Investigating the Role of CK2 in the DNA Damage Response

By
Edward Strenk Miller

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College of Medical and Dental Sciences
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Abstract

Casein Kinase 2 (CK2) is a ubiquitous serine/threonine kinase. Due to its pleiotropic nature CK2 is involved in a multitude of cellular pathways including cell survival, proliferation and apoptosis. Therefore it has come as no surprise that a requirement for CK2 activity has been identified during the repair of DNA damage. Here we have described a novel role for CK2 in the response to DNA double-strand breaks. We have shown the mediator of DNA damage checkpoint 1 (MDC1) is constitutively phosphorylated by CK2, which is required for an interaction with the MRE11/RAD50/NBS1 (MRN) complex, via the FHA domain of NBS1. Moreover, disruption of this interaction resulted in loss of MRN foci following ionizing radiation and a partial G2/M checkpoint defect. Furthermore, the identification of three siblings presenting NBS/Seckel-like phenotypes with a unique mutation in the BRCT domain of NBS1, that phenocopied some of our observations, provided additional evidence for the importance of phospho-dependent interactions within the cell. Lastly, the identification of putative CK2 target residues in MRE11 and our preliminary data suggest that the kinase may play further roles in regulating the activity of the MRN complex that lie outside its activity in DNA double-strand break repair.

This thesis is dedicated to my Mum and Dad for their unconditional support and encouragement and in loving memory of my Nan. You were right I did do it!

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Abbreviations

53BP1	p53-binding protein 1
9-1-1 complex	Rad9-Hus1-Rad1 complex
A-T	Ataxia-telangiectasia
A-TLD	A-T like disorder
aa	Amino acid
AAD	ATR activation domain
ABC	ATP-binding cassette
ACF1	ATP-dependent chromatin-remodelling factor
AID	Activation induced cytidine
alt-NHEJ	Alternative-NHEJ
AOA1	autosomal recessive disorder Ataxia-oculomotor 1
APC/C	Anaphase promoting complex/cyclosome
APE	Apurinic/apyrimidic endonucleases
APLF	Appraxin-and PNK-like factor
APS	Ammonium persulphate
APTX	Aprataxin
ARC	Apoptosis repressor with a CARD
ATCC	American Type Culture Collection
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia mutated and Rad3-related kinase
ATRIP	ATR-interacting protein
BACH1	BRCA1-associated C-terminal helicase 1
BACH1	BRCA1-interacting protein C-terminal helicase 1
BARD1	BRCA1 associated ring domain 1
BER	Base excision repair
BIR	Break-induced replication
BLM	Bloom syndrome protein
BMI1	B lymphoma Mo-MLV insertion region 1 homolog
bp	Base pairs
BRCA1	Breast cancer type 1 susceptibility protein
BRCC36	BRCA1/2 containing complex subunit 36
BRCC45	BRCA1/2 containing complex subunit 45
BRCT	BRCA1 C-terminal
BRIT1	BRCT-repeat inhibitor of hTERT expression
BSA	Bovine serum albumin
CARD	Caspase recruitment domain
CDC	Cell division cycle
CDK	Cyclin-dependent kinase
cDNA	Complimentary DNA
CHK	Checkpoint kinase
CK2	Casein Kinase 2
CKAD	C-terminal Chk1-activating domain
CPT	Camptothecin
CREB	Cyclic AMP response element-binding protein

Abbreviations

CSR	Class Switch Recombination
CtIP	CtBP-interacting protein 1
DAPI	4', 6-diamidino-2-phenylindole
DDR	DNA damage response
DEPC	Diethylpyrocarbonate
dHJs	Double Holliday Junctions
DMEN	Dulbecco modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent kinase
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DNA2	DNA replication helicase 2
dNTP	Deoxyribonucleotide triphosphate
DSBR	Double-strand break repair
DSBs	Double-strand breaks
dsDNA	Double-stranded DNA
DTT	Dithiothretiol
DUB	Deubiquitylating enzyme
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EXO1	Exonuclease 1
FACS	Fluorescence-activated cell sorting
FACT	Facilitating chromatin-mediated transcription
FCS	Foetal calf serum
FHA	Forkhead-associated
FITC	Fluorescein isothiocyante
FRT	Flp recombination target
GAR	Glycine, arginine-rich region
GAR	Glycine-arginine-rich
GFP	Green Fluorescent Protein
GST	Glutathione s-tranferase
Gy	Gray
HAT	Histone acetyltransferase
HECT	Homologous to E6-AP carboxy terminus
HF	High fidelity
HP1-β	Heterochromatin protein 1
HR	Homologous recombination
HU	Hydroxyurea
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IR	Ionising Radiation
KAP1	KRAB-associated protein 1
Kb	Kilo base
KCl	Potassium Chloride
Kda	Kilodalton
LB	Luria Broth

Abbreviations

LCLs	Lymphoblastoid cell lines
MCM	Minichromosome maintenance
MCPH1	Microcephalin
MDC1	Mediator of DNA damage chcekpoint 1
MDM2	Mouse double minute 2 homolog
MERIT40	Mediator of RAP80 interactions and targeting 40kd
MgCl	Magnesium Chloride
MGS	Meier-Gorlin Syndrome
MIUS	Motifs interacting with ubiquitin
MMEJ	Microhomology-mediated end-joinning
MMR	Mismatch repair
MMS	Methyl methane sulphonate
MOF	Males absent on the first
MRE11	Meiotic recombination 1
NaCl	Sodium Chloride
NBS	Nijmegen breakage syndrome
NBS-LD	NBS-like disorder
NBS1	Nijmegen breakage syndrome 1
NES	Nuclear export signal
NETN	NaCl-EDTA-Tris-NP40
NFBD1	Nuclear factor with BRCT domains
NHEJ	Non-homologous end-joining
NLS	Nuclear localisation signal
OD	Optical density
PALB2	Partner and localiser of BRCA2
PARP-1	Poly(ADP-ribose) polymerase-1
pATM	Phosphorylated ATM
PBS	Phosphate buffered saline
PBS-T	PBS-Tween
PCR	Polymerase-chain reaction
PD	Primordial Dwarfism
pfu	*Pyrococcus Furiosus*
PI	Propidium iodide
PIKK	Phosphatidylinositol 3-kinase-related kinases
Plk1	Polo-like kinase 1
PNK	Polynucleotide kinase/phosphatase
PP2A	Protein phosphatase 2A
PRMT	Protein arginine N-methyltransferases
PST	Proline-serine-threonine
PTIP	Pax Transactivation-Domain Interacting Protein
PTM	Post-translational modifications
Rad	Radiation Sensitive
RAG	Recombination activating genes
RAP80	Receptor associated protein 80
RDS	Radio-resistant DNA synthesis

Abbreviations

Repo-man	Recruits PP1 onto mitotic chromatin at anaphase
RIDDLE	Radiosensitivity, Immunodeficiency, Dysmorphic features & Learning Difficulties
Rif1	Rap1-interacting factor 1
RING	Really interesting new gene
RNA	Ribonucleic acid
RNF	Ring finger protein
ROS	Reactive Oxygen Species
RPA	Replication protein A
RT	Room temperature
RTEL1	Regulator of telomere length 1
SCFbTRCP	SKIP1-CUL1-F-box ligase containing the F-box protein bTRCP
SCID	Severe combined immune deficiency
SCKL	Seckel Syndrome
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
SDSA	Synthesis-dependant strand annealing
SIMS	SUMO interacting motifs
siRNA	Small intefering RNA
SIRT1	Sirtuin 1
SMC	Structural maintenance of chromosome (SMC)
SOC	Super optimal catabolite
SRUbL	SUMO-regulated ubiquitin ligase
SSA	Single-strand annealing
SSB	Single-strand break
SSBR	Single-strand DNA break repair
ssDNA	Single-stranded DNA
SSRP1	Structure specific recognition protein 1
SSTE	Sarcosyl-Tris-EDTA
STUbL	SUMO-targeted ubiquitin E3 ligases
SUMO	Small ubiquitin-releated modifier
TBB	4,5,6,7-Tetrabromobenzotriazole
TBE	Tris-orthoboric acid-Na2EDTA
TBS	Tris Buffered Saline
TBS-T	TBS-Tween
TEMED	Tetramethylethylenediamine
TIP60	Acetyltransferase 60 kDa trans-acting regulatory protein of HIV type 1-interacting
Top1	Topoisomerase 1
TopBP1	Topoisomerase II binding protein 1
TP53	Tumour protein 53
TRF2	Telomeric repeat-binding factor 2
UDR	Ubiquitination-dependent recruitment
UIMs	Ubiquitin-interacting motifs
UTR	Untranslated Region
UV	Ulta-violet
V(D)J	Variable Diversity Junction

Abbreviations

WT	Wild-Type
XLF	XRCC4-like factor
XRCC	X-ray cross complimenting
xrs2	X-ray sensitive 2

CHAPTER 1

Chapter 1 General Introduction

1.1 Genomic instability and Cancer

Cancer is a collective term used to describe a range of pathologies resulting from an inability to control cell growth and proliferation. Under normal circumstances, cell division is tightly regulated by a multitude of signalling pathways, that if disrupted can lead to uncontrollable cell growth, giving rise to precancerous lesions and potentially tumours.

Tumourigenesis is a multistep process that requires a complex interplay between numerous genetic and epigenetic events that influence the oncogenic potential of a cell. Genomic stability, which is used to describe the loss or gain of genetic material through mutations or chromosomal rearrangements including translocations, deletions and inversions, can arise from improper repair of damaged DNA in the cell (Hanahan and Weinberg, 2011, Hoeijmakers, 2001). The cellular genetic material is constantly exposed to exogenous and endogenous factors that have the potential to generate a vast array of different DNA lesions. To ensure that DNA is accurately repaired, cells have evolved a plethora of pathways that cooperate in order to prevent the transmission of dangerous DNA lesions to the daughter cell. Pivotal roles in the coordination of the detection, signalling and recruitment of necessary factors as well as accurate and timely repair of DNA lesions is facilitated by a complex protein network termed the "DNA Damage Response" (DDR). The role of the DDR is to sense lesions in the DNA and then halt cell cycle progression in a process known as cell-cycle checkpoint activation. This event allows time for removal and repair of the DNA lesions. If the damage is irreparable, cellular senescence or cell death is triggered. Failure in any of these processes can result in the accumulation and transmission of DNA damage leading to genomic instability.

Previous work has shown elevated levels of unrepaired/persisting DNA damage in precancerous lesions, thought to arise from abortive/problematic replication forks. These replication errors stem from the increased proliferation of these neoplastic cells. This phenomenon has been termed 'replication stress' (Bartkova *et al.*, 2005, Gorgoulis *et al.*, 2005). Consequently, replication stress was shown to trigger activation of the cellular DDR that functions as an 'anti-tumourigenic barrier' through its ability to permanently halt the cell cycle or if the damage is too severe then induce apoptosis/senescence. However, it is clear that prolonged replication stress increases the probability of mutations in critical tumour suppressor genes required to sustain activation of the DDR such as *TP53*, *ATM* and *BRCA1*. This can disrupt their ability to induce senescence or apoptosis, allowing genetically compromised cells to continue to proliferate. This could explain why many genes encoding DDR proteins are mutated in many different human cancers, for instance *TP53* is mutated in over 50% of all sporadic tumours (Gumy Pause *et al.*, 2003, Thorstenson *et al.*, 2003, Vogelstein *et al.*, 2000). Whereas abnormalities within the cellular DDR can predispose to cancer development or accelerate its progression, interestingly, compromising or inactivating the cellular DDR can also be utilised to treat tumours. Cancer cells harbouring mutations in DDR genes are inherently hyper-sensitive to DNA damaging agents due to compromised DNA repair pathways and as such treating these cells with radiation or chemotherapeutic drugs that induce DNA damage, such as Temozolomide, can inflict genetic damage that cannot be repaired inducing cell death. In a similar manner, inhibitors of various DNA repair pathways are now being used in conjunction with different chemotheraputics to increase the efficiency of the treatment (Hastak *et al.*, 2010).

1.2 The DNA damage response (DDR)

The DDR requires a large number of different proteins that are essential for detecting and ensuring accurate recruitment of the repair machinery, which is composed of several classes of enzymes, including nucleases, helicases, kinases and ligases. Before initiation of repair can occur an orchestrated series of events are required to take place. This involves different groups of proteins such as DNA damage sensors and mediators that can amplify the signal as well as transducers that themselves signal primarily through phosphorylation (Figure 1.1). A major role in controlling the cellular DDR is played by members of the phosphatidylinositol 3-kinase-like kinase family (PIKKs), including ATM (ataxia-telangiectasia mutated), ATR (ataxia-telangiectasia mutated and rad3-related kinase) and DNA-PK (DNA-dependant protein kinase).

