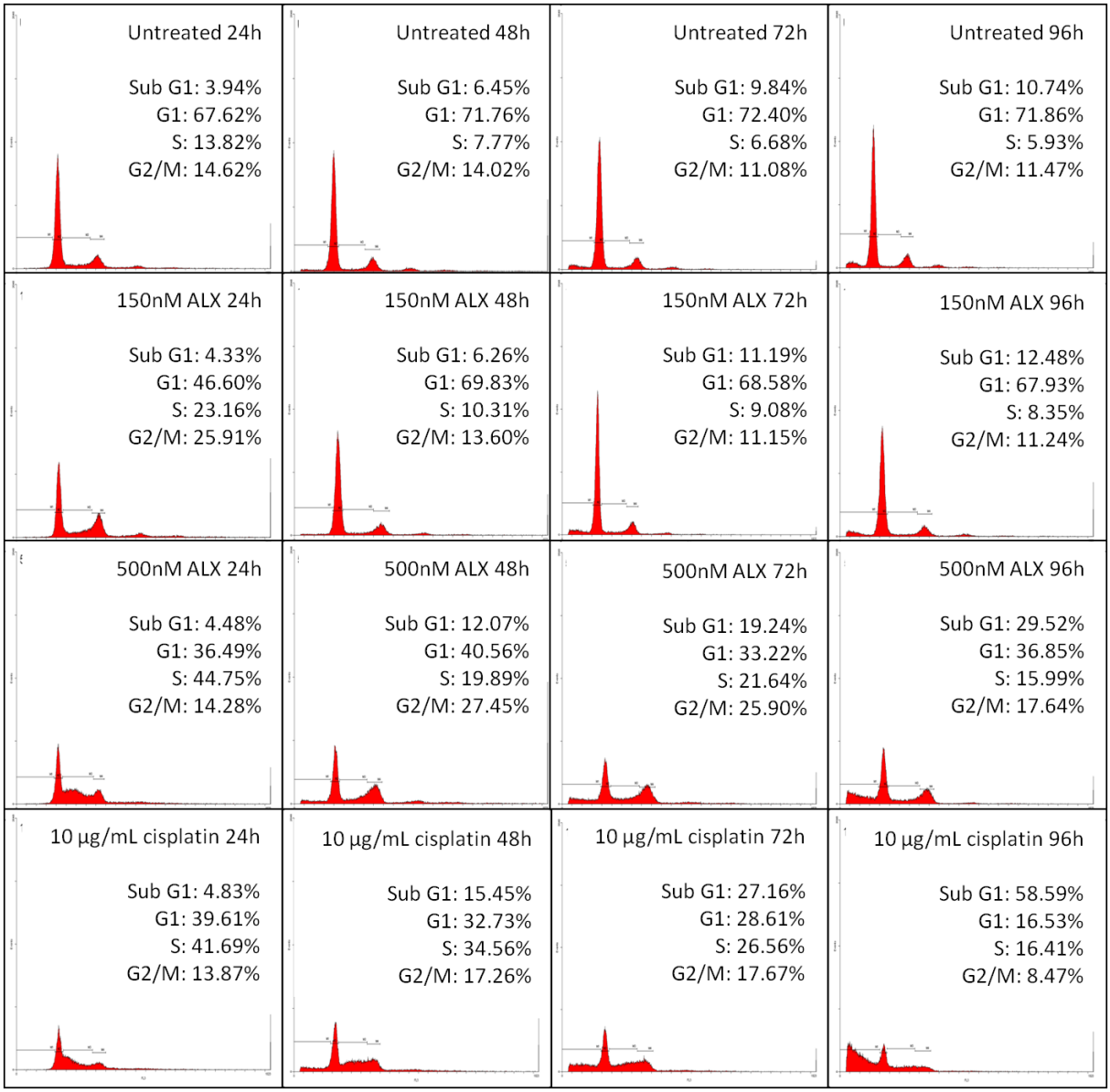

Figure 4.28 Damaged mitotic cells from ALX treatment cause cell death in a p53 null cell line

H1299 cells were treated with 150 nM and 500 nM ALX and harvested every 24 h for 4 days. Samples were analysed by flow cytometry using PI dye to quantify DNA content during the cell cycle. The percentage of cells accumulating in sub G1, G1, S or G2/M is shown for untreated and ALX-treated samples at the times indicated. Cisplatin-treated cells were used as controls.

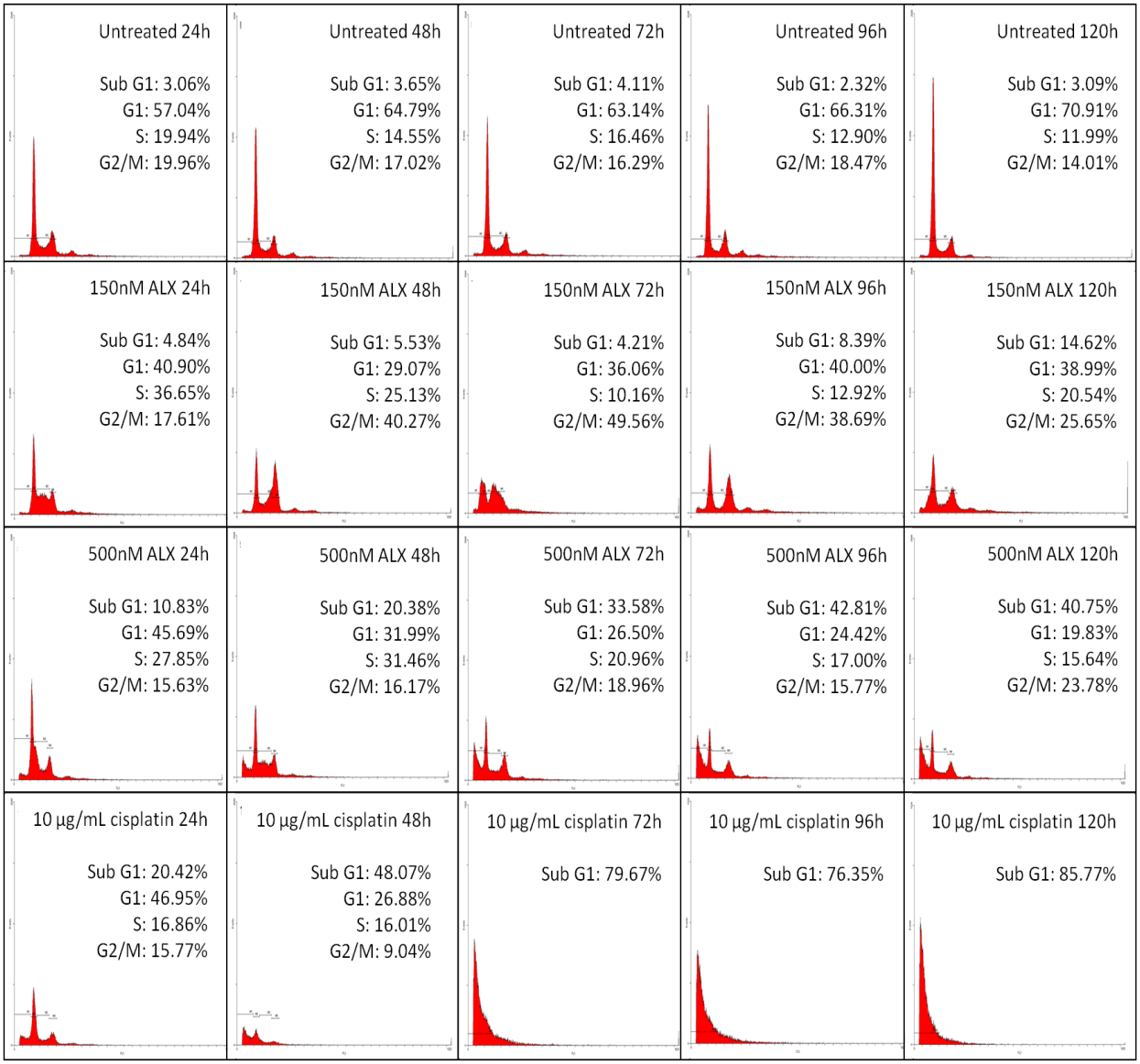

Figure 4.29 Damaged mitotic cells from ALX treatment cause cell death in a p53 proficient cell line

U2OS cells were treated with 150 nM and 500 nM ALX and harvested every 24 h for 5 days. Samples were analysed by flow cytometry using PI dye to quantify DNA content during the cell cycle. The percentage of cells accumulating in sub G1, G1, S or G2/M is shown for untreated and ALX-treated samples at the times indicated. Cisplatin-treated cells were used as controls.

amount of apoptosis was detected. It is clear that the abnormal mitotic cells cause ALX-induced death primarily through mitotic catastrophe.