1.2.1 ATM activation and the ATM-mediated DNA damage response

ATM is primarily required for the detection/repair of DNA double strand breaks (DSBs) in heterochromatin. That is chromatin, which is tightly packed. DNA-PK is also capable of facilitating DNA DSB repair and this will be discussed in detail later in the text. This type of DNA lesion is considered to be the most cytotoxic, since a single unrepaired DSB is sufficient to trigger cell death. A variety of sources can induce the generation of DSBs such as exposure to X-rays, ionising radiation (IR), or radio-mimetic chemicals whereas others can produce DSBs indirectly, for example, reactive oxygen species (ROS) that are often generated as by-products of normal cellular metabolic processes. DSBs can also be generated during replication fork collapse, which occurs after prolonged replication fork stalling or when the replication machinery encounters certain lesions such as DNA single strand breaks (SSBs) or an abortive topoisomerase complexes (Figure 1.2). Moreover, DSBs can also arise in a

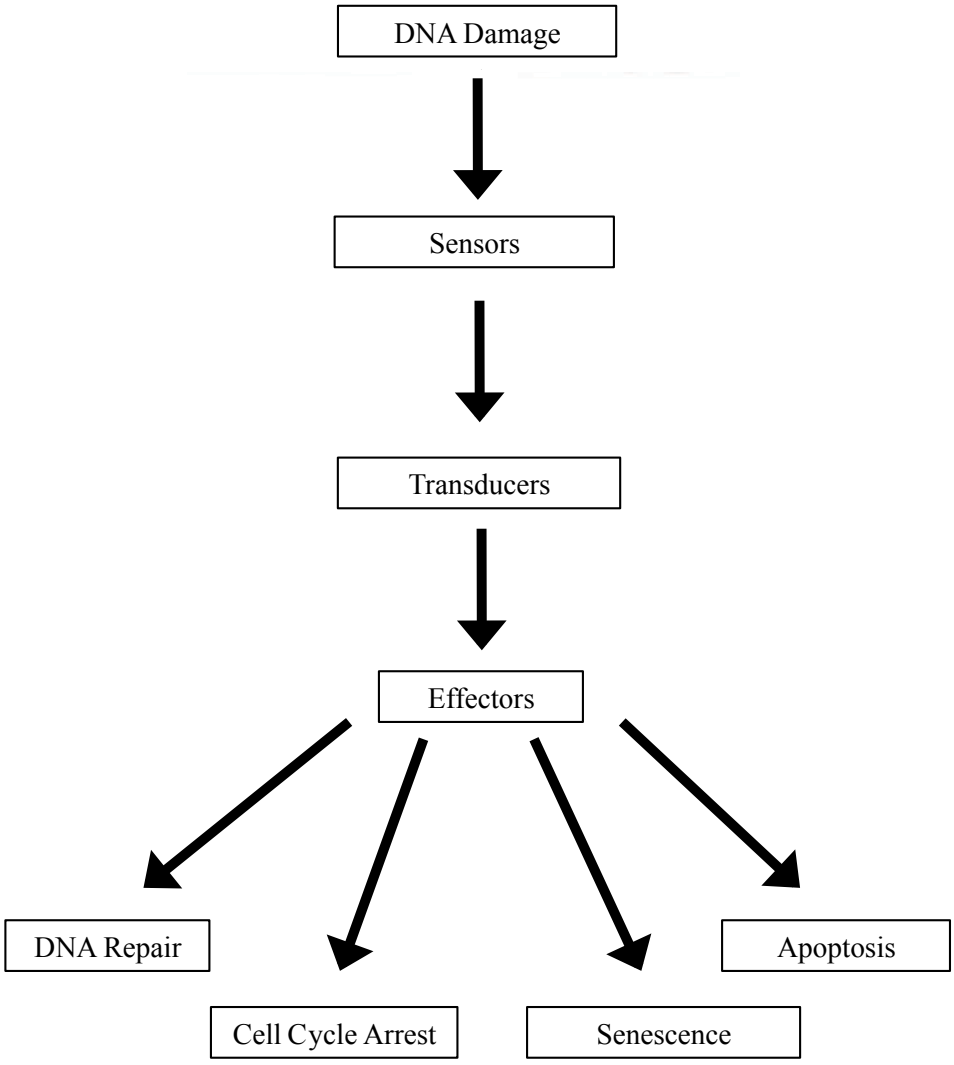


Figure 1.1. ***The DNA damage response.*** *The cellular response to DNA damage involves a tightly orchestrated series of events, termed the DNA damage response. This signalling cascade is dependent on a number of different proteins that can be grouped as sensors, those that sense the damage, transducers, proteins that transmit the DNA damage response signal and effector proteins that facilitate the appropriate response to the lesion.*

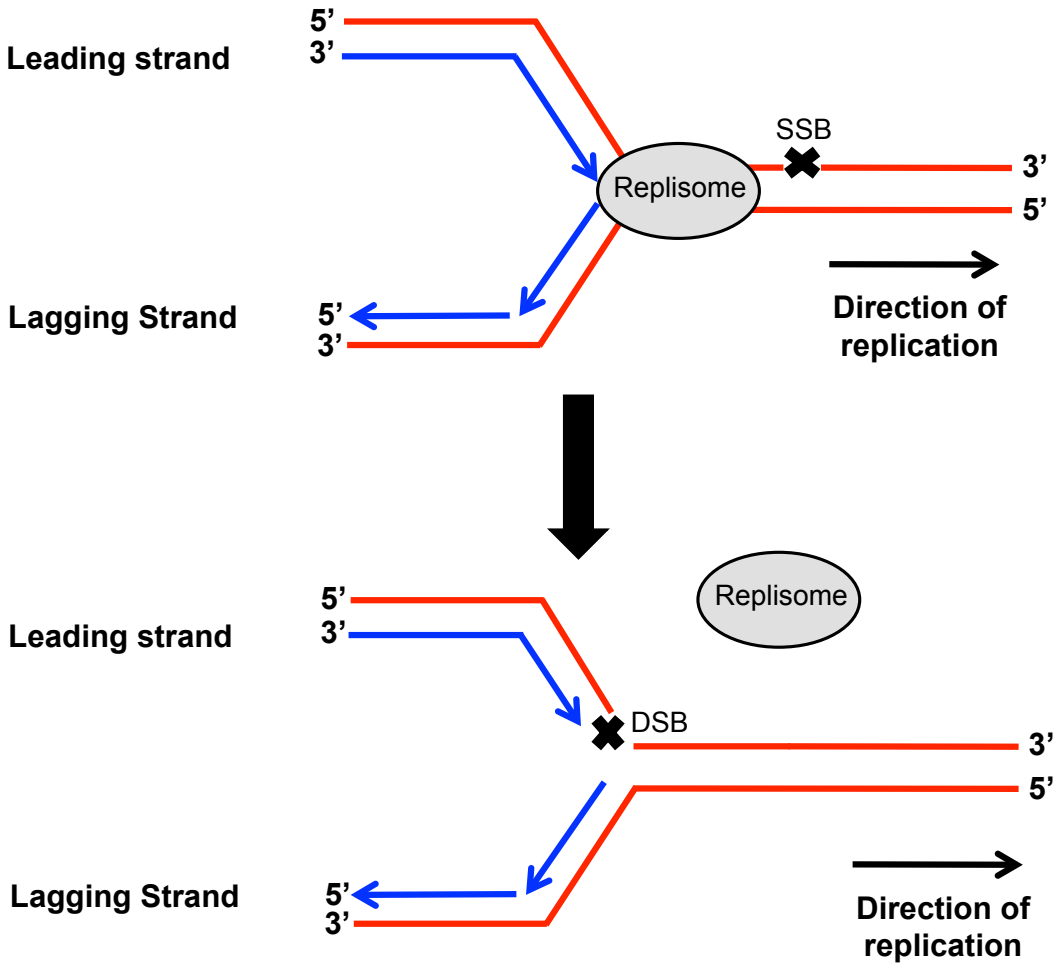


Figure 1.2. ***DNA double-strand breaks generated at the replication forks.*** *A simplified example of one scenario during DNA replication that may result in generation of a DNA double-strand break (DSB). On encountering an endogenous DNA single-strand break (SSB) the replication machinery (replisome) disassociates and the replication fork collapses resulting in a one-ended DSB.*

programmed manner during V(D)J (variable (diversity) and junction) recombination and class switch recombination (CSR) that are essential for development of the immune system (discussed later in the text) (Hiom, 2010, Jackson and Bartek, 2009).

Upon the formation of a DSB, the first factor to sense the damage is the MRE11/RAD50/NBS1 (MRN) complex, which functions to tether as well as stabilise the free DNA ends (Williams *et al.*, 2007). Once bound to a DSB, the MRN complex triggers the activation of ATM via its association with the C-terminus of NBS1 (Falck *et al.*, 2005, Lee and Paull, 2004). In the absence of DNA damage, ATM exists as an inactive dimer. Following break recognition by the MRN complex the inactive ATM dimers dissociate and form active monomers. This damaged-induced event occurs by autophosphorylation of ATM in trans on serine (S) 1981, S367, S1893 and S2996, as well as acetylation on lysine 3016 by the histone acetyltransferase Tip60 (Bakkenist and Kastan, 2003, Kozlov *et al.*, 2011, Kozlov *et al.*, 2006, Sun *et al.*, 2005). These modifications then allow ATM to be activated and exert its kinase activity on numerous downstream targets. One of the first ATM targets is the histone H2A variant (γH2AX), which becomes phosphorylated on S139 (Rogakou *et al.*, 1998). The damage-induced phosphorylation of H2AX on S139 is coupled with a dephosphorylation of a nearby tyrosine, 142, which is constitutively phosphorylated in the absence of damage, by the EYA phosphatase (Cook *et al.*, 2009, Xiao *et al.*, 2009). This rapidly modified histone then serves to coordinate and amplify the damage signal in a highly structured way. The phosphorylation and dephosphorylation of H2AX allows for the recruitment of the mediator protein, mediator of DNA damage checkpoint 1 (MDC1), which interacts directly with the γH2AX, via its BRCA1 C-terminal (BRCT) domain (Lee *et al.*, 2005b, Lou *et al.*, 2006, Stucki *et al.*, 2005). The binding of γH2AX to MDC1 stimulates the recruitment of additional MRN complexes to the site of DNA damage, which serves to recruit

more activated ATM therefore spreading the γH2AX signal along the chromatin either side of the lesion. Using this positive ATM-dependent feedback loop, this process results in amplification of the intracellular signal generated by the DNA damage (Chapman and Jackson, 2008, Lou *et al.*, 2006, Lukas *et al.*, 2004a, Melander *et al.*, 2008, Spycher *et al.*, 2008) (Figure 1.3).

In conjunction with its role as a mediator protein, MDC1 also functions as a scaffold protein recruiting other DDR response proteins along the chromatin flanking the break. Through its interaction with ATM, the N-terminus of MDC1 becomes phosphorylated, creating a binding platform recognised by the forkhead-associated (FHA) domain of the E3 ubiquitin ligase, RNF8 (Huen *et al.*, 2007, Kolas *et al.*, 2007, Mailand *et al.*, 2007). RNF8 catalyses the ubiquitylation of H2A and H2AX surrounding the break. The ubiquitylation of H2A-type histones signals the recruitment of another E3-ubiquitin ligase, RNF168, which interacts with the ubiquitylated histones via two atypical ubiquitin-interacting motifs (termed motifs interacting with ubiquitin or MIUs). RNF168, together with the E2 conjugating enzyme UBC13, catalyses the lysine 63 (K63) -linked poly-ubiquitylation of H2A/H2AX, which is thought to facilitate chromatin relaxation proximal to the break and also create an interaction platform for the recruitment of downstream DNA repair/checkpoint proteins, such as BRCA1 and 53BP1 (Doil *et al.*, 2009, Pinato *et al.*, 2011, Stewart, 2009) (Figure 1.3).

Poly-ubiquitylated H2A and γH2AX are both recognised by the tandem ubiquitin-interacting motifs (UIMs) of RAP80 (receptor associated protein 80) an adaptor protein that is involved in facilitating the recruitment of the BRCA1-A complex to sites of DNA damage via its association with another protein, Abraxas (Kim *et al.*, 2007a, Sobhian *et al.*, 2007, Wang *et al.*, 2007).

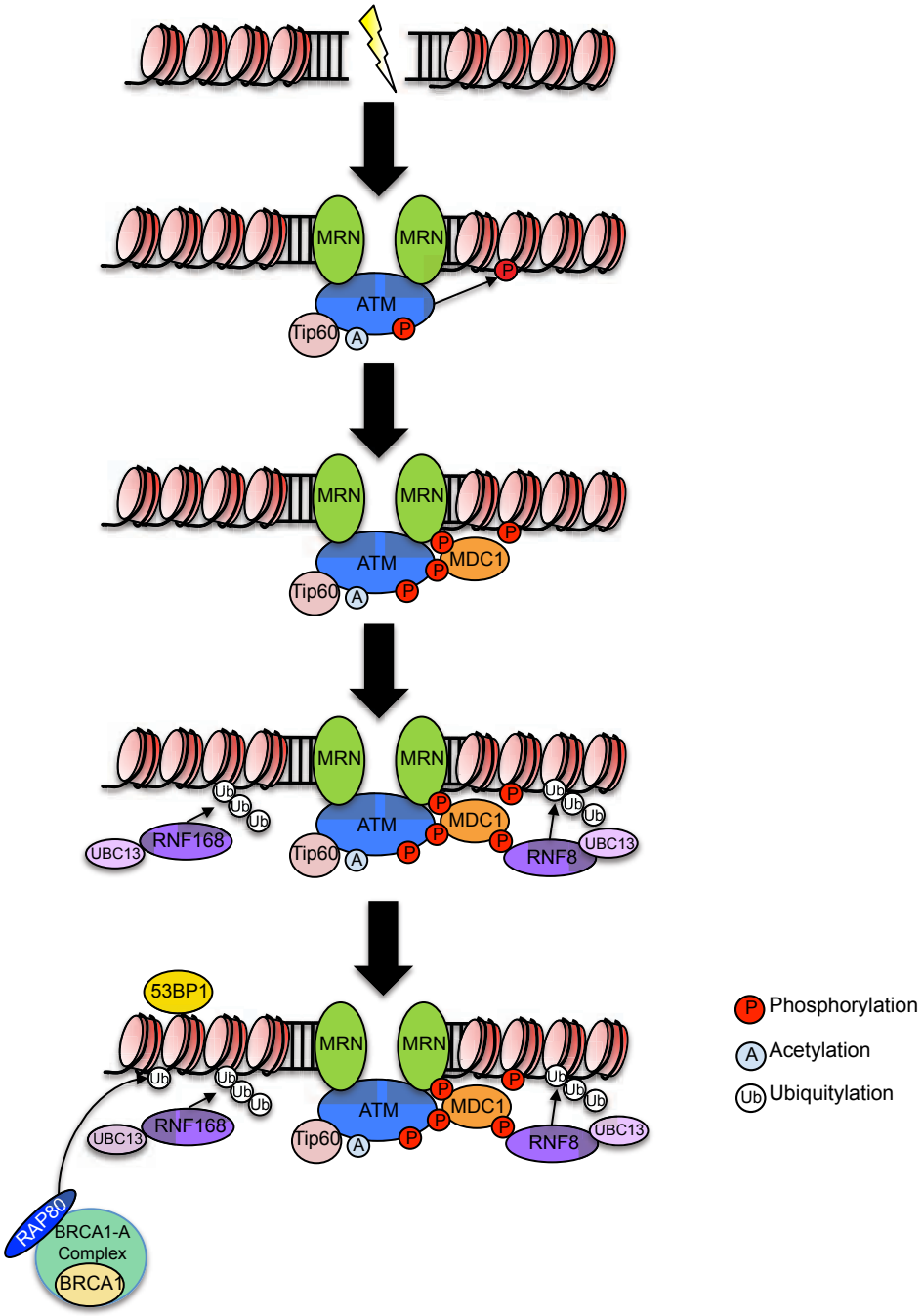


Figure 1.3 ***The ATM-mediated DNA damage response.*** *Following the generation of a DSB the MRN complex is recruited to the lesion, which in turn recruits ATM, which is then activated by MRN and Tip60. ATM then phosphorylates H2AX. γH2AX directly interacts with MDC1. MDC1 is then phosphorylated by ATM, and phosphorylated MDC1 is recognized by RNF8, that along with RNF168 and the E2 UBC13 catalyse the H2A-type histone ubiquitylation which results in chromatin relaxation, permitting the recruitment recruitment of 53BP1 and the BRCA1-A-complex. (Adapted from Ciccia and Elledge 2010).*

Despite a requirement of BRCA1 for homolgous recombination repair (HR) as well as activation of both the intra-S and G2/M cell cycle checkpoints following the induction of DNA damage, the contribution of its E3 ubiquitin ligase to these functions remains elusive.