4.2.7 DNA damage following exposure to ALX is enhanced in cycling cells and can be exacerbated under conditions of replication stress

Since ALX appears to induce a DDR that is primarily mediated by ATR and that the loss of cell viability due to ALX treatment is predominantly through mitotic catastrophe, it is possible that ALX may be acting preferentially in replicating cells. To address whether this was the case, hTERT-immortalised RPE1 cells that were cycling asynchronously or that had been synchronised and arrested in G0/G1 by serum starvation and contact inhibition, were treated with ALX over a 24 h timecourse. Western blotting was subsequently used to determine the DDR induced by ALX in these cells. The percentage of cells arrested in G1 in samples that had been synchronised compared to asynchronous cells was confirmed by flow cytometry (Figure 4.30A). The asynchronously-growing cells exhibited a significantly increased response to ALX compared to the cells that had been arrested in G0/G1 (Figure 4.30B). This can be seen by the increase in levels of phosphorylated DNA-PKcs, SMC1, NBS1, Chk1 and H2AX (Figure 4.30B). In addition, the expression of the key regulatory effector molecule, Chk1, was significantly down-regulated in arrested cells (Figure 4.30B). Consistent with these results, cells that were arrested in G0/G1 displayed a lower signalling response to ALX compared to asynchronous cells (Table 4.1). The percentage of dead cells after treatment with ALX is greatly increased in the asynchronous, cycling cells compared to the percentage in the non-cycling, synchronous cells (Table 4.1). Together, these data

demonstrate that ALX induces a greater DDR in actively-replicating cells rather than those that have exited the cell cycle.

Early stage tumours have been found to display markers suggestive of an enhanced DDR and heightened DNA replication stress (Bartkova *et al.*, 2005). One of the causes of such constitutive stress in tumour cells is the elevated levels of certain oncogenes, including cyclin E, whose increased expression is a common occurrence in carcinomas and leads to genomic instability (Spruck *et al.*, 1999). To further investigate the enhanced activity of ALX in replicating cells, the effect of the drug in cells undergoing replication stress was examined using U2OS-derived cells in which cyclin E could be over-expressed by the removal of tetracycline. To test whether increased replication stress could exacerbate the DDR-induced by ALX, cells with and without tetracycline were treated with ALX over a 24 h time period. Western blot analysis demonstrated that cells over-expressing cyclin E displayed amplified levels of phosphorylated RPA2 and H2AX and, to a lesser extent, increased the levels of phosphorylated Chk1 (Figure 4.31). This enhanced signalling response to ALX in cyclin E-over-expressing cells shows that the anti-tumourigenic activity of ALX may indeed be more effective in cells that replicate faster compared to cells with a normal rate of proliferation (Figure 4.31).

4.3 DISCUSSION

Organisms have evolved a complex array of pathways, collectively known as the DDR, to preserve genomic integrity in response to DNA damage. These pathways can be exploited in cancer therapy since the majority of chemotherapeutic agents function by inducing irreparable DNA damage that forces the tumour cell to enter damage-dependent apoptosis. High levels of

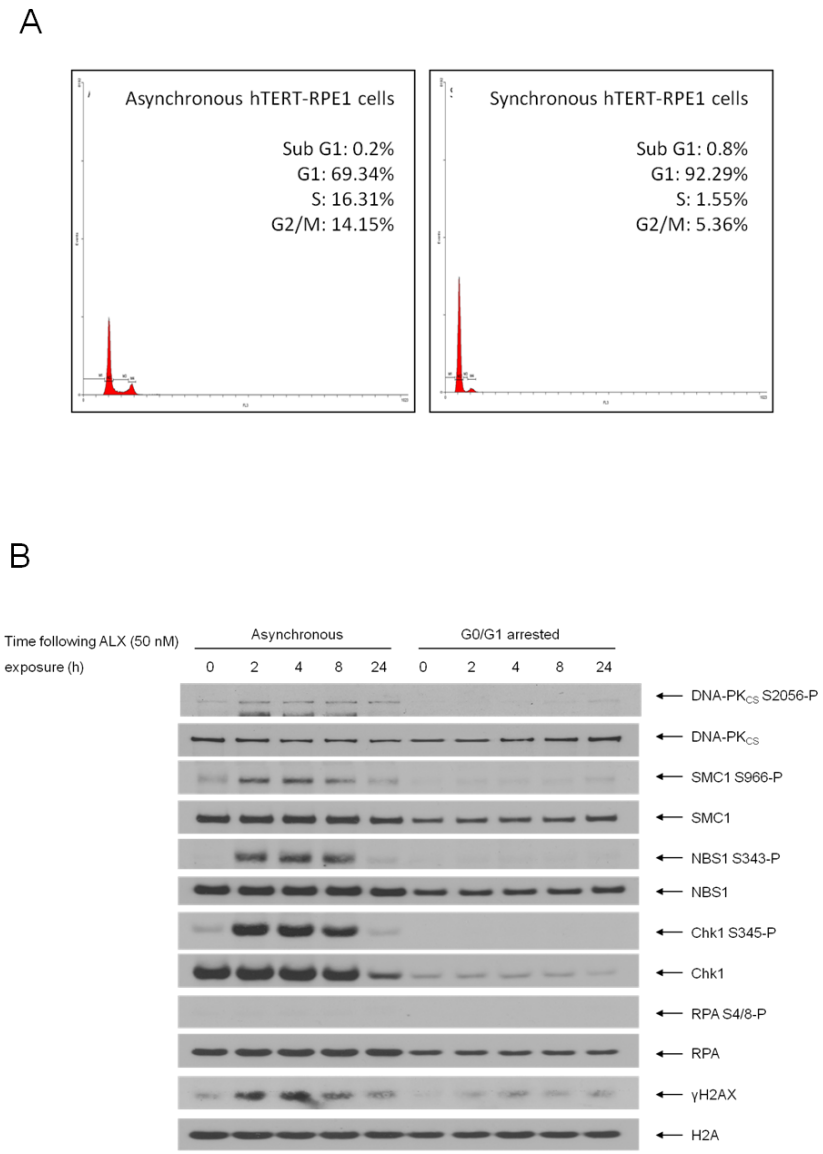

Figure 4.30 Non-cycling cells display a reduced signalling response to ALX
hTERT immortalised RPE1 cells were either arrested in G0/G1 by serum starvation or left to cycle asynchronously. (A) Samples were analysed by flow cytometry using PI dye to quantify DNA content during the cell cycle. The percentage of cells accumulating in sub G1, G1, S or G2/M is shown for synchronous and asynchronous cells; (B) Both cell types were treated with 50 nM ALX and harvested at the times indicated. Whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with the indicated antibodies against DNA damage proteins. Antibodies against total proteins were used as loading controls.

	Synchronous (non-cycling) cells in sub G1 (%)				
	24 h	**48 h**	**72 h**	**96 h**	**120 h**
Untreated	0.99	1.30	4.43	1.64	1.28
150 nM ALX	1.33	1.00	2.61	2.22	0.99
500 nM ALX	1.50	4.77	5.66	5.01	2.24
10 µg/mL cisplatin	6.88	23.99	77.81	80.91	51.85
	Asynchronous (cycling) cells in sub G1 (%)				
	24 h	**48 h**	**72 h**	**96 h**	**120 h**
Untreated	1.3	3	14.5	7.2	6.9
150 nM ALX	8.8	20.6	37.3	63	65.8
500 nM ALX	31.5	42.7	30.9	50	73.9
10 µg/mL cisplatin	18.4	49.9	87.1	94.1	93.9

Table 4.1 ALX causes a higher percentage of cell death in actively-cycling cells
hTERT immortalised RPE1 cells were either arrested in G0/G1 by serum starvation or left to cycle asynchronously. Cells were treated with 150 nM and 500 nM ALX and harvested every 24 h for 5 days. Samples were analysed by flow cytometry using PI dye to quantify DNA content during the cell cycle. The percentage of cells accumulating in sub G1 is shown for untreated and ALX-treated samples at the times indicated.

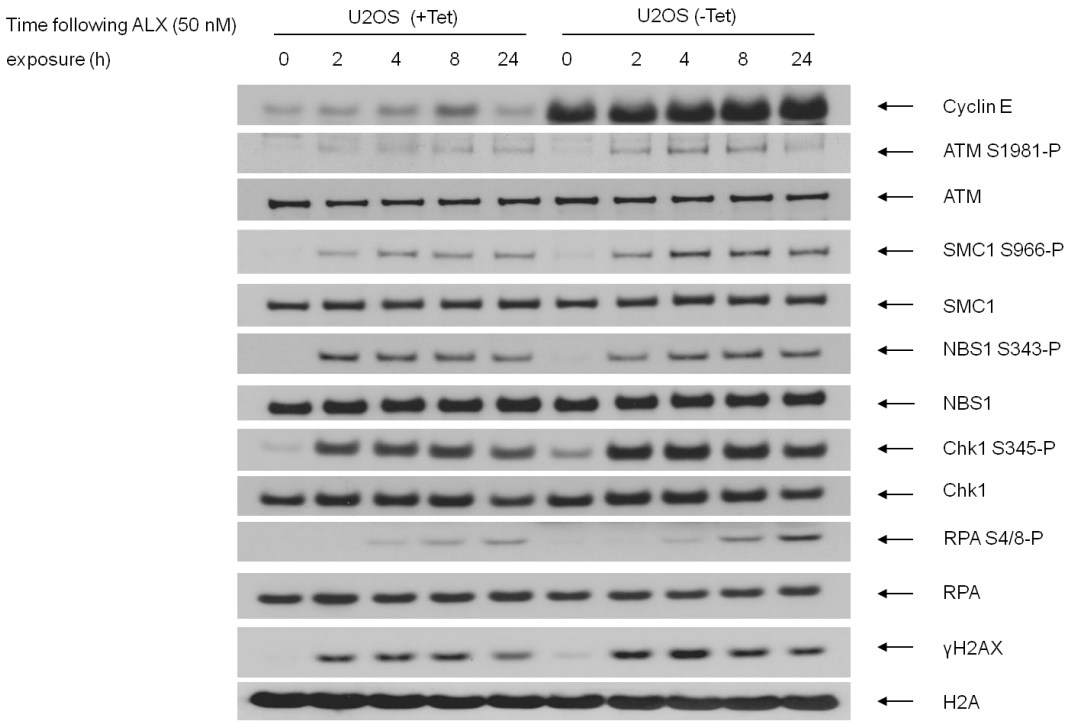

Figure 4.31 ALX exacerbates the DDR in cells with a high rate of replication
U2OS cells with either normal or greatly elevated levels of cyclin E were treated with 50 nM ALX and harvested at the times indicated. Whole cell lysates were prepared, separated by SDS-PAGE and immunoblotted with the indicated antibodies against DNA damage proteins. Antibodies against total proteins were used as loading controls.

DNA damage in S phase can result in stalled replication forks or replication fork collapse, leading to replication-associated DSB formation. Since cancer cells have a higher rate of replication than normal cells, many anti-cancer drugs act by inducing DNA lesions during or following DNA replication (Helleday *et al.*, 2008).

Drug resistance remains a common problem in cancer therapy. To combat this, numerous strategies have been adopted, including the development of derivatives of TOP2 inhibitors. This has resulted in the generation of a hybrid class of compounds that also possess alkylating capabilities, of which ALX was proven to be most effective (Figure 4.1) (Pors *et al.*, 2003).

4.3.1 The DDR in response to ALX

Although ALX has been shown to possess anti-tumour activity, an understanding of the DDR pathways that are activated in response to it remains limited. Preliminary dose-response and timecourse experiments were performed, which established that ALX was capable of inducing a DDR at nanomolar concentrations and in a variety of cancer cell lines that possessed different genetic backgrounds (Figures 4.2-4.7).

It was also observed that longer exposure to ALX generated DSBs; therefore, further characterisation of ALX was required to determine which DDR pathways were activated in response to it (Figure 4.8). Interestingly, the abrogation of ATM or components of the ATM-dependent pathway did not inhibit the ability of ALX to induce a DDR in these cells, despite the fact that ATM primarily responds to DSBs (Figures 4.9-4.12). It has become increasingly apparent that the functional state of ATM (often in combination with the state of p53) in individual patient tumours has a huge impact on identifying those patients who are most likely to respond to a particular chemotherapeutic agent (Jiang *et al.*, 2009). The fact that ATM is

not required in the ALX-induced DDR indicates that a cytotoxic response can potentially be initiated in tumour cells that possess inactive ATM, which would otherwise often be associated with resistance to treatment.

The lack of involvement of the ATM pathway indicates that the signalling response to ALX requires the presence of another PIKK; therefore, the effect of reducing the levels of ATR and components of the ATR pathway on the response to ALX was investigated. The data established a requirement of ATR and Chk1 to mediate the activation of the ALX-induced cell cycle checkpoint since the loss of this DDR pathway exacerbated the formation of DSBs (Figure 4.13). Notably, the abrogation of Chk1 has been of particular interest in order to sensitise cancer cells to a number of anti-cancer agents, including mitomycin C and cisplatin (Akinaga *et al.*, 1993, Bunch and Eastman, 1996). It has also been found that the depletion of ATR renders cancer cells extremely sensitive to alkylating agents, a finding that is consistent with the results observed in this study since ALX possesses alkylating capabilities. The increased generation of DNA damage in cells with reduced ATR levels treated with ALX highlights the therapeutic potential of ALX in combination with inhibitors of the ATR pathway to potentiate its effects (Figure 4.13) (Wilsker and Bunz, 2007).

The enhanced H2AX response after ALX treatment in DLD1-Seckel cells could be due to the fact that ATR-deficient cells contain an increased level of genomic instability from high levels of replication fork stalling, their subsequent collapse and the accumulation of DSBs (Cimprich and Cortez, 2008). Therefore, it is likely that another PIKK is activated in response to ALX and that this kinase can function in a redundant manner to ATR. Consistent with this suggestion, the pre-treatment of the DLD1-Seckel cells with a DNA-PK inhibitor reduced the levels of phosphorylated DDR proteins, indicating that ALX treatment induces a DNA-PKcs-

dependent cellular response that can function as well as ATR (Figure 4.15B). This is consistent with the observations that that DNA-PK can phosphorylate a subset of DDR proteins involved in the chromatin remodelling response, such as H2AX (Mukherjee *et al.*, 2006, Stiff *et al.*, 2004, Tomimatsu *et al.*, 2009). Since it was also observed that levels of phosphorylated Chk1 and SMC1 were reduced following both ATR depletion and DNA-PK inhibition, it would be interesting to determine whether DNA-PK plays a role in the phosphorylation of other DDR proteins in response to ALX and to ascertain whether DNA-PK is actually involved in the cell cycle response to ALX (Figure 4.15B). In addition, since the efficiency of DNA-PK inhibition was only moderate, the use of siRNA to deplete the levels of DNA-PK would be a better approach to study this effect on the response to ALX.

4.3.2 The mode of action of ALX

4.3.2.1 ALX and topoisomerase

It appears that the alkylating moiety of ALX does elicit a DSB response, since the signalling response is greatly reduced when the alkylating side-chain is removed (Figure 4.16). However, as the DNA damage induced by ALX also requires the presence of TOP2, it is possible that the alkylating damage after treatment with ALX is limited to regions where TOP2 is inhibited (Figure 4.18B). This is consistent with the lack of PCNA mono-ubiquitylation after ALX treatment, indicating that the base damaging activity of ALX may be restricted to TOP2-bound replication forks (Figure 4.17). This is an attractive hypothesis since it implies the specific targeting of base damage to certain regions of DNA rather than it being indiscriminate as with other commonly used alkylating agents. Region-specific DNA damage is a concept that has already been developed to localise drug-induced damage to

critical nucleotide sequences; for example, drugs like bizelesin, target AT-rich fragile sites that are unstable regions found in leukaemias and lymphomas (Woynarowski, 2002, Woynarowski *et al.*, 2001).