H2A and H2AX ubiquitylation also serves to facilitate the accumulation of 53BP1 to sites of damage. The 'opening up' of the chromatin structure by the ubiquitylation of histones is thought to allow recognition of an epigenetic mark on histone H4 (H4K20me2), which normally remains buried within nucleosomes in undamaged chromatin, by the Tudor domain of 53BP1 (Botuyan *et al.*, 2006). Once recruited, one proposed function of 53BP1 is to promote the repair of DSBs by non-homologous end-joining (NHEJ) through its ability to limit DNA end resection and as a consequence, HR (Bothmer *et al.*, 2010, Chapman *et al.*, 2012).

In addition to activating and/or stimulating proteins required to repair DNA DSBs, ATM also serves to halt cell cycle progression through its ability to phosphorylate a number of key cell cycle regulatory proteins e.g. CHK1, CHK2, p53 and BRCA1 (Banin *et al.*, 1998, Canman *et al.*, 1998, Cortez *et al.*, 1999, Khanna *et al.*, 1998, Matsuoka *et al.*, 2000, Saito *et al.*, 2002). As a consequence cells derived from patients with ataxia-telangiectasia (A-T) display a defect in their ability to activate the G1/S, intra-S and G2/M phase cell cycle checkpoints following the induction of DNA DSBs.

Given the multitude of the cellular roles of ATM, it was an unexpected observation that approximately only fifteen percent of DSB are repaired in an ATM-dependent manner (Riballo *et al.*, 2004). Furthermore, the repair of this subset of breaks was found to have "slow" kinetics compared to the rest of the DSB repair in the cell. It has been proposed that this "slow" component of repair is not only related to the biochemical complexity of some

DNA breaks but also to the cells ability to detect the damage in the context of the localised chromatin structure e.g. heterochromatin versus euchromatin (Goodarzi *et al.*, 2010).

In addition to the MRN-dependent activation of ATM in response to DNA damage, ATM can also be activated in an MRN-independent manner in the absence of DNA damage. A number of reports have shown that in response to hypoxia, ATM is both active and phosphorylated, and this activation does not require the presence of detectable DNA damage and is MRN-independent (Bencokova *et al.*, 2009, Hammond *et al.*, 2003a). Hypoxia is used to describe low oxygen conditions in the cellular environment and is a common feature of many solid tumours. Hypoxic conditions do not generate DSBs, but instead are thought to cause replication stress in the form of stalled replication forks, due to depletion of deoxyribonucleotides (dNTPs) (Loffler *et al.*, 1997). This replication stalling leads to an increase in ATR activity potentially regulating cell cycle checkpoints and repair (Hammond *et al.*, 2002). Since hypoxia does not generate detectable DSBs the precise mechanisms involved in ATM activation and signalling in this cellular environment is still unclear. Although it has been hypothesised that ATR activation, as a result of single-stranded DNA at the stalled replication fork may be a contributing factor (Bencokova *et al.*, 2009). However, it may be that ATM plays a more important role in signalling a cellular response during reoxygenation, a process that often occurs following transient periods of hypoxia. It is likely that ROS produced during reoxygenation induces sufficient DNA damage to trigger an ATM-dependent response although the collapse of stalled forks attempting to restart may also contribute to this (Freiberg *et al.*, 2006, Hammond *et al.*, 2003b). The ability of ATM to initiate a G2 phase cell cycle arrest following reoxygenation may allow time for any DNA damage incurred to be repaired.

1.2.2 ATR activation and the ATR-mediated DNA damage response

Another PIKK kinase, Ataxia telangiectasia mutated and rad3-related (ATR), is thought to be activated by exposed ssDNA that can be generated by the stalling of replication fork progression due to the presence of lesions across which the replicative polymerases are unable to synthesise DNA. These can be photoproducts generated by ultraviolet (UV) light, depletion of the cellular pool of dNTPs induced by treatment with compounds that inhibit ribonucleotide reductase e.g. hydroxyurea (HU) or DNA base alkylation. For this reason, ATR is active during every S-phase, where it functions to regulate replication origin firing, stabilise stalled forks and prevent the untimely entry into mitosis (Cimprich and Cortez, 2008). In the presence of replication stress ssDNA is thought to occur due to the uncoupling of the DNA helicase from the replicative polymerases (Byun *et al.*, 2005). This leads to a situation where the level of DNA unwinding is greater than synthesis, producing stretches of unwound ssDNA, resulting in ATR activation. ATR can also be activated by the production of ssDNA generated through end resection of a DSB (see homologous recombination section) (Jazayeri *et al.*, 2006, Myers and Cortez, 2006).

The ssDNA rapidly becomes coated with the ssDNA binding protein, replication protein A (RPA) and evidence suggests that this is one of the key events for ATR activation and its re-localisation to sites of damage (Zou and Elledge, 2003). The RPA coated ssDNA is recognised by the ATR-interacting protein, ATRIP, through direct interaction, and it is this association that mediates the recruitment of ATR (Ball *et al.*, 2007, Cortez *et al.*, 2001). Although the ATR signalling pathway requires RPA-coated ssDNA, this alone is not adequate for ATR activation. Adjacent to the ssDNA is dsDNA, which is not coated by RPA and this structure also plays a part in ATR activation. This junction of ssDNA and dsDNA is recognised by the replicative sliding clamp complex, RAD9-RAD1-hus1 (9-1-1) complex,

and the RAD17-replication factor C (RAD17-Rfc) complex, which serve to recruit other ATR regulators (Bermudez *et al.*, 2003, Parrilla-Castellar *et al.*, 2004, Zou *et al.*, 2002). One such regulator is topoisomerase-binding protein-1 (TopBP1), which binds to the 9-1-1 complex, via the phosphorylated C-terminal of RAD9 and hyper-activates ATR through a direct interaction with ATRIP (Kumagai *et al.*, 2006, Mordes *et al.*, 2008). Once bound, TopBP1 acts by stimulating ATR kinase activity (Figure 1.4).

The assembly of an activated ATR complex on the DNA allows for the coordination of the necessary response to the lesion, including the stabilisation and restart of DNA replication forks as well as the activation of cell cycle checkpoints. The latter is the best characterised of these responses and involves activation of the effector serine/threonine kinase CHK1, a substrate of ATR. Activation of CHK1 requires its phosphorylation on Ser317 and Ser345 by ATR (Liu *et al.*, 2000). The mediator/adaptor protein Claspin is also essential for this activation, bringing ATR and CHK1 together (Kumagai and Dunphy, 2000). In addition to Claspin, phosphorylation of CHK1 by ATR is also facilitated by the Timeless-Tipin complex, which interacts with RPA, via Tipin. This stable complex is responsible for the recruitment of Claspin to promote CHK1 activation (Kemp *et al.*, 2010). Following its phosphorylation, CHK1 can then phosphorylate substrates such as the phosphatases CDC25A and CDC25C, inhibiting their activity and as a result preventing CDK activity required for cell cycle progression (Chen *et al.*, 2003, Uto *et al.*, 2004).

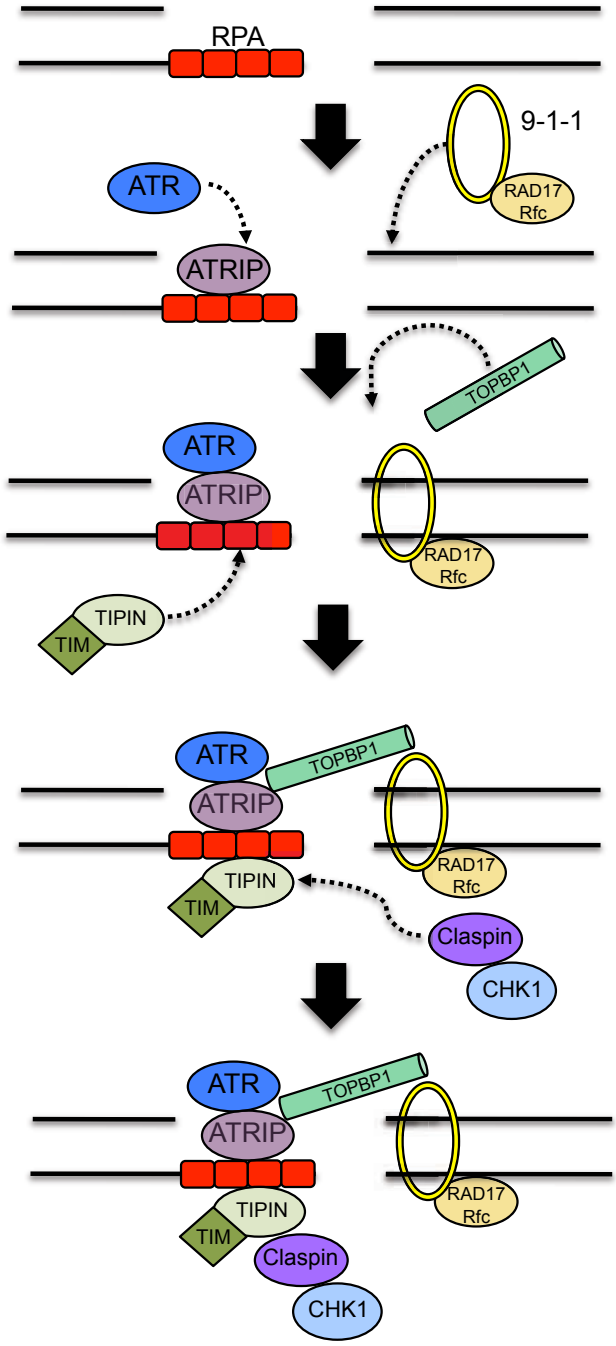


Figure 1.4 ***The ATR-mediated DNA damage response****. ssDNA generated either by DNA damage or replication stress is rapidly coated with RPA. The RPA coated ssDNA is recognised by ATRIP/ATR. The double-stranded DNA adjacent to the break recruits the 9-1-1 complex and RAD17 Rfc. TopBP1 then binds to the C-terminus of Rad9 and stimulates ATR activation. The Timeless/TIPIN complex interacts with RPA and recruits Claspin which promotes the CHK1 phosphorylation by ATR (Adapted from Nam & Cortez 2011, Kemp et al 2010)*

1.3 Cell cycle checkpoints in response to DNA damage

Cell cycle checkpoints function to ensure the integrity of the genome is maintained. This is achieved by halting the progress of the cell cycle in response to DNA damage, allowing time for repair. The checkpoints are activated by the PIKKs, and associated effector kinases, including CHK1 and CHK2. There are four main cellular checkpoints: the G1/S checkpoint prevents cells with damaged DNA entering S phase. The intra-S checkpoint/replication checkpoint controls replication origin firing and stabilises stalled DNA replication forks. The G2/M checkpoint prevents cells with damaged DNA from entering mitosis and the Spindle Assembly Checkpoint, which delays cell division in the event of problems during chromosome segregation.

Cyclin-dependent kinases (CDKs) in conjunction with regulatory subunits called Cyclins are central to controlling the transition between each phase of the cell cycle. Phosphorylation of the CDKs on threonine 14 and tyrosine 15 by the WEE1/MIK1/MYT1 kinases keeps these proteins in an inactive state (Chow *et al.*, 2003). Dephosphorylation of these residues by members of the CDC25 protein phosphatase family activates the CDK-Cyclin complex and as such promotes cell cycle progression. In mammalian cells there are three related CDC25 proteins, CDC25A, CDC25B and CDC25C, which despite having a similar function, all have specific roles in the regulation of cell cycle (Donzelli and Draetta, 2003, Galaktionov and Beach, 1991). CDC25A regulates both the G1/S phase transition with its associated Cyclins E/A and CDK2 complex activity, as well as the progression into mitosis involving the Cyclin B/Cdk1 complex (Hoffmann *et al.*, 1994). In contrast, CDC25B and C are involved in activating Cyclins A/B with CDK1 and ultimately controlling the transition through G2/M (Lammer *et al.*, 1998, Perdiguero and Nebreda, 2004).

If substantial DNA damage is present in the genome, CDC25A becomes phosphorylated by CHK1 (and possibly CHK2), and this phosphorylation marks the CDC25A for ubiquitin-mediated degradation via the proteasome (Falck *et al.*, 2001, Mailand *et al.*, 2000). Two ubiquitin ligases are involved in degrading CDC25A depending on the cell cycle phase. If CDC25A is phosphorylated during the mitotic exit, then the anaphase promoting complex (APC/C) plays a role, whereas the SKIP1-CUL1-F-box ligase containing the F-box protein βTRCP ($SCF^{\beta TRCP}$) is responsible for ubiquitylating CDC25A throughout the cell cycle as well as after DNA damage (Busino *et al.*, 2003, Donzelli *et al.*, 2002). In contrast, phosphorylation of CDC25C by CHK1 (and CHK2) creates a binding site for 14-3-3 proteins, that promotes the transport of CDC25C out of the nucleus, thereby preventing activation of the CDK1-Cyclin B complex and progression of cells from G2 into mitosis (Lopez-Girona *et al.*, 1999). It has been shown that the *in vivo* phosphorylation of CDC25B by p38, which is activated in response to UV damage, similarly creates a 14-3-3 binding site on CDC25B, suggesting that this phosphatase may also be exported from the nucleus to prevent cell cycle progression in response to damage. However, whether this is the case *in vivo* is unclear (Bulavin *et al.*, 2002).

CHK1 and CHK2 phosphorylation by ATR/ATM following DNA damage leads to the activation of the CDK inhibitory kinases (WEE1/MIK1/MYT1) providing further regulation on cell cycle progression (Kastan and Bartek, 2004). Lastly, activation of p53 by ATR/ATM, results in the upregulation of p21, which has an inhibitory role on several Cyclin-CDKs complexes (Bunz *et al.*, 1998, Spurgers *et al.*, 2006).

It is clear that the cell possesses various strategies to halt the cell cycle if genomic integrity is compromised, and it is the activation of these checkpoints that allow time for the controlled repair/re-organisation of the DNA.