As noted above, the response induced by ALX does require the presence of TOP2 (Figure 4.18B). However, in contrast to previously published data showing that ALX acts specifically to inhibit the alpha isoform, the depletion of TOP2α did not alleviate the cytotoxicity of ALX to the same extent as etoposide (Figures 4.18 and 4.19). Interestingly, it required the combined depletion of both TOP2 isoforms to reduce the phosphorylation of H2AX after ALX treatment, indicating that ALX functions by inhibiting both TOP2α and TOP2β (Figure 4.18B). Mutations in TOP2 enzymes have been linked to resistance to many anti-cancer drugs and can be categorised into 3 groups: those that decrease the affinity of the drug for the enzyme, mutations that change the DNA-binding affinity of the enzyme and mutations in the active site residues (Dingemans *et al.*, 1998, Vassetzky *et al.*, 1995). Therefore, if both TOP2α and TOP2β were mutated or depleted in cancer cells, resistance to ALX might, conceivably, develop. However, it is notable that the combined depletion of both isoforms does not lead to decreased phosphorylation of Chk1 in response to ALX, indicating that the ATR-mediated checkpoint response remains unaffected (Figure 4.18B).

The results also highlight the fact that it may be the alkylating function of ALX that is responsible for the continued checkpoint response in the absence of TOP2 expression since it has already been shown that the alkylating side-chain is importance for the ability of ALX to induce a DDR (Figure 4.16). This is of particular interest since it reduces the possibility of developing resistance to ALX if the cellular levels of both topoisomerases were to be

depleted. This is, therefore, another example of potential drug resistance that would not seem to affect the DNA response induced by ALX. Notably, the use of some catalytic inhibitors of TOP2 often potentiates the cytotoxicity of chemotherapeutic compounds like alkylating agents (Larsen *et al.*, 2003). The mechanism by which this occurs remains controversial, although there have been reports that TOP2 is involved in DNA repair, therefore, the reduced activity of TOP2 results in increased sensitivity to alkylating agents (Tan *et al.*, 1987). Another possibility is that some catalytic inhibitors of TOP2 also inhibit drug efflux pumps through their ability to block the ATP binding site, thus enabling cytotoxic agents to remain at their target sites for longer (Larsen *et al.*, 2003). In this regard, it is possible that the inhibition of TOP2 by ALX may be rendering the cells sensitive to the alkylating effect of this agent.

4.3.2.2 ALX and the MMR and FA pathways

MSH2 is an important component of the MMR pathway, which mediates the toxicity of a number of chemotherapeutic agents (Fink *et al.*, 1998). Not only has the loss of MMR pathway in tumours been linked to resistance to alkylating agents and platinum-based compounds, it has also been reported to result in resistance to TOP2 inhibitors such as etoposide and doxorubicin (Fedier *et al.*, 2001, Fink *et al.*, 1998). Notably, the specific loss of MSH2 was shown to result in resistance to the TOP2 poisons (Fedier *et al.*, 2001). Contrary to these findings, the loss of MSH2 in this study actually exacerbates the signalling response to ALX, indicating that the abrogation of the MMR pathway in tumours will not affect the activity of ALX and that it evades this particular resistance mechanism (Figure 4.20).

There is also the possibility that tumours that harbour deficiencies in this repair process may be hypersensitive to the cytotoxic effects of ALX. This is consistent with the capacity of ALX

to induce a DDR in cells known to be deficient in another component of the MMR pathway, MLH1 (Pors *et al.*, 2003).

Interestingly, catalytic inhibitors of TOP2 exhibit toxicity by inhibiting the activity of the enzyme and do not rely on MMR-mediated apoptosis to potentiate their effects since the mode of death caused by these inhibitors is through mitotic catastrophe. The fact that ALX does not specifically inhibit TOP2α and does not mirror the activity of etoposide means that there is a possibility that ALX is acting in a similar manner to catalytic inhibitors of TOP2 and not mimicking the activity of TOP2 poisons (Figures 4.18 and 4.19). Another reason why MMR deficiency does not affect ALX activity might also be due to the types of alkyl lesions it generates since these will determine the repair pathway that is activated. For example, the DNA adducts resulting from treatment with the nitrogen mustard, melphalan, do not seem to be recognised by the MMR pathway (Fink *et al.*, 1998). In addition, ALX may induce a number of different alkyl lesions; therefore, if one type is repaired, others could remain cytotoxic, regardless of the MMR status of the tumour cell.

FANCF is one of the components of the E3 ubiquitin ligase complex that ubiquitylates FANCD2 in the FA pathway. The FA pathway is the repair pathway that is responsible for recognising and removing lesions encountered during replication such as DNA ICLs. These are lesions induced by platinum-based chemotherapeutics like cisplatin and some bifunctional alkylating agents (Fu *et al.*, 2012, Kee and D'Andrea, 2010). Indeed, tumours with an intact or restored FA pathway display drug-induced ICL resistance (Taniguchi *et al.*, 2003). Since the FA pathway appears to be dispensable for the ALX-induced DDR (Figure 4.21), this suggests that ALX does not generate DNA ICLs, which is surprising considering it contains two functional alkylating moieties (Figure 4.1). It is also unexpected since ALX contains

chloroethyl moieties that render it able to modify N^7 and O^6 of guanine bases to generate chloroethyl adducts; O^6Cl-ethylG adducts can undergo rearrangement to result in G-C ICLs (Fu *et al.*, 2012). However, earlier work demonstrates that ALX, although it does possess a bis-alkylating function, does not covalently cross-link DNA, which is consistent with the results shown here (Pors *et al.*, 2004).

Similar to alkylating agents like the nitrogen mustards, ALX is likely to target N^7 of guanine to form bulky N-monoadducts in addition to its potential to form O^6Cl-ethylG adducts. It would be interesting to determine whether the abrogation of components of other repair pathways would affect the DNA damage generated by ALX as this would give a better indication of the lesions that ALX induces. It is clear that ALX contains properties that circumvent both MMR and FA deficiency.

4.3.3 Mechanism of ALX-induced cell death

The mechanism by which ALX induces cell death appears complicated. This is perhaps not surprising considering that ALX has the ability to induce a number of cytotoxic DNA lesions, including alkyl lesions, stalled replication forks and DNA DSBs as discussed above.

The primary mode by which ALX appears to induce cell death is through mitotic catastrophe. The exact mechanism of mitotic catastrophe is not fully understood; however, it mostly likely represents a mechanism to remove cells with severe chromosomal abnormalities (Roninson *et al.*, 2001). It has been suggested that mitotic catastrophe can be caused by cells that enter mitosis with extensive levels of unrepaired DNA damage. Whilst it is clear that ALX induces a robust G2/M checkpoint, the presence of aberrant mitotic cells with unrepaired DNA damage suggests that the cells can bypass this blockade in the cell cycle (Figures 4.22 and

4.23). Since it is known that the progression of cells from G2 to M phase of the cell cycle is driven by the activation of the CDK1/cyclin B1 complex, the high levels of cyclin B1 in cells treated with ALX after 24 h may be the underlying driving force behind this premature entry in mitosis (Figure 4.22B). Indeed, it has been found that aberrant mitotic entry is often associated with the over-expression and premature entry of CDK1/cyclin B1 into the nucleus (Jin *et al.*, 1998).

There is a likelihood that the transient G2/M arrest could be caused by the low level of expression of p53 in the HeLa cells used in the aforementioned experiment since cells with deficient p53 are able to initiate a G2/M checkpoint but are not able to sustain it (Bunz *et al.*, 1998). However, a similar transient G2/M arrest can also be observed in both p53-null and p53-proficient cell lines that had been treated with ALX over the same period of time (Figures 2.28 and 2.29). These results indicate that the p53 status does not affect the transient nature of the observed G2/M checkpoint arrest and this is consistent with the assumption that p53 is not required for the onset of mitotic catastrophe (Roninson *et al.*, 2001), and nor for any of the effects of ALX seen in this study.

The occurrence of mitotic catastrophe in cells exposed to ALX for long periods of time could also be explained by the prolonged growth arrest of these cells. There are studies that show that p21-induced growth arrest results in the inhibition of a number of mitotic proteins, thereby causing abnormal chromosome segregation and abnormal mitosis (Chang *et al.*, 2000). The G2/M checkpoint arrest that was observed in ALX-treated cells could result in the inhibition of the transcription of genes that are required for correct mitotic function; therefore, when some cells bypass the checkpoint and enter mitosis, they will be forced to undergo abnormal mitosis. Notably, DNA-PK has been implicated in mitotic catastrophe from

observations that its inhibition results in prolonged G2/M arrest and aberrant spindle formation, eventually accumulating in mitotic catastrophe (Shang *et al.*, 2010b). Since ALX-induced damage partially depends on DNA-PK, it would be interesting to determine whether the response is increased following DNA-PK depletion and longer exposure to ALX.

It was observed that ALX could induce a low level of apoptosis, associated with partial PARP1 cleavage in the HeLa cell line expressing low levels of p53, although procaspase 3 and lamin B1 levels remained unchanged in these samples compared to those treated with cisplatin (Figure 4.25A). Several studies have found that mitotic catastrophe is often accompanied by the release of pro-apoptotic proteins as well as other molecular events that define apoptosis (Castedo *et al.*, 2004a, Castedo *et al.*, 2004b). In addition, the mode of cell death can often depend on the anti-cancer agent itself and the dose that is used; in this way, one form of cell death could occur before the second is initiated. This is clearly demonstrated when cells treated with the anthracycline antibiotic, doxorubicin, exhibit apoptotic morphology at earlier times of treatment before eventually dying through mitotic catastrophe (Eom *et al.*, 2005).

Although ALX induces its stabilisation, p53 only appears to be transcriptionally competent at the later time of exposure to ALX (Figure 4.27). This coincides with the formation of DSBs at later treatment times as well as the onset of aberrant mitosis (Figures 4.8 and 4.23). Therefore, it is conceivable that ALX will induce cell death through mitotic catastrophe and/or apoptosis depending on the presence of an intact p53 pathway.

TOP2 is critically involved in removing catenated sister chromatids during chromosome segregation and in removing DNA supercoils during replication. The catalytic inhibitors of TOP2 exert their cytotoxicty by inhibiting the activity of the enzyme, which inevitably results

in abnormal mitosis and mitotic catastrophe (Giménez-Abián *et al.*, 2000). Since ALX is a known TOP2 inhibitor, it is possible that it displays similarities with the non-DNA-damaging catalytic TOP2 inhibitors rather than the TOP2 poisons. In this regard, it is likely that cells that enter mitosis following ALX treatment fail to undergo proper chromosome decatenation after DNA replication.

Despite a poor understanding of the mechanistic details of mitotic catastrophe, it is becoming preferable, from a clinical point of view, that chemotherapeutic drugs are designed to induce modes of death other than apoptosis since many tumour cells are refractory to the induction of apoptosis caused by many of these agents. Indeed, the primary target of anti-microtubular agents, such as Vinca alkaloids and the taxanes, is the mitotic spindle, which results in the disruption of mitosis and the induction of mitotic catastrophe (Roninson *et al.*, 2001). Interestingly, mitotic catastrophe was found to be the predominant mechanism of cell death in different cell lines treated with one of the taxanes, Docetaxel (Morse *et al.*, 2005). More importantly, the exposure of some cancer cells to ICRF-193, a compound belonging to the catalytic TOP2 inhibitors, causes cellular effects that are similar to those observed from exposure to agents that interfere with the mitotic spindle (Larsen *et al.*, 2003). In addition, incomplete chromatid decatentation was shown to occur after treatment with certain catalytic TOP2 inhibitors; some were also demonstrated to activate the catenation checkpoint, although a number of different agents were able to overcome this checkpoint (Larsen *et al.*, 2003).

4.3.4 Conclusions

The activation of an ATR-dependent checkpoint in response to ALX results in the accumulation of cells in S phase, which is indicative of ALX interfering with DNA

replication (Figure 4.22A). The response to ALX then appears to culminate in a G2/M checkpoint arrest, which is consistent with the fact that if cells are damaged in S phase they exit this stage of the cell cycle and undergo arrest at the G2/M checkpoint (Bartek *et al.*, 2004).

Observations throughout this study suggest that the primary DDR following exposure to ALX requires ATR and that the loss of cell viability after treatment is predominantly mediated through mitotic catastrophe. Thus, it is clear that the ability of ALX to induce a DDR is cell cycle dependent. Consistent with this notion, non-cycling cells displayed a severely attenuated DDR after ALX treatment as well as lower percentage cells in sub G1 compared to cycling cells (Figure 4.30B and Table 4.1). In addition to this specificity for cycling cells, ALX was also found to induce an enhanced signalling DDR in cells undergoing replicative stress (Figure 4.31). A sustained proliferative capacity is one hallmark of cancer cells and it is becoming increasingly apparent that early stage tumours harbour elevated levels of DNA damage (Bartkova *et al.*, 2005, Gorgoulis *et al.*, 2005, Hanahan and Weinberg, 2000). Therefore, it appears that ALX exacerbates the DDR in tumour cells that are characterised by dysregulated DNA replication and high levels of replication stress. This is further demonstrated by the earlier findings that ALX treatment resulted in a greatly heightened H2AX response in cells undergoing replicative stress due to the depletion of ATR (Figure 4.13A). Tumours that display dysregulated DNA replication and high levels of replication stress may, therefore, be more sensitive to treatment with ALX.

The response to chemotherapeutic agents does not rely on a single pathway to mediate its effects; rather, it is the combined outcome of many different signalling pathways, be they defective or normal, which determine the fate of the tumour cell. The status of key signalling

pathways are, therefore, vital to the potential of the drug in question and may lead to resistance, exacerbated side effects or ineffectual treatment.

As an example of a DNA-directed alkylating agent, ALX is a 'combination' on its own, requiring TOP2α and TOP2β, as well as its ability to alkylate DNA. Furthermore, it is possible that ALX-induced alkylating damage is initiated at regions where TOP2 binds to DNA. Functionally, ALX seems to cause irreparable S phase damage that is cytotoxic as the cells progress into mitosis; tumour cells that are undergoing replication stress are therefore likely to be particularly sensitive to ALX-mediated cytotoxicity. It is capable of inducing a response in tumour cells independently of a number of components that are often considered necessary for the action of chemotherapeutic agents, including ATM, p53, proteins involved in the MMR and FA pathways and the apoptotic pathway.

This attribute of ALX also reduces the requirement for combination therapy. Drug-induced DNA damage along with repair pathway inhibitors has been successful and many such combinations are in development. However, it is often a concern that the increase in the number of drugs given to an individual, could lead to elevated side effects. The fact that the response to ALX is unaffected by components of the repair pathways observed in this study means that inhibitors of these pathways are not needed, although other pathways will require investigation. There is, therefore, a potential for ALX to be used as a novel anti-cancer agent in resistant secondary tumours that have acquired mutations.

CHAPTER 5

FINAL DISCUSSION AND FUTURE WORK

CHAPTER 5 FINAL DISCUSSION AND FUTURE WORK

5.1 FINAL DISCUSSION

5.1.1 The DDR and cancer

The DDR comprises a series of pathways that function together to maintain genomic integrity (Ciccia and Elledge, 2010). This involves numerous signal transduction pathways that sense and repair DNA damage, as well as those that determine the fate of the cell. The abrogation of proteins involved in the DDR leads to the loss of genomic stability, which is a characteristic of cancer cells (Hanahan and Weinberg, 2011). Conversely, defects in components of the DDR have been exploited through the use of chemotherapeutic agents, which induce DNA damage in tumour cells (Aziz *et al.*, 2012). However, tumour cells exhibit differing sensitivities to DNA damaging anti-cancer agents primarily due to selective alterations in pathways regulating cell cycle checkpoints, DNA repair and apoptosis. The underlying genetic defects of a tumour cell can be the cause of resistance to many existing anti-cancer treatments.

In this thesis an attempt has been made to address these two aspects of cancer biology: firstly, looking at certain properties of the p53 tumour suppressor and one of the mechanisms by which its transcriptional activity can be regulated and, secondly, characterizing, in detail, the mode of action of a novel chemotherapeutic agent, ALX.

5.1.2 A role for hnRNPUL-1 as a p53 co-repressor

Previous findings have implicated hnRNPUL-1 in the regulation of transcription, although the exact mechanism by which this occurs was unknown (Barral *et al.*, 2005, Kzhyshkowska *et*

al., 2003). Data presented in this study confirm a role for hnRNPUL-1 in the transcriptional regulation of p53, one of the most extensively studied and important component of the DDR.