1.4 DNA double-strand break repair

DNA DSBs can be repaired in different ways. The two main mechanisms involved are Non-homologous end-joining (NHEJ) and Homologous recombination (HR). Repair of DNA lesions by the NHEJ machinery is thought to be performed with "fast" kinetics, and can take place throughout the cell cycle, but predominates during G1. The NHEJ pathway involves re-ligating the DNA ends at the lesion. This method of repair is termed 'error-prone', as there is a potential for nucleotide loss surrounding the break. In contrast, HR is considered 'error-free' as it requires the presence of a sister chromatid, which acts as a template to repair the damaged DNA. Although, some evidence suggests that uncontrolled/excessive HR especially at repetitive DNA regions can also be deleterious for the cell. For instance, increases in the HR protein RAD51 seen in some tumour cells may result in unwarranted recombination resulting in potential genetic instability (Richardson *et al.*, 2004, Vispe *et al.*, 1998). The dependency on the presence of a sister chromatid means that HR is primarily restricted to the S and G2 phases of the cell cycle, where it serves to repair lesions arising during or after DNA replication.

Once a DSB has been detected, the early assembly of certain DDR proteins facilitates the choice of the appropriate repair strategy and this depends on several factors including the cell cycle phase, the structure of the damaged DNA ends and presence of DNA homologous to the damaged sequence (Bunting *et al.*, 2010, Shibata *et al.*, 2011, Shrivastav *et al.*, 2008, Takata *et al.*, 1998). Two proteins coupled to this selection of repair are 53BP1 and BRCA1. Evidence has suggested that both these proteins serve as molecular switches governing the competition between the two repair pathways for the repair of DSBs (Bunting *et al.*, 2010, Escribano-Diaz *et al.*, 2013). Following damage, occurring in cells in G1 phase, the accumulation of BRCA1 is inhibited by 53BP1 and another protein, RIF1, allowing NHEJ to

be the preferred repair pathway. Whereas in S/G2 cells, RIF1 is suppressed by BRCA1 and CtBP-interacting protein 1 (CtIP), favouring HR as the pathway for the repair of DSB (Chapman *et al.*, 2013a, Chapman *et al.*, 2013b, Di Virgilio *et al.*, 2013, Escribano-Diaz *et al.*, 2013, Feng *et al.*, 2013, Zimmermann *et al.*, 2013).

1.4.1 Homologous recombination

BRCA1 has been shown to promote repair by HR if conditions favouring this type of repair are present (Moynahan *et al.*, 1999). BRCA1 acts as a mediator during HR, recruiting and activating a number of proteins. Prior to the repair of DSBs, HR requires resection of the DNA end, which exposes single stranded DNA 3'termini, allowing for strand invasion into the sister chromatid (Mimitou and Symington, 2011). Resection of the DNA is tightly controlled and in vertebrates is instigated by CDKs via the phosphorylation of CtIP (Huertas and Jackson, 2009, Ira *et al.*, 2004). Keeping CtIP at low levels during G1 phase via proteosomal degradation provides further regulation of resection, ensuring that it only takes place during S and G2 phases (Buis *et al.*, 2012, Germani *et al.*, 2003). In addition, the phosphorylation of CtIP on serine 327 by CDKs, governs complex formation with other key HR proteins, such as the MRE11/RAD50/NBS1 (MRN) complex and BRCA1 in the S/G2 phase of the cell cycle (Chen *et al.*, 2008, Yu and Chen, 2004). A second CDK-dependent phosphorylation on theronine 847 of CtIP activates resection (Huertas and Jackson, 2009). Once CtIP forms a complex with MRN and BRCA1, BRCA1 can then ubiquitylate CtIP, a modification that serves to retain the protein at the sites of DNA damage (Yu *et al.*, 2006). The phosphorylation and expression of CtIP is also thought to be governed by the MRE11 subunit of the MRN complex, by an interaction with both MRE11 and CDK2 (Buis *et al.*, 2012). This interaction (between CtIP and the MRN) is necessary for resection to initiate and has no impact on the other characterised roles of the MRN, such as sensing of DSBs and

ATM re-localisation/activation. Lastly, phosphorylation of CtIP by ATR on threonine 818 contributes to CtIP chromatin association and robust resection (Peterson *et al.*, 2013). ATM is also thought to be required for CtIP localisation, all though whether this is through a phosphorylation event is still unclear (Peterson *et al.*, 2013, You *et al.*, 2009).

Whilst the mechanism of resection was first elucidated from studies in yeast, revealing a two-step process, a similarly conserved process has also been observed in higher eukaryotes (Mimitou and Symington, 2008, Zhu *et al.*, 2008). Firstly, MRE11 cooperates with CtIP (sae2) to process the 5' DSB end via the MRE11 exonuclease activity. This initial processing then paves the way for long range resection catalysed by the exonuclease EXO1 (exonuclease 1) and DNA2 (DNA replication helicase 2) together with BLM (Bloom syndrome protein), a helicase, which acts to unwind the DNA and promote resection (Gravel *et al.*, 2008, Nimonkar *et al.*, 2011, Nimonkar *et al.*, 2008). The exposed single stranded DNA is then rapidly coated with RPA, which activates ATR and checkpoint signalling, allowing time for repair to occur (Figure 1.5). This is then followed by displacement of RPA by RAD51 with assistance from RAD52 (Sugiyama and Kowalczykowski, 2002). RAD51 is recruited directly by BRCA2 and its binding partner PALB2 (partner and localiser of BRCA2), which are the primary mediators of RAD51 nucleoprotein filament formation (Sy *et al.*, 2009, Xia *et al.*, 2006, Yuan *et al.*, 1999). The accumulation of these factors results in the formation of the RAD51 nucleoprotein filament, which along with the ATPase RAD54, can invade the homologous sister chromatid and form a displacement loop (D-loop) structure in order to switch from strand invasion to DNA synthesis (Kiianitsa *et al.*, 2006, Li *et al.*, 2007b) (Figure 1.6). Once the D-loop is formed, the invading strand can be extended by DNA polymerases using the homologous strand as a template. A number of polymerases have been proposed to be involved in HR-DNA synthesis including Polε (Polymerase epsilon), Polη (eta), Polδ

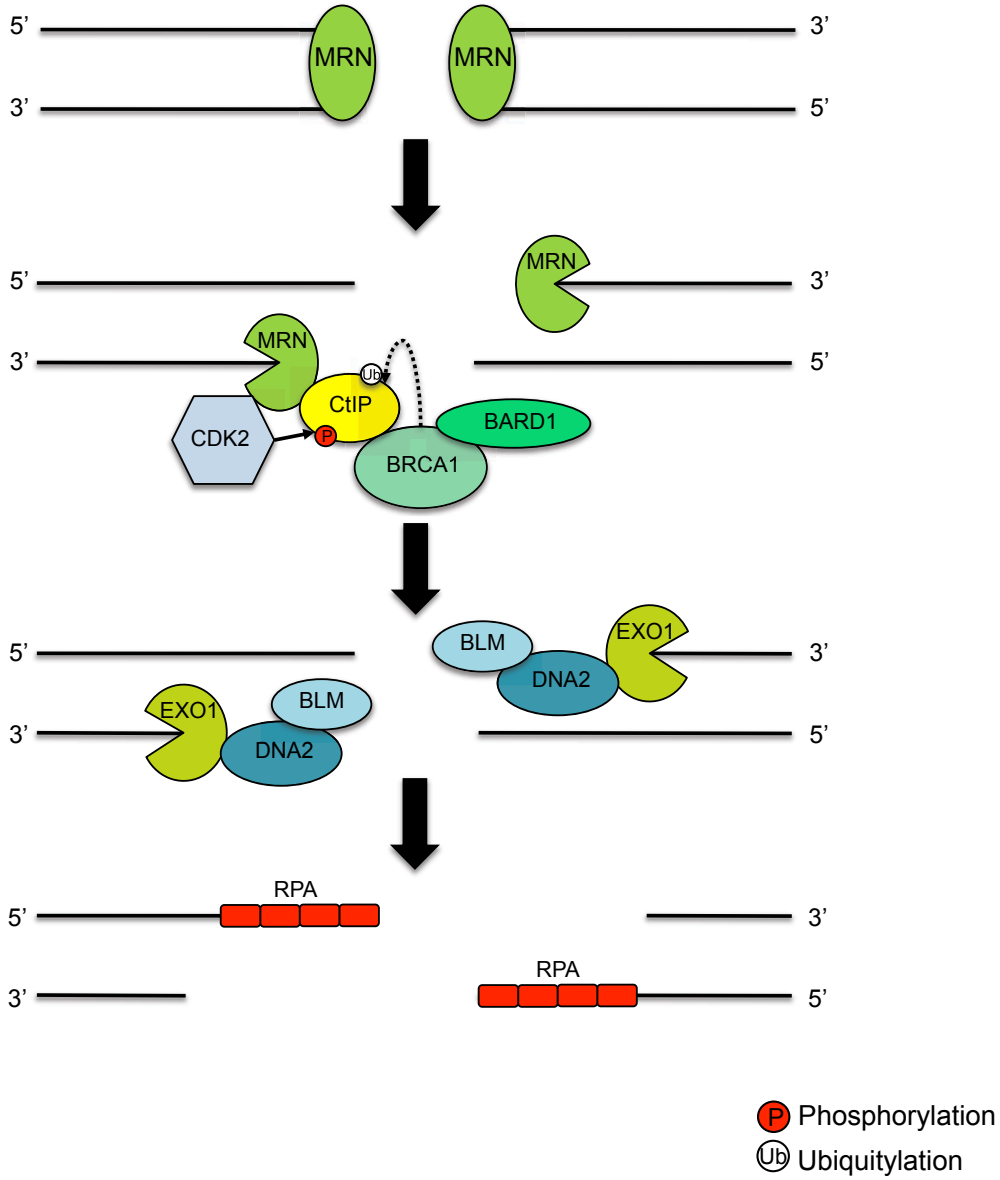


Figure 1.5 ***DNA end-resection****. MRN is recruited to the DSB. Where in complex with Cdk2 phosphorylated CtIP and BRCA1, initiates resection through the nuclease activity of MRE11. This early processing then allows the nucleases EXO1 and DNA2 to perform long range resection at the break, in conjunction with the BLM helicase, which acts in stimulatory capacity. The single-stranded resected DNA is then coated with RPA. (Adapted from Ciccia & Elledge 2010, Polo et al 2012)*

(delta) and more recently Polζ (zeta) (Kawamoto *et al.*, 2005, Maloisel *et al.*, 2008, Sebesta *et al.*, 2013, Sharma *et al.*, 2012).

Synthesis of a new DNA strand instigates the "removal" of the D-loop, which can occur in a number of ways. The predominant method is synthesis-dependent strand annealing (SDSA), whereby the newly synthesised strand is displaced from the D-loop and annealed to the resected DNA strand at the other end of the break (Paques and Haber, 1999) (Figure 1.5). This method does not lead to crossovers, and thus, genomic rearrangements, and is thought to be promoted by the helicase regulator of telomere length 1 (RTEL1) possibly by dissolution of the D-loop (Barber *et al.*, 2008). Alternatively, in a process called second-end capture, the second resected DNA strand is annealed to the displaced strand of the D-loop (Nimonkar *et al.*, 2009). If this scenario occurs, a Holliday junction is formed which can be resolved by a number of structure-specific endonucleases (Chapman *et al.*, 2012). One such structure-specific endonuclease is the resolvase MUS81 and its partner EME1 (Boddy *et al.*, 2001, Chen *et al.*, 2001, Ciccia *et al.*, 2003).

MUS81-EME1 is not specific to any HR outcome and could, therefore, be considered as promiscuous, eliciting its activity on a variety of intermediates possibly formed during HJ resolution, although, it does have a preference toward nicked Holliday junctions (Hollingsworth and Brill, 2004, Osman *et al.*, 2003). The branch migration of the D-loop, as a consequence of second strand capture, can produce double Holliday junctions (dHJs) (Figure 1.6). A fraction of structure-specific endonucleases identified are thought to be specific to resolving dHJs. GEN1 and SLX1/SLX4 promote the resolution of dHJs by symmetrically cleaving the four-way nucleotide junction, producing either a crossover or non-crossover

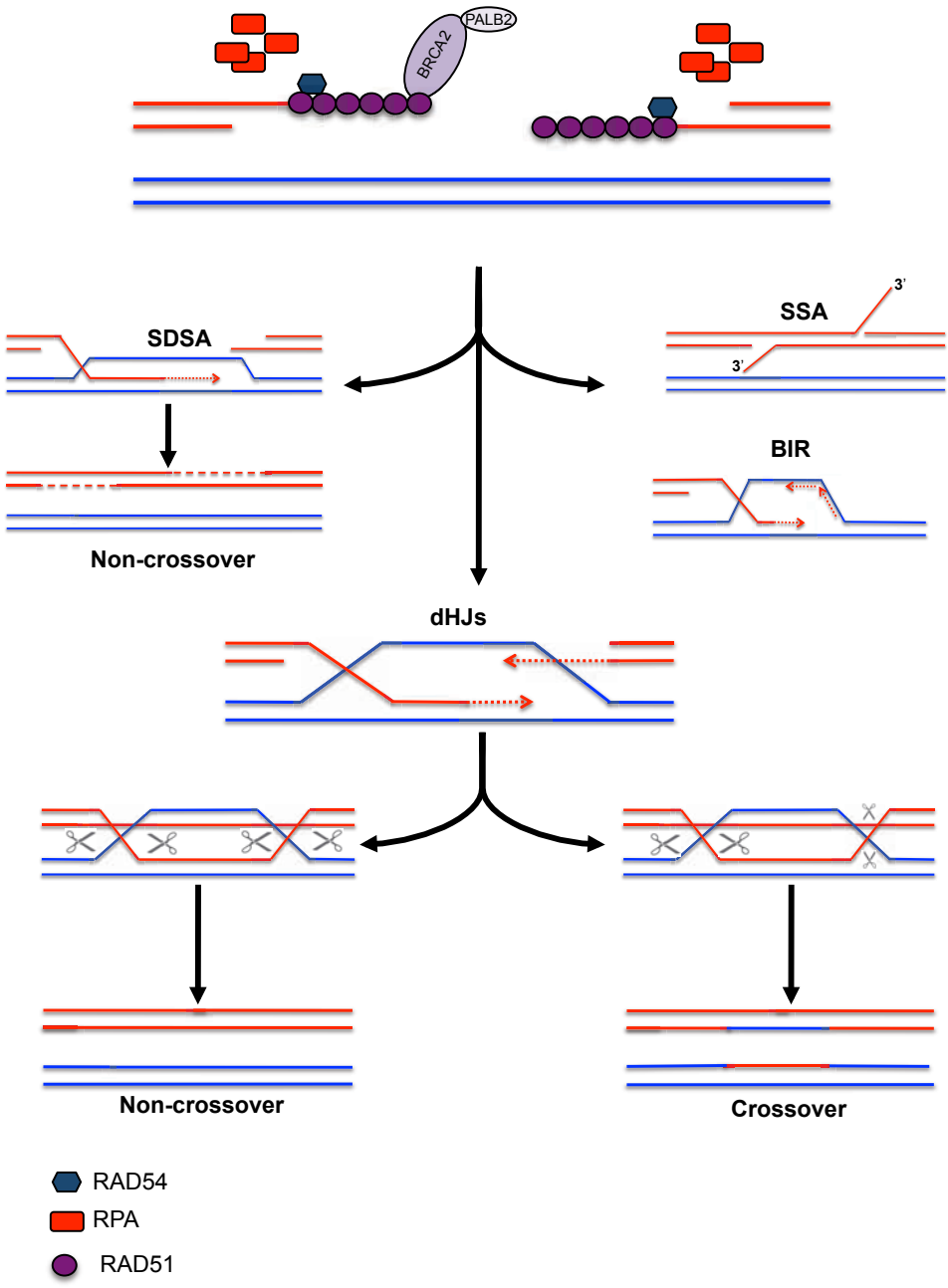


Figure 1.6 ***Homologous recombination repair of DSBs.*** *RAD51, recruited by BRCA2 and PALB2, displaces RPA from the ssDNA, generated during resection. The RAD51 nucleoprotein filament, aided by RAD54 can then invade the sister chromatid, where several scenarios can arise to repair the DSBs that result in crossover and non-crossover events (Adapted from San Filippo et al 2008).*

recombination outcome (Fekairi *et al.*, 2009, Ip *et al.*, 2008, Munoz *et al.*, 2009, Svendsen *et al.*, 2009) (Figure 1.6). Recent evidence has shown that SLX4 acts as a scaffold recruiting SLX1, XPF and MUS81 to dHJ (Fekairi *et al.*, 2009). Lastly, the BLM helicase in complex with TOPOIIIα can dissolve dHJs by migration of the two HJs towards each other, followed by decatenation of the resulting structure; a process, which yields non-crossover products (Wu and Hickson, 2003). As the resolution of dHJs by structure-specific endonucleases can produce crossover events, they must be tightly regulated as this can lead to loss of heterozygosity and genomic rearrangements in mitotic cells (Ciccia and Elledge, 2010).

1.4.1.1 Single-strand annealing and break-induced replication

Another homology related DNA repair pathway is single-strand annealing (SSA). SSA can be utilised in two possible situations. Firstly, if HR components such as BRCA2 or RAD51 are absent, the latter being required to promote strand invasion, a shift will be observed from HR to SSA (Stark *et al.*, 2004). Secondly, SSA can occur if the DSB arises near regions of sequence homology within the same strand. The initial step of SSA requires the same group of proteins required for HR, as the DNA either side of the lesion requires resection. Resection occurs until the two regions of homology are exposed, where RAD52 facilitates the annealing of the two repeat regions (Singleton *et al.*, 2002). As a product of resection, 3' prime tails are created which are cleaved by the flap endonucleases, excision repair cross complementation (ERCC1)/Xeroderma Pigmentosum F (XPF) complex, after which the two strands can then be ligated (Al-Minawi *et al.*, 2008). Repair via SSA is potentially mutagenic, compared to canonical HR, as it involves loss of large regions of genetic information due to resection (Figure 1.6). One study revealed that >2kb of resection had to occur in order for SSA to happen (Stark *et al.*, 2004).

Break-induced replication (BIR) occurs if a second end is lacking, which results in the single end invading either the sister chromatid or a homologous chromosome (Malkova *et al.*, 1996, San Filippo *et al.*, 2008). Again like SSA, the break is resected, followed by invasion of the free 3'tail, which then invades the donor strand. BIR is highly mutagenic, maybe due to the lack of a second end, resulting in the invading strand undergoing repeated rounds of homology searching during synthesis, possibly encountering regions of non-homologous sequence (Llorente *et al.*, 2008, Smith *et al.*, 2007) (Figure 1.6).

1.4.2 Non-homologous end-joining

NHEJ is the primary method for repairing DSBs. Following the generation of a DSB, the KU70/80 heterodimer binds to the DNA ends and serves as a dock for other NHEJ proteins at the break (Blier *et al.*, 1993, Mimori and Hardin, 1986). The binding of the KU complex onto the DNA on either side of the break changes its conformation allowing for the interaction of the serine/theronine kinase, DNA-dependent protein kinase catalytic subunit (DNA-PKcs). It has been observed that Ku does not form a stable interaction with DNA-PKcs in the absence of DNA (Yaneva *et al.*, 1997). Recent evidence has suggested the ATP-dependent chromatin-remodelling factor (ACF1) is required for the localisation of KU to DNA via a direct interaction (Lan *et al.*, 2010). A molecular bridge is then created between the DNA-PKcs molecules bound to each DNA end, termed a synapsis (DeFazio *et al.*, 2002, Dobbs *et al.*, 2010). Interaction of DNA-PKcs with both KU and DNA activates its kinase activity, leading to the phosphorylation of a number of substrates including KU70/80, X-ray cross complimenting 4 (XRCC4) and Artemis, though it is unclear what impact these modifications have on the regulation of the NHEJ process (Douglas *et al.*, 2005, Goodarzi *et al.*, 2006, Yu *et al.*, 2003). Interestingly, DNA-PKcs also phosphorylates itself, on a number of autophosphorylation sites, and studies have demonstrated that this is required for efficient

NHEJ (Chan *et al.*, 2002, Ding *et al.*, 2003). The DNA ends at the break must become ligatable for repair to continue, as the two ends are rarely compatible for re-ligation and often harbour 3'-5' overhangs. In mammalian NHEJ, Artemis is the key end-processing enzyme and its recruitment to DSBs is likely mediated through an interaction with DNA-PKcs (Ma *et al.*, 2002, Moshous *et al.*, 2001). Artemis possesses DNA-PKcs-dependent endonuclease and DNA-PKcs-independent 5'-3' exonuclease activity and where necessary, the protein can trim the DNA ends (Ma *et al.*, 2005). In addition to Artemis, if the processing of the lesion is complex, gaps may need to be filled by polymerases. Members of the polymerase X family, which include polymerase μ (mu) and polymerase λ (lambda) are thought to facilitate this process during NHEJ (Ma *et al.*, 2004, Mahajan *et al.*, 2002). The last step of NHEJ requires the ligation of the DNA ends and is coordinated by XRCC4/DNA ligase IV/XRCC4-like factor (XLF)/Cernunnos complex (Grawunder *et al.*, 1997, Wu *et al.*, 2007) (Figure 1.7). XRCC4 lacks any enzymatic activity but is thought to act as a protein scaffold, bringing DNA Ligase IV and XLF interacting partners into proximity with Ku and the DNA (Nick McElhinny *et al.*, 2000). It has also been shown that XRCC4 is phosphorylated by CK2, which promotes its interaction with polynucleotide kinase/phosphatase (PNKP), a bifunctional DNA end-processing enzyme that can repair damaged DNA termini possessing 3'phosphate and a 5'hydroxyl groups, thereby facilitating DNA ligation (Figure 1.7) (Koch *et al.*, 2004, Mani *et al.*, 2010).

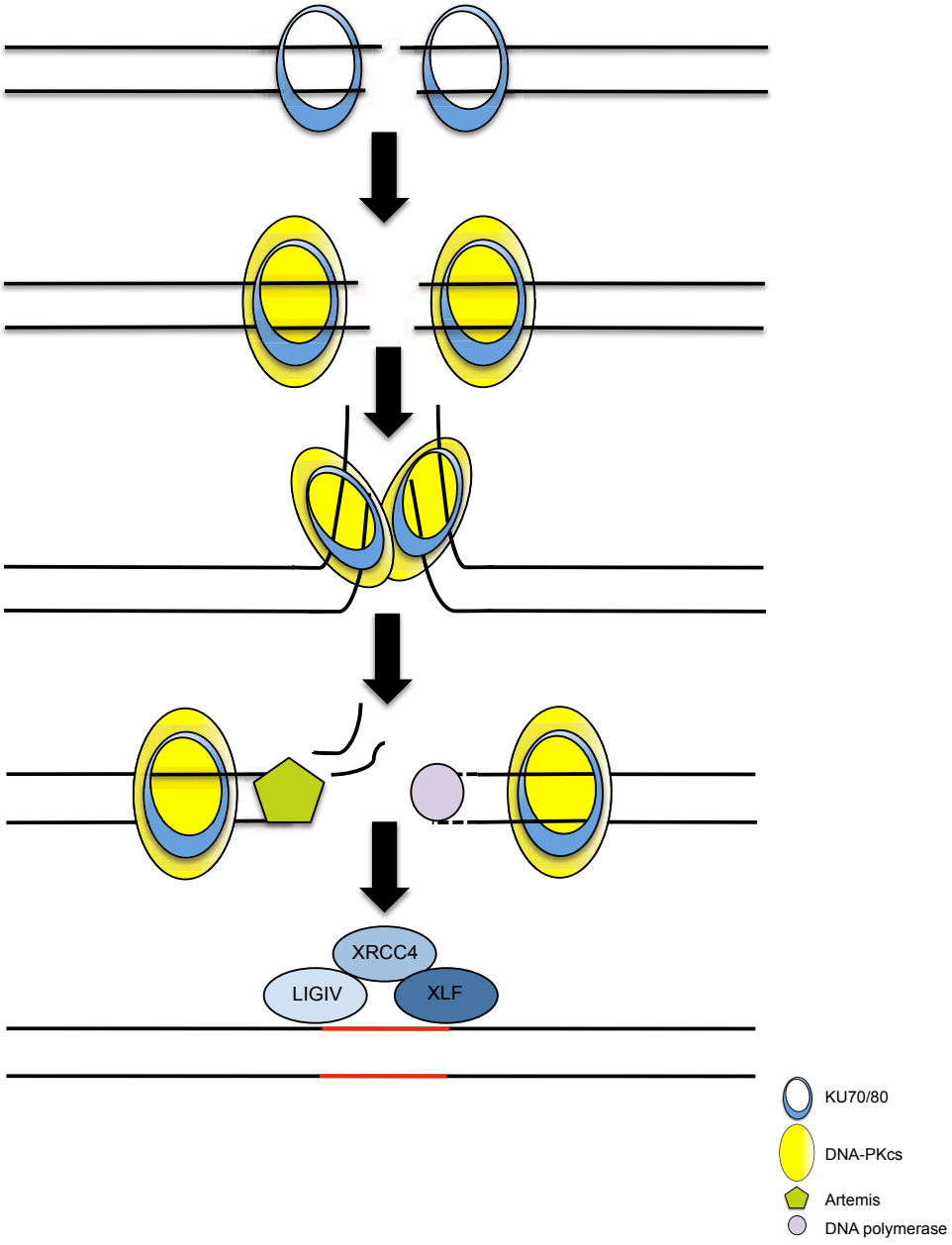


Figure 1.7 ***Non-homologous end-joining.*** *The KU70/KU80 heterodimer binds to the DSB, changing its conformation, which allows the binding of DNA-PKcs, and activates its kinase activity. Both KU70/KU80 and DNA-PKcs create a molecular bridge between the two DNA ends. The DNA nuclease Artemis is then recruited to process the DNA ends, or if gaps need to be filled then polymerases are recruited. Once the DNA ends have been processed the break is ligated by the XRCC4/DNA ligase IV/XRCC4-like factor (XLF) complex (Adapted from Weterings & Chen 2008).*

1.4.2.1 Alternative end-joining

Alternative end-joining (Alt-EJ) occurs when classical NHEJ proteins, for instance Ku, XRCC4 or DNA ligase IV are absent (Hartlerode and Scully, 2009). Alt-EJ is also reliant on short regions of sequence homology at the sites of damage and its usage has been associated with the formation of chromosomal deletions and translocations (Bennardo *et al.*, 2008, Della-Maria *et al.*, 2011, Guirouilh-Barbat *et al.*, 2007). Microhomology-mediated end-joining (MMEJ) is just one of the possible means of repairing DNA breaks via Alt-EJ, however, the two are mutually exclusive (McVey and Lee, 2008). Like SSA, which also relies on regions of homology, repair of the break by Alt-EJ requires resection of the DNA by MRN/CtIP, albeit over a shorter region than during HR (Della-Maria *et al.*, 2011, Rass *et al.*, 2009). However, Alt-EJ differs from SSA in that RAD52 partially inhibits and ERCC1 only partially promotes repair by Alt-EJ, whereas both of these proteins promote SSA (Bennardo *et al.*, 2008). Resection serves to reveal the short regions of homology allowing for ligation to occur. Error-prone polymerases may also be involved in Alt-EJ, where they are required to process the resected DNA ends. MMEJ, requires Polη and Polζ in *Saccharomyces cerevisiae*, and Polymerase θ (theta) in *Drosophila melanogaster* (Chan *et al.*, 2010). Which polymerases are required for re-synthesis across DNA gaps during this repair process in mammalian cells is still unclear. Ligation of the DSB during Alt-EJ occurs independently of DNA ligase IV and instead is thought to require DNA ligase I and DNA ligase IIIα/XRCC1, although their relative contributions to this process requires further investigation (Boboila *et al.*, 2012, Liang *et al.*, 2008). Most likely, the absence of NHEJ proteins allows access for other factors to be recruited to the DSB. For example, Poly(ADP-ribose) polymerase-1 (PARP-1), normally associated with DNA single-strand break repair (SSBR), is thought to promote recruitment of the DNA ligase IIIα/XRCC1 complex to lesions undergoing Alt-EJ (Audebert *et al.*, 2004).

The MRN complex also associates with DNA ligase IIIα/XRCC1, in cells deficient in classical NHEJ machinery, where they function together to join DNA ends (Della-Maria *et al.*, 2011).

1.4.3 Programmed double-strand breaks

As discussed previously, DSBs caused by endogenous or exogenous agents are highly toxic to the cell and need to be dealt with in a quick and coordinated manner. In spite of this, the development of the immune system requires the DDR cascade and interestingly, creation of DSBs in a controlled and highly restrictive manner.