Data in Chapter 3 corroborate previous reports that hnRNPUL-1 can interact with p53 and inhibit its transcriptional activity, indicating that hnRNPUL-1 functions as a p53 co-repressor (Figures 3.1-3.5). The ability of p53 to function as a transcriptional regulator is essential to its role as a tumour suppressor since the majority of tumour-derived mutations in p53 reside within its DBD and these render it incapable of binding and activating target promoters (Kato *et al.*, 2003).

Findings within Chapter 3 show that, from the p53-activated genes examined in this study, the mRNA and protein levels of p21 were significantly increased compared to other p53 target genes such as MDM2 and Bax following hnRNPUL-1 depletion (Figures 3.6A and 3.7). Interestingly, the *p21* promoter requires BRD7 for efficient transcriptional activation, whereas pro-apoptotic genes do not (Drost *et al.*, 2010). BRD7 knockdown also resulted in the reduction of p300 and acetylated p53 bound to the *p21* promoter (Drost *et al.*, 2010). Previous studies have confirmed that both hnRNPUL-1 and BRD7 interact with each other and with the C-terminal region of p53 (Barral *et al.*, 2005, Drost *et al.*, 2010). Therefore, it is conceivable that, through direct competitive binding, hnRNPUL-1 inhibits the interaction between p53 and its co-activator, BRD7, to specifically inhibit a subset of p53 target genes. The findings here and by Drost *et al.* (2010) are not the only examples of specificity conferred by BRD7: another has been described by Harte *et al*. (2010), where BRD7 recruits BRCA1 to specific promoters of BRCA1 targets (Drost *et al.*, 2010, Harte *et al.*, 2010). It would be interesting to examine, using ChIP analysis, whether hnRNPUL-1 affects the p53-dependent recruitment of BRD7 to a specific subset of p53 target promoters.

The ability of p53 co-factors to determine the ability of p53 to transactivate different target genes that affect p53-mediated cellular outcome is apparent in many proteins (Beckerman and Prives, 2010). The negative regulator of p53, APAK, binds to a region on the pro-apoptotic gene *p53AIP1* that overlaps with the p53 responsive element (p53RE) that is, in turn, responsible for the ability of p53 to transactivate this gene (Yuan *et al.*, 2012). Similarly, ZBTB2 utilises a similar DNA-binding competition mechanism to selectively block the p53-dependent transactivation of cell cycle arrest genes (Jeon *et al.*, 2009). Data presented in Chapter 3 provide evidence that hnRNPUL-1 may also function as a co-factor that regulates selective target gene activation by p53, specifically repressing a growth inhibitory gene. ChIP analysis would need to be conducted to determine whether hnRNPUL-1 binds to p53-binding sites of specific promoters and whether this binding subsequently represses p53-dependent transactivation of a subset of genes.

Results in Chapter 3 also show that p53 binds to the BBS region on hnRNPUL-1 (Figures 3.10B and 3.11). Since the deletion of this region increases the ability of hnRNPUL-1 to repress basic transcription, it is possible that the binding of p53 to this site on hnRNPUL-1 inhibits the binding between the latter protein and BRD7 (Kzhyshkowska *et al.*, 2003). This would, subsequently, enable hnRNPUL-1 to function as a repressor of transcription as is depicted in this study.

It is also possible that hnRNPUL-1 may be functioning to inhibit p53 transcriptional activity by recruiting additional co-repressors or by inhibiting elements of the transcriptional machinery. The latter is supported by evidence that identifies the interaction between hnRNPUL-1 and TATA-binding protein (TBP), a transcription factor that is a component of the transcriptional machinery (Barral, 2004). Since TBP recruits RNA polymerase II and a

host of other transcription factors to the transcription start site, it is possible that the binding of hnRNPUL-1 to TBP inhibits the initiation of transcription (Rhee and Pugh, 2012).

Previous data has identified the interaction between hnRNPUL-1 and other proteins that are involved in transcriptional regulation, including CBP, Elf-1 and pRB (Barral, 2004). Incidentally, hnRNPUL-1 was also shown to interact with PLZF, which, in turn binds to p53 (Barral, 2004). Although the biological consequences were not investigated, the interaction of hnRNPUL-1 with these proteins may affect the transcriptional regulation of p53.

5.1.3 A role for hnRNPUL-1 in disease progression

There is evidence that other members of the hnRNP family are involved in disease progression (Carpenter *et al.*, 2006). Recent work has shown that patients containing a deletion in the chromosomal region that encompasses the *hnRNPU* gene display developmental abnormalities, seizures and low muscle tone (Caliebe *et al.*, 2010). Notably, this is the hnRNP member with which hnRNPUL-1 shares the most sequence homology. Mutations in hnRNPUL-1 itself have been associated with early-onset myocardial infarction (Shiffman *et al.*, 2006).

It has been shown that some hnRNP proteins are involved in the onset of tumourigenesis; for example, transgenic mice overexpressing hnRNPD were found to develop sarcomas (Gouble *et al.*, 2002). In addition, the levels of hnRNPA2 and B1 have been proposed to be markers of cancer, particularly lung cancer, and have also been linked to cell proliferation (Fielding *et al.*, 1999, He *et al.*, 2005b). Data presented in Chapter 3 demonstrate that hnRNPUL-1 represses p53 transcriptional activity and inhibits p21 transactivation (Figure 3.4). It is possible that the inhibition of p53 by hnRNPUL-1 is sufficient to aid cellular proliferation.

This is further supported by the cellular response elicited after DNA damage in which there is an increase in cells arrested in G1 following hnRNPUL-1 depletion (Figures 3.8 and 3.9A). Therefore, the repression of p53 by hnRNPUL-1 may facilitate the propagation of damaged cells due to checkpoint loss. The repression of genes required for growth inhibition is a characteristic of oncogenes; therefore, if hnRNPUL-1 contains growth-promoting effects through its inhibition of p53, it may have oncogenic potential. To date, however, there have been no reports of over-expression of hnRNPUL-1 in tumour cell lines, although as endogenous levels are already high, definitive conclusions are difficult to draw.

BRD7 was identified as a tumour suppressor gene required for p53-dependent oncogene-induced senescence (OIS) (Drost *et al.*, 2010). It is conceivable that the ability of BRD7 to act as a p53 co-factor is inhibited by hnRNPUL-1, which would lead to a loss of p53-dependent OIS and the subsequent onset of oncogene-induced transformation. Indeed, low levels of BRD7 expression were reported in nasopharyngeal carcinoma tumours as well as in a subset of breast tumours harbouring WT p53 (Drost *et al.*, 2010, Liu *et al.*, 2008).

5.1.4 A role for ALX in the treatment of apoptotic-resistant tumours

While the mutation or loss of p53 is a major contributing factor to the onset of genomic instability, p53-deficient tumours are particularly refractory to chemotherapeutic treatment. *TP53* is mutated in nearly 30% of B-cell chronic lymphocytic leukaemia (CLL) chemo-resistant tumours, despite being mutated in only 5-10% of cases at the time of diagnosis (Cordone *et al.*, 1998, el Rouby *et al.*, 1993). However, from the data presented in Chapter 4, ALX, a substituted anthraquinone with intercalating and alkylating properties, has been found

to be unaffected by p53 status and, since p53 does not appear to mediate its toxicity, ALX has the potential to treat tumours independently of p53 (Figures 4.7 and 4.28).

Similarly, ALX-induced DNA damage was found to occur irrespective of ATM status (Figures 4.9-4.12). ATM is strongly implicated in p53 activation and the abrogation of ATM can result in defective p53 activity since mutations in *ATM* have been found to be an alternative mechanism by which p53 function is disabled in CLL (Pettitt *et al.*, 2001). Patients with dysfunctional ATM have also been shown to have shorter survival times and those CLL patients with a loss of ATM function respond poorly to chemotherapy (Austen *et al.*, 2007, Starostik *et al.*, 1998). The ATM-p53 pathway is critical for the induction of apoptosis after DNA damage.

A number of chemo-resistant tumours with mutations in ATM and p53 frequently display defective apoptotic pathways. Therefore, there is a need for novel approaches to kill cancer cells that possess mutations in ATM or p53 and where drug resistance is present. The observation that ALX does not induce cell death mainly through apoptosis reduces the likelihood of drug-induced resistance (Figure 4.25A). The culmination in cell death by checkpoint slippage-induced mitotic catastrophe means that ALX has the potential to cause death in apoptosis-resistant cancer cells and reduces the clonogenic survival of any apoptosis-resistant cells (Figure 4.26). This is of particular importance since repeated exposure of many tumours to anti-cancer drugs can result in the selection of chemo-resistant cancer cells that subsequently possess enhanced tumourigenic and metastatic potential and means that the use of chemotherapeutics can often be counterproductive. For example, repeated exposure of melanoma cells to dacarbazine, a non-classical alkylating agent, results in a more aggressive phenotype (Lev *et al.*, 2004).

5.1.5 A role for ALX in the treatment of genetically-different tumour types

One of the challenges that face chemotherapeutic intervention is the targeting of specific therapies to genetically-distinct types of tumours. As mentioned before in 4.3.1, the reduced expression of ATM is often associated with resistance to chemotherapeutic agents; however, the altered level of ATM and its downstream components do not affect the signalling response elicited after treatment with ALX, confirming a lack of involvement of the ATM pathway in mediating its effects (Figures 4.9-4.12). In contrast, the loss of ATR potentiates the effects of ALX (Figure 4.13A). However, loss of ATR is particular rare in human tumours, although it has been reported in a very limited number of cases (Tanaka *et al.*, 2012).

As demonstrated in this thesis, ALX-induced DNA damage was not attenuated by defects in *MSH2*; indeed, its effects were exacerbated (Figure 4.20). Colorectal tumours are almost always associated with defects in the MMR pathway and mutations in *MSH2*, *MLH1*, *MSH6* and *PMS2* are linked to a familial form of colorectal cancer, hereditary non-polyposis colorectal cancer (Chapelle, 2004). The genetic defects in the MMR pathway are not confined solely to colorectal cancers: MSI linked to MMR abnormalities has also been found in sporadic leukaemias and bladder cancers (Gu *et al.*, 2002a, Gu *et al.*, 2002b). Conversely, defects in MMR components can lead to the resistance to many chemotherapeutic agents, because of an inability of the cells to detect drug-induced adducts or because MMR mediates the toxicity of some anti-cancer drugs (Pors and Patterson, 2005).

Although they initially respond well to treatment, patients with ovarian cancer frequently relapse and, very often, die. The restoration of FA genes in ovarian tumour cells leads to resistance to chemotherapeutic drugs, such as cisplatin (Taniguchi *et al.*, 2003). From data presented here, ALX-induced damage in the 2008 ovarian carcinoma cell line is unaffected by

defects in the FA pathways (specifically the loss of *FANCF* in this cell line), which reduces the chances that this type of tumour will become refractory to ALX treatment (Figure 4.21). This is important since in early tumour progression and in expanding tumour cells, *FANCF* is inactive, so cells remain sensitive to cisplatin; whereas in some cells, demethylation of *FANCF* leads to the restoration of the repair pathways and subsequently to chemo-resistance (Taniguchi *et al.*, 2003). Therefore, if ALX-induced lesions are not recognised by the FA pathway to begin with, ALX has the potential to be used to treat tumours like ovarian tumours without the fear of drug resistance occurring in this manner.

TOP2-targeting drugs often have limited effectiveness due to mutations in TOP2 itself that lead to the reduced or altered expression of this enzyme (Dingemans *et al.*, 1998, Vassetzky *et al.*, 1995). This is a particular problem since many TOP2 inhibitors are widely used as chemotherapeutic agents for the treatment of solid tumours such as breast and lung cancers as well as haematological malignancies. Data in Chapter 4 show that the reduced expression levels of both isoforms of TOP2 did not alter the checkpoint response to ALX, suggesting that this particular resistance mechanism may not reduce the ability of ALX to exert its anti-cancer activity (Figure 4.18). This is also supported by the observation that ALX can utilise its alkylating ability to initiate a DDR and does not rely on one function alone (Figure 4.16).

If high enough, the dose of a particular chemotherapeutic agent could overwhelm the repair capacity of the cancer cell in question. However, higher therapeutic doses could potentially lead to more serious side-effects. If the damage induced by ALX is not recognised by specific repair pathways a reduced dose of the drug may be sufficiently effective.

It is important to note that, because cancer cells are heterogeneous, different mechanisms of drug resistance can occur. Although the restoration or abrogation of DNA repair pathways is

one mechanism, other modes of drug resistance include increased drug efflux and the impaired ability of the cell to undergo apoptosis (Longley and Johnston, 2005). Previous findings have shown that ALX is not a substrate for P-glycoprotein and data here has established that ALX is not affected by the *ATM* or *TP53* status and does not cause cell death by apoptosis. Since this study has also shown that ALX is unaffected by defects in notable repair pathways that are often implicated in chemo-resistance, there is great scope to develop ALX further, potentially to treat resistant tumours.

5.2 FUTURE WORK

5.2.1 Mechanisms behind the co-repressor function of hnRNPUL-1

Since data in Chapter 3 show that hnRNPUL-1 and p53 interact and that hnRNPUL-1 negatively regulates p53, it is possible that the interaction between the two inhibits the recruitment of p53 to its target gene promoters. To determine whether hnRNPUL-1 is itself recruited to promoters of p53-responsive genes, ChIP analysis would have to be conducted. If it does not directly bind to these promoter regions, a similar technique would need to be utilised to establish whether the depletion of hnRNPUL-1 relieves any inhibition of p53 being recruited. Once the ChIP assay is optimised, it will be possible to examine the effect of hnRNPUL-1 on the ability of p53 to bind to the promoter elements of its growth arrest genes compared to its pro-apoptotic targets. This would give a better understanding of any specificity that hnRNPUL-1 has in conferring the ability of p53 to transactivate its target genes.

It is also possible that hnRNPUL-1 functions in an indirect manner by repressing p53 co-activators. BRD7 is a p53 co-activator and has also been implicated in the ability of

hnRNPUL-1 to regulate transcription (Drost *et al.*, 2010, Kzhyshkowska *et al.*, 2003). Since BRD7 is required to activate a subset of p53-dependent target genes, not just p21, it would be interesting to determine whether hnRNPUL-1 affects the expression levels of these other genes in a p53-dependent manner (Drost *et al.*, 2010).

To establish whether hnRNPUL-1 associates with BRD7, co-immunoprecipitation experiments will need to be conducted. A co-immunoprecipitation between p53 and BRD7 could be performed, in the presence and absence of hnRNPUL-1, to ascertain whether the interaction between p53 and BRD7 is enhanced in cells depleted of hnRNPUL-1.

Although the binding of hnRNPUL-1 to CBP has been identified, any interaction between another acetyltransferase, p300, will also need to be determined by using co-immunoprecipitation assays (Barral, 2004). These enzymes function as p53 co-activators by acetylating p53 itself as well as surrounding histones (Gu and Roeder, 1997, Lill *et al.*, 1997). Initial luciferase reporter assays have already been optimised to measure p300/CBP activity after the down-regulation of hnRNPUL-1. Preliminary findings show that the depletion of hnRNPUL-1 results in an increase in the activity of p300/CBP, although the transfection efficiency appeared to be poor. Further assays will need to be performed, possibly using a different cell line that possesses higher transfection efficiency.

It would be interesting to determine the consequence of hnRNPUL-1 depletion on other p53 responses, since the effects on the cell cycle have been examined in this study. Initial Western blot analysis has been conducted to look at the effects of the depletion of hnRNPUL-1 on known apoptotic proteins after DNA damage; however, no difference in the levels of these proteins could be discerned in samples treated with hnRNPUL-1 siRNA compared to those treated with control siRNA. Further doses of DNA damaging agents will need to be

examined, as well other means of determining the apoptotic response using, for example, the Annexin V assay. However, it is possible that hnRNPUL-1 does not inhibit the expression of apoptotic genes, and will, therefore, not affect the p53-dependent apoptotic response.

Data in Chapter 3 also show an increase in the mRNA levels of the repair protein, DDB2, after the down-regulation of hnRNPUL-1. The effect of hnRNPUL-1 on other p53-dependent repair genes will be significant, especially in light of recent advances implicating hnRNPUL-1 in DNA-end resection and the promotion of DSB repair by HR (Polo *et al.*, 2012).

5.2.2 The effect of the abrogation of other repair pathways on ALX-induced damage

BER and NER are important major pathways that act to repair damage induced by alkylating agents; however, these pathways have not been investigated in this study. Abdallah *et al.* (2012) recently used the DRAG assay to determine the growth inhibitory effects of ALX in Chinese hamster ovary (CHO) cells with defects in NER genes (Abdallah *et al.*, 2012). For the purpose of completeness in this study, it would be beneficial to obtain cells derived from XP patients, such as XP12RO, that contain genetic defects in NER. Exposure of these cells to ALX and the subsequent effect on the levels of DDR proteins would need to be conducted. The effect of the abrogation of components in the BER and direct reversal pathways will also need to be investigated.