V(D)J recombination produces diversity in T-cell receptors and immunoglobulins (Ig) through genetic recombination in developing lymphocytes (Malu *et al.*, 2012). V(D)J recombination achieves this by joining combinations of variable (V), diversity (D) and junction (J) regions of DNA sequences and utilises NHEJ to join the breaks (Figure 1.8). The target regions for this process are the Ig and T-cell receptor (TCR) genes, which have recombination signal sequences (RSS) embedded within them (Jung and Alt, 2004). This process involves cleavage of the DNA by recombination activating genes 1 and 2 (RAG1, RAG2), which recognise the RSSs, inducing a DSB adjacent to the V, J and D sequences and creating a hairpin structure at coding ends. The KU heterodimer is able to bind to the cleaved DNA end and begin the repair phase. DNA-PKcs can then localise and bridge the coding ends, where it also serves to recruit and activate the nuclease required to open the hairpin generated by RAGs. Whereas *in vitro* studies have implicated the RAG complex, MRN and Artemis in DNA hairpin opening, data from knockout mouse models suggests that Artemis plays a major role in opening the hairpin *in vivo* (Besmer *et al.*, 1998, Ma *et al.*, 2002, Malu *et al.*, 2012, Paull and Gellert, 1999, Rooney *et al.*, 2002, Shockett and Schatz, 1999). Once

open, the hairpin coding ends may require further processing, either from the addition of nucleotides by TdT, known as N-additions, or the removal of nucleotides by an as yet unidentified nuclease(s) (Desiderio *et al.*, 1984, Malu *et al.*, 2012). Both the removal and addition of nucleotides serves to increase the diversity within the antigen binding regions of these surface receptors and as a consequence, the range of putative antigens recognised. Finally, the coding ends can be ligated by the XRCC4/DNA ligase IV/XLF complex, generating a new variable region (Figure 1.8).

Class-switch recombination (CSR) takes place both inside and out of germinal centres (GC). This process functions to promote antibody maturation by replacing the constant regions (C_H) within the antibody heavy chain, resulting in the production of different antibody isotypes that can interact with different effector molecules (Alt *et al.*, 2013, Chaudhuri *et al.*, 2007, Xu *et al.*, 2012). The activation induced cytidine deaminase (AID) initiates CSR, by deaminating cytidine residues in S (switch) regions, converting them to uracils, creating a U/G DNA mismatch (Figure 1.9) (Soulas-Sprauel *et al.*, 2007b). This mismatch is recognised by the base excision repair (BER) protein uracil-N-glycosylase (UNG), which removes the uracil from the DNA generating an abasic site (Imai *et al.*, 2003, Rada *et al.*, 2002). The abasic site is then recognised by apurinic/apyrimidic endonucleases (APE1 and APE2) that induce the formation of a ssDNA nick (Stavnezer *et al.*, 2008). As AID is able to target both strands of DNA within the S region, two closely located ssDNA nicks can become a DSB (Soulas-Sprauel *et al.*, 2007b, Stavnezer, 2011, Xu *et al.*, 2012). If the ssDNA nicks are close to each other, DSBs will form spontaneously. After the DSB has been correctly processed, recombination between the donor and acceptor regions can initiate (Figure 1.9) (Stavnezer *et al.*, 2008). Members of the NHEJ machinery KU70/KU80 and XRCC4/DNA-ligase IV are important for the completion of CSR (Casellas *et al.*, 1998, Manis *et al.*, 1998, Soulas-Sprauel

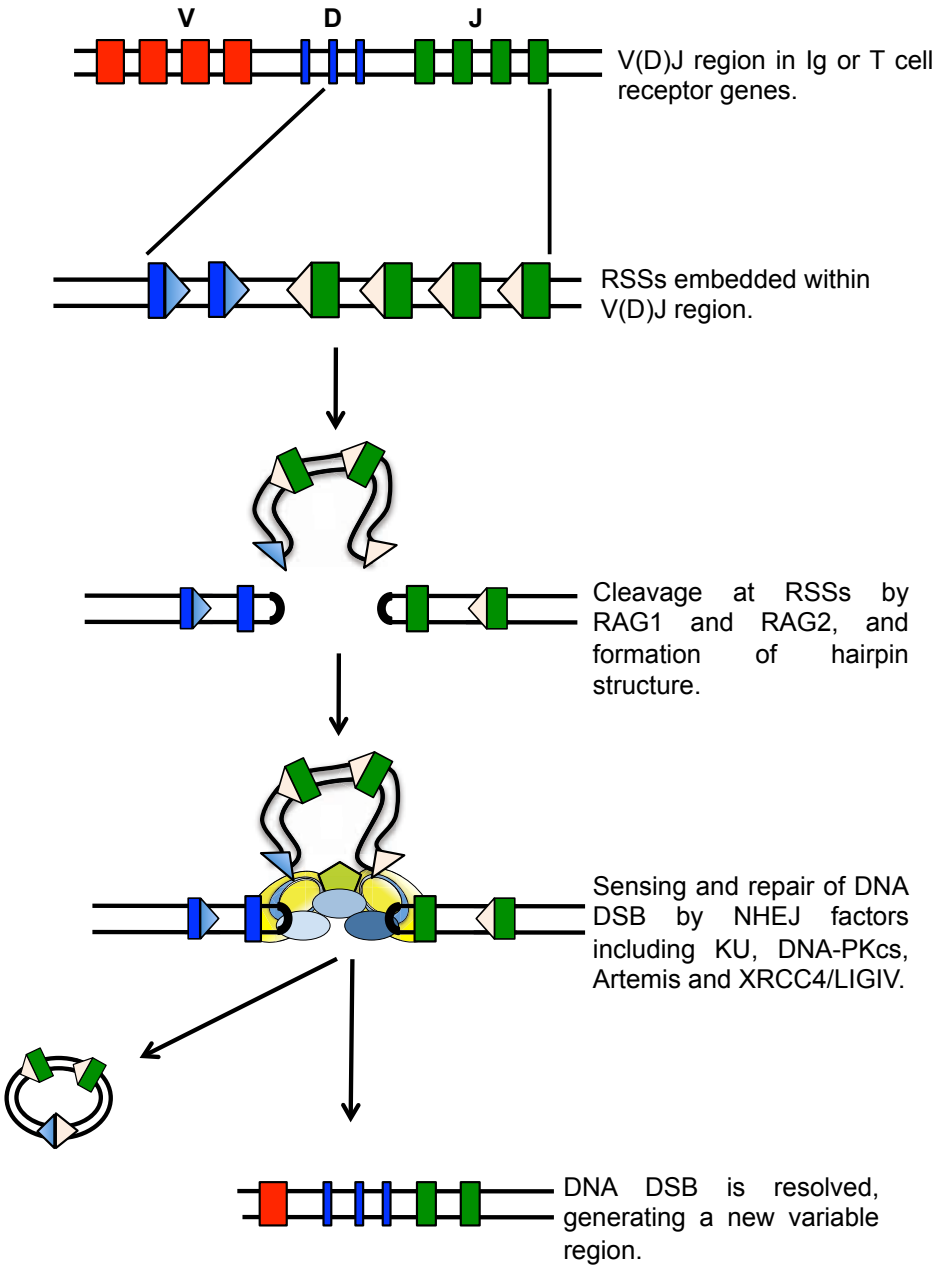


Figure 1.8 ***V(D)J Recombination.*** *V(D)J recombination occurs in developing lymphocytes. It is the process by which diversity in T-cell receptors and immunoglobulins (Ig) is achieved. Briefly DNA DSBs are created in the variable region of Ig or T-cell receptor genes, resulting in cleavage of a specific segment of DNA . The DNA DSBs is then processed and repaired using NHEJ proteins, creating a new variable region and therefore increasing antigen recognition (Adapted from Posey et al 2004).*

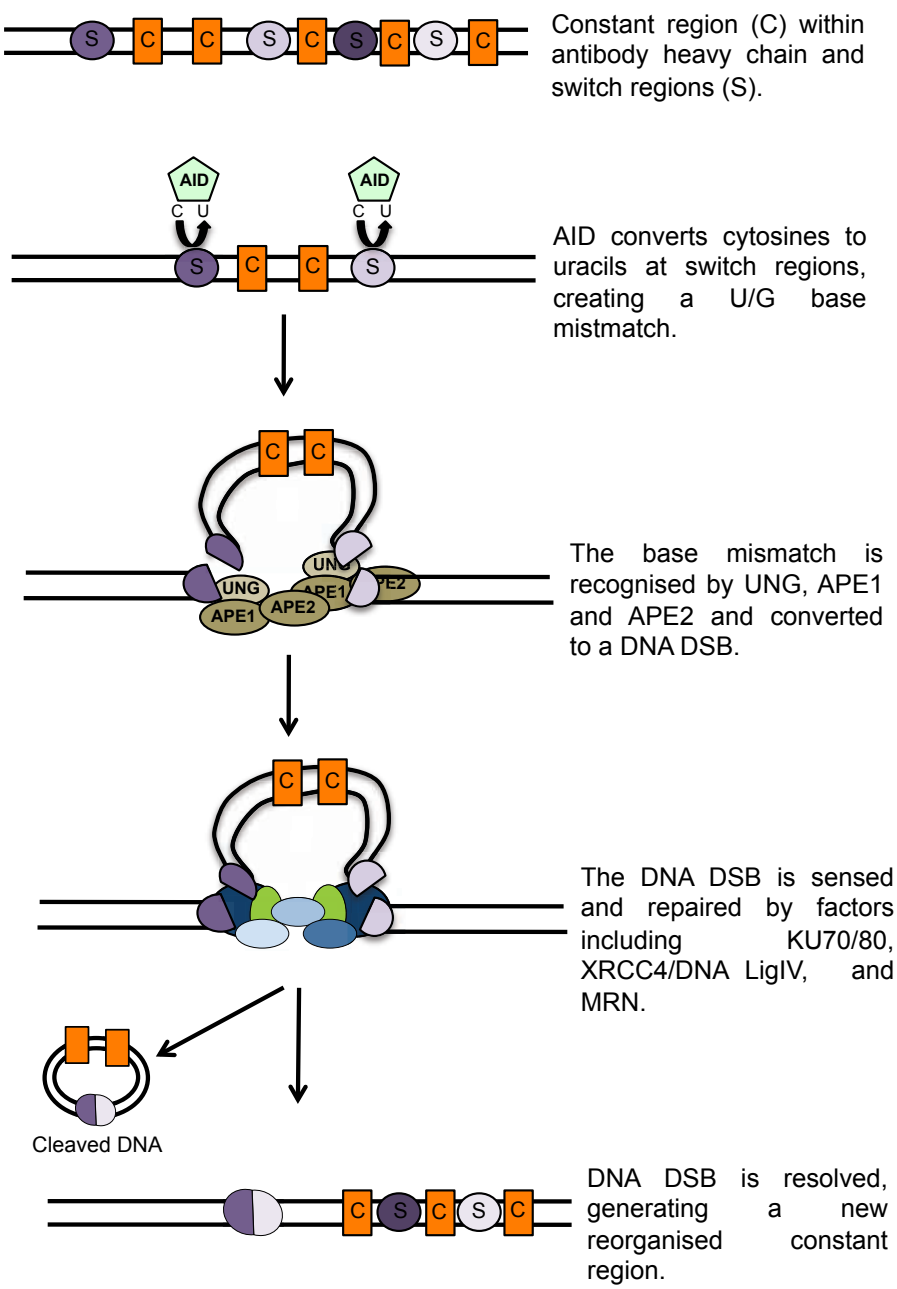


Figure 1.9 ***Class-switch Recombination.*** *CSR takes place both in and out of the germinal centres. It is required for antibody maturation by rearranging the constant regions within the antibody heavy chain. This is achieved by creating DNA DSBs at specific switch regions (S) embedded in the constant region (C). The DSB is then processed by NHEJ proteins in addition to other repair factors such as the MRN complex, resulting in rearrangement of the constant region and production of different antibody isotypes (Adapted from Posey et al 2004).*

et al., 2007a, Yan *et al.*, 2007a). Surprisingly, studies have revealed that in the absence of NHEJ factors, CSR is still active suggesting that alternative pathways may compensate (Boboila *et al.*, 2010a, Yan *et al.*, 2007a). Alt-EJ during CSR is thought to rely heavily on microhomologies, and the type of Alt-EJ required depends on which NHEJ factor is missing. In the absence of XRCC4/DNA ligase IV, KU is still activated and alternative ligases (either DNA ligase I or III) are used to join the break (Boboila *et al.*, 2010b). In this situation, resection of the DNA is a prerequisite prior to ligation, to reveal regions of homology, and possibly involves the nucleolytic activity of MRE11 (Dinkelmann *et al.*, 2009, Lahdesmaki *et al.*, 2004). Cells deficient in either KU70 or KU80 can still undergo CSR, and this scenario also relies on regions of microhomology.

The contribution of DDR proteins in the development of the immune system becomes more apparent when studying genetic syndromes that result from mutations in these proteins.

1.5 Human DSB repair deficiency syndromes

Intact DSB repair pathways and cell cycle checkpoints are integral in preventing genomic instability. The cellular and clinical features of syndromes caused by deficiencies in these pathways often overlap, including radiosensitivity, immunodeficiency and neurological phenotypes. Below describes a selection of diseases associated with mutations in DSB signalling and repair pathways.

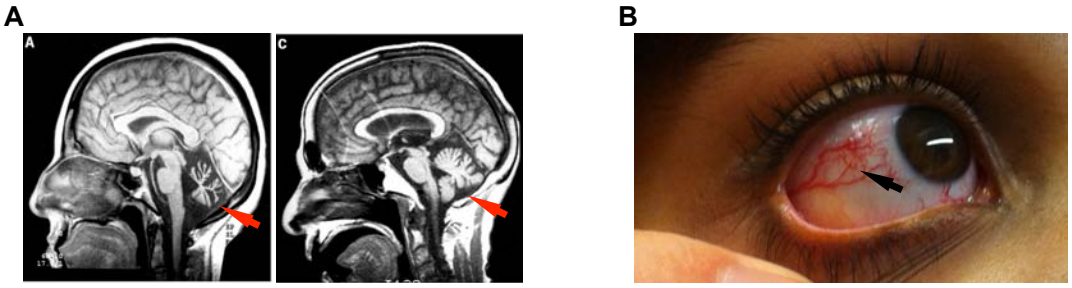
1.5.1 Ataxia-Telangiectasia

Ataxia-Telangiectasia (A-T) is an autosomal-recessive disease, with the biological cause of the disease pinned to mutations in the *ATM* gene (Savitsky *et al.*, 1995a, Savitsky *et al.*, 1995b). Typical clinical features are of early onset, and include progressive cerebellar cortical

degeneration, specifically from loss of Purkinje and granule cells, leading to ataxia (loss of motor control and balance) (Figure 1.10). In addition to neuropathologies patients also exhibit oculomotor apraxia (problems in eye coordination), dysarthria (impaired articulation-speech), ocular telangiectasia (dilated blood vessels), immunodeficiency and a predisposition to the development of cancers, including leukemia and breast (Figure 1.10) (Chun and Gatti, 2004, Frappart and McKinnon, 2006, Kerzendorfer and O'Driscoll, 2009, McKinnon, 2004, Stankovic *et al.*, 1998, Taylor and Byrd, 2005). Cellular features of A-T include increased radiosensitivity, presumed to occur due to defects in ATM-dependent DSB repair involved in a sub-set of breaks associated with heterochromatin and defects in cell cycle checkpoint activation (Figure 1.10) (Goodarzi *et al.*, 2008, Houldsworth and Lavin, 1980, Painter and Young, 1980). The vast majority of patient mutations in the *ATM* gene cause instability in the protein resulting in a complete absence of ATM (Becker-Catania *et al.*, 2000, Demuth *et al.*, 2011, Gilad *et al.*, 1998, Stankovic *et al.*, 1998, Verhagen *et al.*, 2012). The lack of expression of the ATM protein in these patients does not appear to be governed by the type of mutation or position in the gene. However, some mutations associated with a milder disease course have been documented to give rise to detectable levels of mutant protein expression that retains some kinase activity (Chun *et al.*, 2003, Frappart and McKinnon, 2006, Lakin *et al.*, 1996, Stewart *et al.*, 2001).