If a specific repair pathway is disabled in some way, other repair pathways can compensate, thereby enabling tumour cells to survive (Al-Ejeh *et al.*, 2010). Alkyl lesions can be repaired by a number of pathways, including compensating pathways such as HR, NHEJ and TLS (Fu *et al.*, 2012). Although the disruption of major repair systems does not appear to affect ALX-

mediated cytotoxicity, the effect of abrogating components of secondary repair pathways will need to be investigated. In this manner, the effect of combined defects of two repair pathways on ALX-induced DNA damage would be particularly interesting.

5.2.3 The use of mouse models to determine the chemotherapeutic potential of ALX

Studies in Chapter 4 highlight the importance of genetic heterogeneity in influencing the response to anti-cancer therapy. Human-derived cancer cell lines have been used throughout this study; however, mouse tumour xenograft systems provide a more physiologically relevant study of the response to particular drugs. The non-obese diabetic/severe combined immunodeficient (NOD/SCID) xenograft mouse model is one of the most successful models in which to study haematological malignancies such as B cell chronic lymphocytic leukaemia (B-CLL) and acute lymphoblastic leukaemia (ALL) (Macor *et al.*, 2008); our preliminary studies have made use of this (Appendix 6.1 and 6.2).

The results in Chapter 4 have provided evidence that ALX is capable of inducing a response regardless of *ATM* or *TP53* gene status. Therefore, the use of B cell chronic lymphocytic leukaemia (B-CLL) as a model tumour system will be of the utmost importance since this type of cancer often displays resistance to conventional treatment due to mutations in *ATM* or *TP53*. Preliminary findings have indicated that the administration of ALX to NOD/SCID mice engrafted with the *TP53* mutant MEC-1 cell line resulted in a reduction in the relative tumour volume compared to the vehicle-treated mice (Figure 6.1A). The reduction of the solid tumour after treatment with ALX in these mice can also be observed (Figure 6.1B). Further experiments using the *ATM* mutant mantle cell lymphoma cell line, Granta, also need to be conducted and this will be completed in the near future.

The engraftment of primary leukaemic cells in NOD/SCID mice will be an ideal model to recapitulate primary disease and to subsequently study the effects of ALX. A mouse xenograft model system with patient T-ALL cells has been established (Appendix 6.2). Preliminary data in these mice have shown that the administration of ALX caused a reduction in tumour volume as assessed by the shrinking of the spleen compared to the larger spleen weight of the vehicle-treated mice (Figure 6.2).

Thus, we have shown that it is feasible to examine the efficacy of ALX in the treatment of human leukaemias using this mouse model. In future we will use it to examine the effects of ALX derivatives as well as cell lines and tumours with differing combinations of mutations.

CHAPTER 6

APPENDIX

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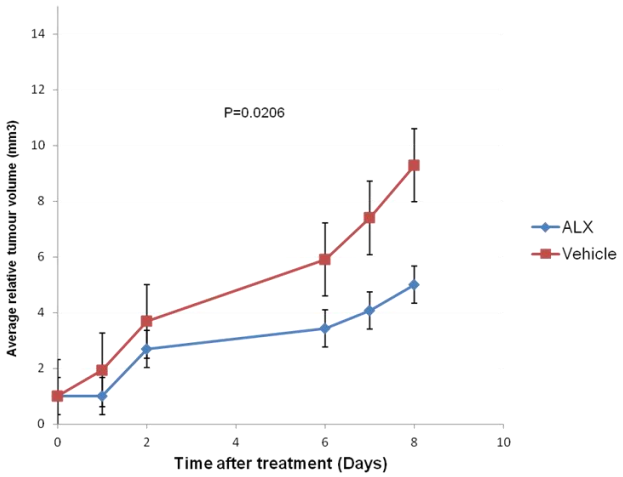
6.1 APPENDIX 1 – MEC-1 XENOGRAFT MOUSE MODEL

MEC cells cultured were harvested and resuspended at a density of $5x10^7$ cells/ml. NOD/SCID mice (aged 5-9 weeks; Biomedical Services Unit) were injected subcutaneously with 5×10^6 MEC cells. At week 6, all mice had solid tumours and were randomised into two groups: treatment and vehicle. A stratified randomisation was conducted to achieve similar average tumour volumes of $250mm^3$ in each group. On the day of randomisation, animals received either 8mg/kg ALX or 0.1% DMSO in water via intraperitoneal injection. Animals were weighed and tumours measured every other day over a period of 8 days. On day 8, animals were culled because tumour volumes were $>1200mm^3$. Tumour volumes were measured manually using a calliper.

6.2 APPENDIX 2 – PRIMARY ALL XENOGRAFT MOUSE MODEL

Primary T-ALL cells were resuspended at a density of 1×10^7 cells/ml in PBS. 100µl of the cell suspension was injected into the 4-8 week old sub-lethally irradiated NOD/SCID mice. Tumour cell engraftment was confirmed by FACS analysis of human anti-CD45 (cell surface marker to confirm that the cells that engrafted were human), mouse anti-CD45 and human anti-CD3 (T-cell marker to confirm the engraftment of a T-ALL cell line) (all eBioscience). At week five following infusion of cells, at least 1% of cells in the peripheral blood of mice were human CD45-positive. Mice were then randomised into two groups: treatment and vehicle and treated as before by intraperitoneal injection. Mice were culled at day 8 and tumour load in the spleen was assessed by FACS analysis of human CD45-positive cells.

A



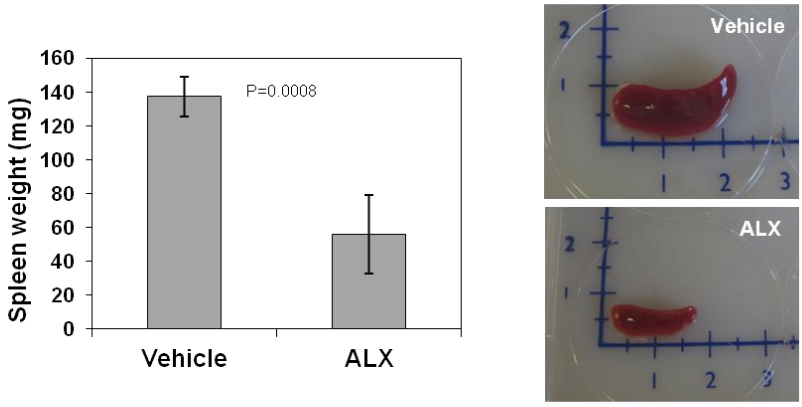
B

Figure 6.1 ALX impedes the growth of p53 mutant cells *in vivo* in a MEC-1 mouse xenograft model

(A) The effect of ALX treatment on subcutaneous tumour volume generated by MEC-1 engraftment compared with vehicle alone over a period of 8 days; (B) Subcutaneous tumour size in mice treated with ALX compared to vehicle alone after 8 days of treatment. Each group contained n=4.

(Experiments performed and figures provided by Tracey Perry).

A



B

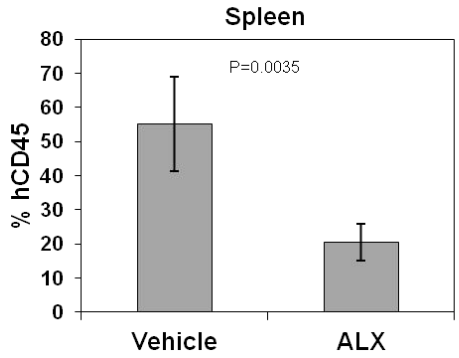


Figure 6.2 ALX reduces the tumour volume in a primary ALL xenograft mouse model.
NOD/SCID mice were engrafted with primary ALL cells. (A) After 8 days of either ALX- or vehicle-treatment, spleens from these mice were removed and weighed; (B) The percentage of human CD45-positive cells in the spleen of mice engrafted with ALL cells after treatment with ALX or vehicle alone. Standard deviations from three individual experiments are presented. Each group contained n=4.
(Experiments performed and figures provided by Tracey Perry).

CHAPTER 7

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