1.5.2 Syndromes associated with mutations in the MRN complex

Described formally in 1981 as an A-T variant, due to a number of overlapping features with A-T, Nijmegen Breakage Syndrome (NBS) is a result of mutations in the *NBN* gene (Shiloh, 1997, Varon *et al.*, 1998, Weemaes *et al.*, 1981, Weemaes *et al.*, 1993). Unlike the genetic causes of A-T, more than 90% of all NBS results from a single 5bp hypomorphic mutation,



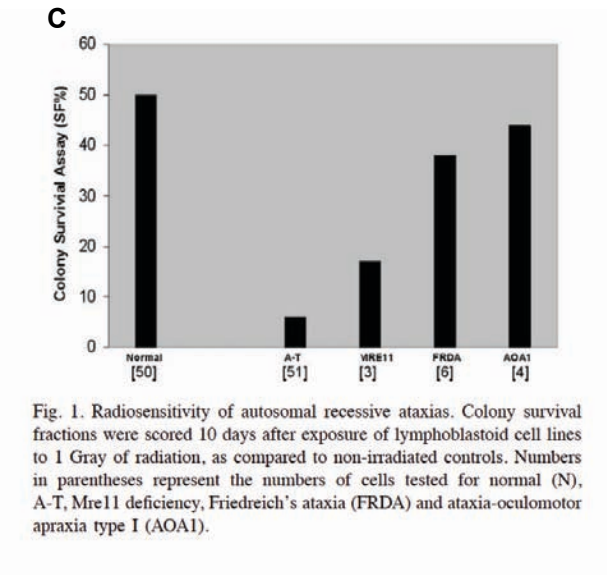


Fig. 1. Radiosensitivity of autosomal recessive ataxias. Colony survival fractions were scored 10 days after exposure of lymphoblastoid cell lines to 1 Gray of radiation, as compared to non-irradiated controls. Numbers in parentheses represent the numbers of cells tested for normal (N), A-T, Mre11 deficiency, Friedreich's ataxia (FRDA) and ataxia-oculomotor apraxia type I (AOA1).

Figure 1.10 ***Examples of clinical and cellular phenotypes associated with Ataxia-telangiectasia. (A) Neurodegeneration.*** *Reduced cerebellum (left panel) of an A-T patient, compared to an unaffected individual (right panel).* ***(B) Ocular telangiectasia.*** *Dilated blood vessels in the eye of an A-T patient.* ***(C) Radiosensitivity.*** *Colony survival assay of A-T (A-T) patient derived and normal lymphoblastoid cell lines (LCL), 10 days post 1 Gy of IR, compared to unirradiated control. Taken from Chun & Gatti 2004.*

termed 657del5 (Digweed and Sperling, 2004, Maser *et al.*, 2001). Carriers of the 657del5 mutation produce two truncated forms of NBS1. An N-terminal fragment encompassing the FHA and BRCT domains, p26 and a larger 70kDa protein named p70 incorporating the C-terminus of NBS1 (Figure 1.11) (Maser *et al.*, 2001).

As mentioned, NBS shares many cellular and clinical traits with A-T, including a hyper-sensitivity to ionising radiation, cell cycle checkpoint defects, immunodeficiency, both at the cellular and humoral level and cancer predisposition (Digweed and Sperling, 2004). The cancers associated with affected individuals are lymphomas. Unlike A-T patients, NBS patients do no suffer from progressive neurodegeneration (loss of neural tissue) and instead exhibit microcephaly, defined as a head circumference 3 SD (Standard deviations) below the norm, intellectual deficiency and delayed growth (Digweed and Sperling, 2004).

Even though ATM and NBS1 both function in the repair of DSBs and damage signalling, it is unclear why these two syndromes exhibit such different neurological pathology. A recent theory suggests the explanation may be linked to discrepancies in the activation of ATM-dependent apoptosis during neurological development, resulting in microcephaly and progressive cerebellar degeneration, in NBS and A-T patients respectively (Shull *et al.*, 2009). In A-T patients the inability of cells to undergo ATM-dependent apoptosis may cause an accumulation of un-repaired DSBs. Despite this, fairly normal brain development can occur. Eventually a threshold of unrepaired breaks is reached and the neurological cells begin apoptosing in an ATM-independent manner, leading to the cerebellar cortical degeneration associated with these patients. It is thought NBS patients however, can undergo ATM-dependent apoptosis, therefore NBS1 deficient cells harbouring elevated levels of unrepaired DNA damage are eliminated. The removal of these cells during early stage development of the brain reduces the overall number or neuronal cell populations, resulting in microcephaly.

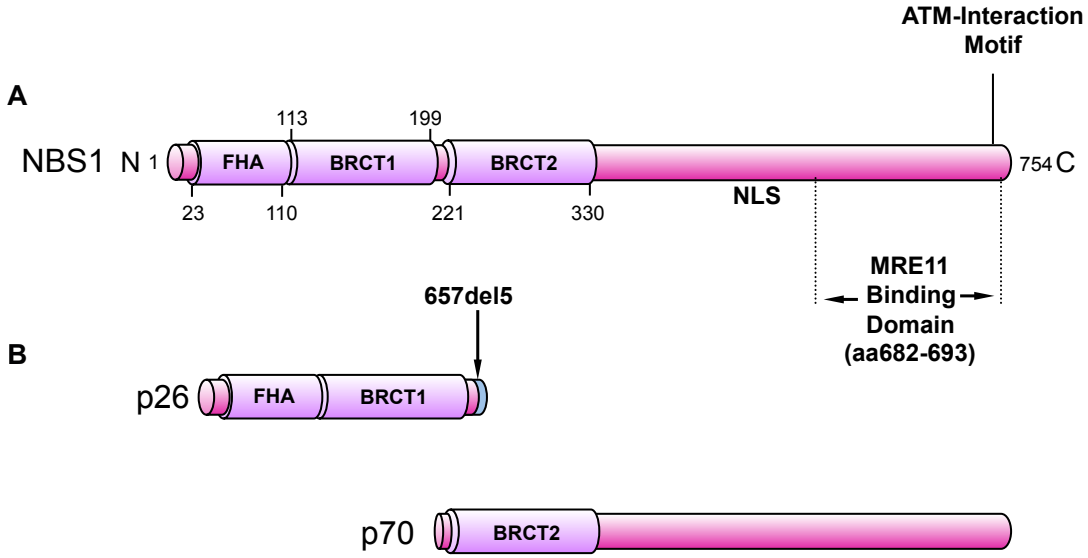


Figure 1.11 ***NBS1 protein fragments arising from the NBS 675del5 mutation. (A)*** *Schematic of the full length NBS1 protein. Highlighting functional domains (purple) with amino acid positioning.* ***(B)*** *Two NBS1 protein fragments (p26 & p70) produced as a result of the 657del5 mutation occurring in 90% of NBS patients.*

Whilst there is experimental evidence supporting this hypothesis, it is clear that other factors contribute to the type of neurological deficit exhibited by patients with either A-T or NBS. This is highlighted by the identification of two NBS patients with typical mutations in the *NBN* gene that exhibit severe microcephaly but no overt defects in DNA DSB repair (Seemanova *et al.*, 2006). Similarly, two classical A-T patients expressing no ATM protein were found to present with progressive ataxia as well as microcephaly and mental retardation (Curry *et al.*, 1989).

Like NBS, Ataxia-Telangiectasia-Like Disorder (A-TLD) also results from mutations in a member of the MRN complex. In this instance the syndrome arises from hypomorphic mutations in the *MRE11* gene (Stewart *et al.*, 1999). Apparent from its title, A-TLD presents with a similar clinical and cellular phenotypes to A-T, including occulomotor apraxia, dysarthria, although not as severe, with a later onset of ataxia and slower neurodegeneration, radiosensitivity and a failure to active both the intra-S and G2/checkpoints (Taylor *et al.*, 2004). Surprisingly, none of the identified patients display immunodeficiency. However, translocations are observed in chromosomes 4 and 17 which contain genes linked to the immune system (Frappart and McKinnon, 2006). To date only 9 A-TLD families have been documented, totaling 20 patients, indicating that this syndrome is relatively rare in comparison to both A-T and NBS (Reynolds and Stewart, 2013). Unlike A-T and NBS patients, a cancer predisposition phenotype in ATLD individuals is still inconclusive, which may be a reflection of the low incidence of this syndrome. Although, recently two A-TLD patients have been identified that developed lung adenocarcinoma, but whether this is due to the patients disorder is still unclear and to be determined (Uchisaka *et al.*, 2009).

Given the MRN complexes role in ATM activation it is not surprising mutations in *MRE11* present similar clinical phenotypes to A-T patients. However, the underlying cause why there

are disparities between the neurological conditions exhibited by patients with ATLD and NBS remains unclear. It could be inferred that the microcephaly exhibited by NBS patients is related to the restricted set of mutations in the *NBN* gene causing this disorder. Furthermore, since a complete loss of NBS1 is lethal, it is likely that the residual NBS1 protein expressed in patient derived cells is clearly sufficiently functional to prevent cerebellar degeneration. Interestingly, most ATLD patients express extremely low levels of Nbs1 protein, due to destabilising effect of *MRE11* mutations, and it has been reported that some patients do present microcephaly and mental retardation in addition to cerebellar ataxia, providing evidence for an overlap of symptoms (Fernet *et al.*, 2005, Uchisaka *et al.*, 2009). Conversely, NBS-like phenotypes, but with no cerebellar degeneration were reported in two unrelated individuals with mutations in *MRE11* (Matsumoto *et al.*, 2011). This implies that different mutations in ATLD patients can give rise to neurological abnormalities similar to that of A-T and/or NBS patients. Again, the clinical outcome of this variation maybe based on the type of mutation and its impact on ATM-dependent apoptosis in neurological tissue.

Lastly a single patient has been identified with a mutation in *RAD50*, the third component of the MRN complex, and this has been termed NBS-Like Disorder (NBS-LD) (Waltes *et al.*, 2009). As indicated from the name the clinical features exhibited by this individual are similar to those of NBS, as opposed to those seen in A-T and the majority of A-TLD patients. The mutation in *RAD50* gives rise to low levels of protein, which disrupts the interaction of RAD50 with NBS1 and MRE11. Interestingly, levels of MRE11 in RAD50-deficient cells are more stable than levels of NBS1, which may explain clinical similarity with NBS patients (Waltes *et al.*, 2009). The cellular features of NBS-LD include radiosensitivity, impaired ATM activation and signaling, and defects in cell cycle checkpoint activation (Waltes *et al.*, 2009). Despite its namesake the cellular features are not as severe as those seen in NBS-

derived patient cells. Similarly, this mirrors ATLD cells, which present milder cellular phenotypes compared to A-T cells. The varying traits exhibited by these three syndromes caused by mutations in such closely interacting proteins serves as a reminder to the many functions of the MRN complex in maintaining genomic instability.

1.5.3 Syndromes associated with mutations in RNF168

To date only two patients have been identified with pathological mutations in the E3 ubiquitin ligase *RNF168* gene (Devgan *et al.*, 2011, Stewart *et al.*, 2009, Stewart *et al.*, 2007). Disruption of RNF168, impacts on the ubiquitin-dependent arm of DNA DSB repair required for the accumulation of repair factors including 53BP1 and BRCA1. The first patient described (15-9BI) exhibited heterozygous mutations (c.397dupG & c.1323-1326 delACAA), that were predicted to encode for two truncated forms of the protein, named A133fsX and Q442fsX (Figure 1.12) (Stewart *et al.*, 2009, Stewart *et al.*, 2007). The second patient (RS66), haboured nonsense homozygous mutations, which ablated RNF168 protein production (Devgan *et al.*, 2011). Although both patients presented with growth retardation, radiosensitivy and immunodeficiency, patient RS66 also displayed microcephaly, ataxia and telangiectasia (Devgan *et al.*, 2011, Stewart *et al.*, 2009, Stewart *et al.*, 2007). The severity of clinical phenotypes observed in patient RS66, compared to patient 15-9BI, could be attributed to absence of the RNF168 protein in the former. In addition, whilst it is unknown whether deficiencies in RNF168 predispose to cancer, the father of patient 15-9BI developed chronic B-cell lymphocytic leukemia, that is also associated with A-T (Stewart *et al.*, 2009).

1.5.4 Seckel Syndrome

Seckel Syndrome (SCKL) is an inherited autosomal-recessive disorder, and was first described in 1960, by the German pediatrician Helmut Paul George Seckel (Seckel, 1960).

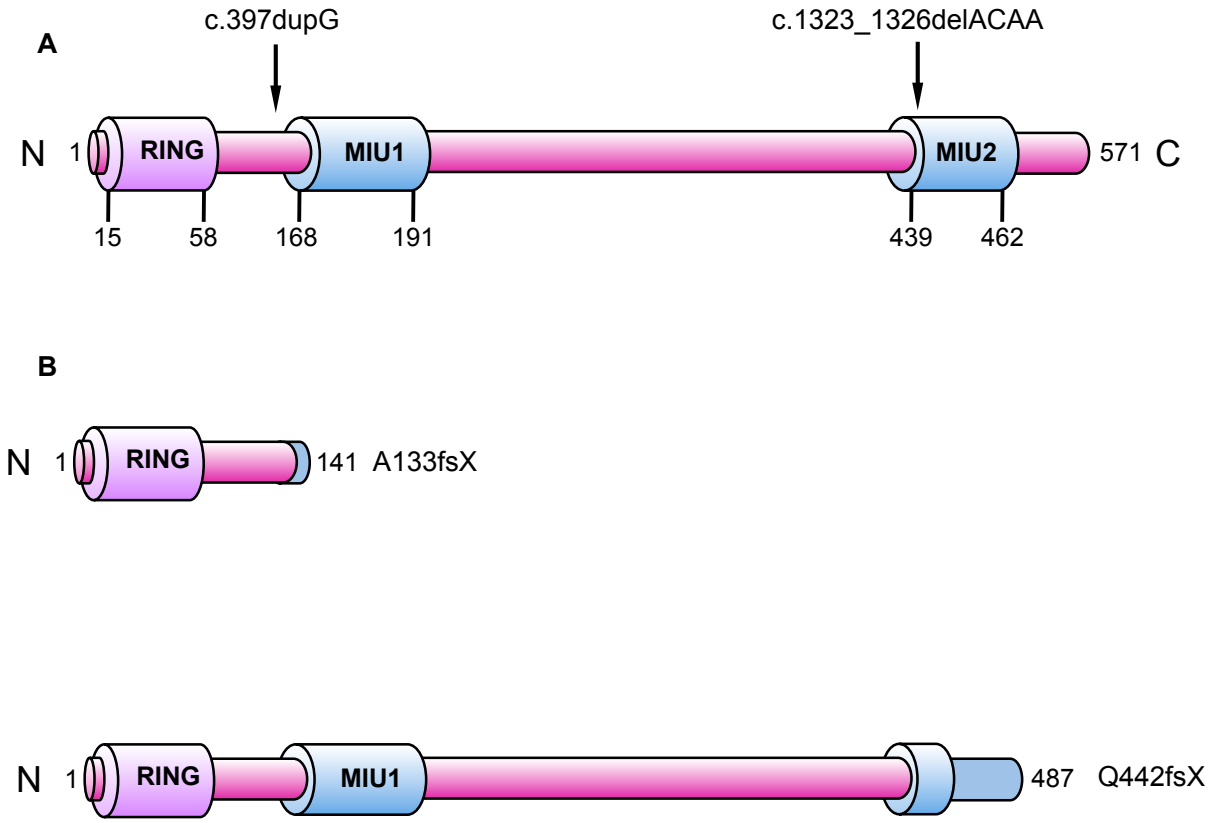


Figure 1.12 ***RNF168 mutations in patient 15-9BI.*** ***(A)*** *Schematic of the full length RNF168 protein. Functional domains are highlighted with corresponding amino acid position. Also highlighted are the heterozygous mutations identified in patient 15-9BI, c.397dupG and c.1323_1326delACAA.* ***(B)*** *Predicted proteins produced as a result of patient 15-9BI RNF168 mutations. (Adapted from Stewart et al 2009).*

Via genetic mapping, causation of the syndrome has been accounted to at least five genomic loci, termed SCKL1-5 with four genes identified (Borglum *et al.*, 2001, Goodship *et al.*, 2000, Griffith *et al.*, 2008, Kalay *et al.*, 2011, O'Driscoll *et al.*, 2003, Ogi *et al.*, 2012, Qvist *et al.*, 2011). Therefore, unlike the previous conditions it is a genetically heterogeneous disorder. Patients with SCKL syndrome clinically display severe intrauterine growth retardation, intellectual disabilities, microcephaly, skeletal abnormalities, including joint laxity and dislocation and characteristic facial dysmorphology, such as receding forehead and chin, and a pronounced nose (Majewski and Goecke, 1982). Some patients also have been reported to have lymphoma.

A single mutation in the *ATR* gene (A>G 2101), named ATR-Seckel syndrome (SCKL1), was identified as the first causal defect in two related families (O'Driscoll *et al.*, 2003). The mutation was shown to be hypomorphic and impact on ATR-dependent damage signalling (O'Driscoll *et al*., 2003). Recently, two unrelated UK Seckel patients have also been identified with the same heterozygous mutation in ATR (Ogi *et al.*, 2012). In addition to ATR, mutations have been found in other genes within the reported SCKL loci. For instance mutations in the centrosomal protein CEP152 have been found in several SCKL patients (Kalay *et al*., 2011). The recent identification of mutations in the *CTIP* gene as the underlying cause for a SCKL-like disorder provides the first molecular link between this disorder and DSB repair (Ogi *et al.*, 2012, Qvist *et al.*, 2011). One common characteristic between individuals with SCKL syndrome is defects in ATR-dependent damage signaling, suggesting that the genes mutated in these patients all participate within the same cellular pathway. Indeed the most recent gene mutation reported in a Seckel patient was discovered in the ATR interacting protein ATRIP, supporting this notion (Ogi *et al*., 2012). The clinical features of SKCL patients often overlap with other related disorders, such as Microcephalic Primordial

Dwarfism, primary microcephaly and radiosensitive SCID. To highlight this, mutations in another centrosomal protein, Pericentrin (*PCNT1*) have been identified in a large cohort of patients exhibiting clinical features consistent with SCKL syndrome (Griffith *et al*., 2008). The identification of further mutations in *PCNT1* in the primordial dwarfism microcephalic osteodyplastic primordial dwarfism type II (MOPDii) suggests Seckel syndrome and MOPDii appear to be sub-classifications of a clinical spectrum with an overlapping underlying pathobiology (Rauch *et al.*, 2008).

1.5.5 Ligase IV syndrome

DNA Ligase IV syndrome (LIG4 syndrome) arises from mutations in the gene coding for the NHEJ protein Ligase IV (O'Driscoll *et al.*, 2001). Clinically, subjects affected by this mutation share phenotypes with other DNA damage syndromes, such as NBS and Seckel syndrome. These include severe immunodeficiency, growth retardation, microcephaly, and developmental delay (Chistiakov *et al.*, 2009, Murray *et al.*, 2014, O'Driscoll *et al.*, 2001). Cells from these patients are extremely radiosensitive but show normal cell cycle checkpoint activation (O'Driscoll *et al.*, 2001). The radiosensitivity documented in these cells stems from defects in the NHEJ pathway, in which DNA ligase IV is required to ligate the DNA ends. Since, NHEJ represents a major pathway for repair of DSBs in mammalian cells, unsurprisingly loss of DNA ligase IV in knockout mouse models results in embryonic lethality (Frank *et al.*, 1998). In contrast, mouse models with a hypomorphic mutations in *LiGIV* recapitulate many of the features exhibited by LIG4 patients, such as a severe DSBR defect, abnormal brain development and immune system abnormalities caused by compromised V(D)J recombination (Nijnik *et al.*, 2007). As the NHEJ machinery functions in immune system development, LIG4 patients also present unusual V(D)J recombination phenotypes including reduced joining events (O'Driscoll *et al*., 2001). It has also been

suggested that LigIV has a role in lymphocyte proliferation that extends beyond V(D)J recombination, and this may explain why a number of patients display a high number of B- and T- cell tumors (Buck *et al.*, 2006a, Buck *et al.*, 2006b, Enders *et al.*, 2006, O'Driscoll *et al.*, 2001, Riballo *et al.*, 1999, Toita *et al.*, 2007).

1.5.6 RS-SCID (Associated with Artemis or XLF deficiency)

Patients diagnosed with severe combined immune deficiency (SCID), show marked defects in T and B cell production. A subset of these patients display radiosensitivity and are named radiosensitive-SCID (RS-SCID). The condition is autosomal recessive, and can be further divided again into Artemis-associated RS-SCID and XLF-associated RS-SCID, where presentation is a result of mutations in the *ARTEMIS* and *XLF* genes respectively (Ahnesorg *et al.*, 2006, Buck *et al.*, 2006b, Moshous *et al.*, 2001).

Mutations in the *ARTEMIS* gene give rise to radiosensitive T-B-SCID (absence of both T and B cells) (Dvorak and Cowan, 2010, Moshous *et al.*, 2001). Patients present with immunological defects due to the repair of RAG induced DSB being impaired, in support of the role that Artemis plays as part of the NHEJ machinery. However, it should be noted that unlike cells with hypomorphic *LIGIV* mutations, Artemis deficient cells display repair defects in a small percentage of IR-induced DSB comparable to those observed in A-T/NBS cells (Deckbar *et al.*, 2007, Kerzendorfer and O'Driscoll, 2009, Riballo *et al.*, 2004, Rooney *et al.*, 2003, Wang *et al.*, 2005b). A variety of different mutation types have been identified in Artemis-associated RS-SCID patients, ranging from exon deletions to truncations of the protein (Kobayashi *et al.*, 2003, Li *et al.*, 2002c). Potential hypomorphic missense mutations have also been found in some patients that interestingly have been noted to have an increased association with the formation of B cell lymphomas (Moshous *et al.*, 2003).

Unlike Artemis-associated RS-SCID, individuals with XLF-associated RS-SCID, as well as having NHEJ defects and thus problems with V(D)J recombination, also exhibit microcephaly, growth retardation, bone malformations and 'bird-like' facial features (Dvorak and Cowan, 2010). The presentation of microcephaly in these patients suggests a role for XLF in nervous system development, similar to other syndromes associated with the defective repair of DSBs e.g. LIGIV syndrome and NBS. What is clear for both Artemis/XLF-associated RS-SCID is that these are solely DNA repair disorders as cell cycle checkpoints are functional in both (Deckbar *et al.*, 2007, Kerzendorfer and O'Driscoll, 2009).

1.6 Mediators of DNA repair

Mediator proteins involved in the signalling of DNA damage are downstream substrates of the main kinases, ATM and ATR. Upon recruitment and activation, mediators serve to attract repair factors to the lesion. In doing this they provide scaffolding for protein complexes localised at the site of DNA damage which are required for chromatin remodelling, protection of the area surrounding the lesion, amplification of the DDR, checkpoint activation and repair. To date a number of mediators have been identified that participate in the DDR.

MDC1, 53BP1, BRIT1 (BRCT-repeat inhibitor of hTERT expression 1)/MCPH1 (Microcephalin), PTIP, BRCA1 and NBS1 operate as mediators in response to ATM activation. Whereas, Claspin and TopBP1 respond to ATR activation (Harper and Elledge, 2007). All these mediators contain phospho-binding domains (either FHA domains and/or BRCT domains) that can interact with phosphorylated proteins. These domains act as binding platforms that can facilitate the assembly of phosphorylation-dependent DNA repair/checkpoint protein complexes (Bork *et al.*, 1997, Callebaut and Mornon, 1997, Durocher *et al.*, 1999, Mohammad and Yaffe, 2009, Polo and Jackson, 2011). FHA domains

tend to bind to proteins containing phospho-threonine residues, whereas in contrast BRCT domains target proteins with phosphorylated serine residues (Durocher *et al.*, 1999, Li *et al.*, 2002b, Liao *et al.*, 2000, Manke *et al.*, 2003, Mohammad and Yaffe, 2009). With the exception of BRCA1 that displays E3 ubiquitin ligase activity, these proteins have no known enzymatic activity and are thus thought to function as molecular scaffolds ((FitzGerald *et al.*, 2009, Li and Zou, 2005, Lukas and Bartek, 2004)

1.6.1 Claspin

Claspin was originally identified as a CHK1 interacting protein in *Xenopus* egg extracts (Kumagai and Dunphy, 2000). This protein of 1332 amino acids is involved in the ATR-dependent intracellular signalling in response to DNA damage or replication stress. It is required for the effective phosphorylation of CHK1 by ATR via an association with CHK1 (Chini and Chen, 2003, Kumagai and Dunphy, 2000). It has been shown that casein kinase 1 gamma 1 (CK1γ1) phosphorylates Claspin within its C-terminal CHK1-activating domain (CKAD) that facilitates both its binding to and the subsequent activation of CHK1 (Meng *et al.*, 2011). In addition to CK1γ1 phosphorylation, Claspin is also phosphorylated in response to DNA damage on threonine 916 and serines 945/982 and these damage-dependent phosphorylations are also required for CHK1 interaction and activation (Chini and Chen, 2006, Clarke and Clarke, 2005). The kinase responsible for this phosphorylation has not been identified but it has been suggested that it may be CHK1 itself, therefore producing a positive regulatory feedback loop (Chini and Chen, 2006). Claspin has also been found to associate with replication forks in undamaged cells, suggesting a potential function in monitoring both normal replication progression as well as checkpoint control (Lee *et al.*, 2005a, Petermann *et al.*, 2008, Scorah and McGowan, 2009). In addition to its contribution to checkpoint

activation in the presence of DNA damage, the participation of Claspin in DNA replication through interactions with the replication machinery in a DNA damage-independent manner, may also contribute to its role in promoting genetic integrity (Sar *et al.*, 2004, Scorah and McGowan, 2009, Sercin and Kemp, 2011). It has been previously shown that the N-terminus of Claspin is important for its interaction with the replication MCM helicase co-factor Cdc45, in contrast to its CKAD domain being located at its C-terminus suggesting that its involvement in the regulation of perturbed and unperturbed replication are separable (Chini and Chen, 2006, Clarke and Clarke, 2005, Jeong *et al.*, 2003, Kumagai and Dunphy, 2003).

1.6.2 TopBP1

Similar to Claspin, TopBP1 also participates in regulating CHK1 activity during the ATR-dependent response to DNA replication stress. However, unlike Claspin, TopBP1 is required to promote the activation of ATR directly and as a consequence, the phosphorylation of most downstream targets, rather than being specific to CHK1 (Delacroix *et al.*, 2007, Greer *et al.*, 2003, Lee *et al.*, 2007, Mordes *et al.*, 2008). TopBP1 was first identified in a two-hybrid screen as a binding partner of topoisomerase IIβ (Garcia *et al.*, 2005, Kumagai *et al.*, 2006, Yamane *et al.*, 1997). This 1522 amino acid protein contains eight BRCT domains and has roles in both replication initiation and checkpoint signalling (Garcia *et al*., 2005). Upon replication stress, which activates ATR-dependent responses, TopBP1 is recruited by the phosphorylated C-terminus of RAD9, a member of the 9-1-1 complex (Delacroix *et al.*, 2007, Greer *et al.*, 2003, Lee *et al.*, 2007, Mordes *et al.*, 2008). It should be noted that the recruitment of TopBP1 by RAD9 is independent of ATR/ATRIP (Kondo *et al.*, 2001, Melo *et al.*, 2001). However, TopBP1 also interacts directly with ATRIP, and this is required for its ability to stimulate ATR activation, since disruption of this interaction results in an inability of cells to recover from replication stress or to activate cell cycle checkpoints (Mordes *et al.*,

2008). Importantly, it has been shown that a region between the sixth and seventh BRCT domain of TopPBP1, termed the ATR Activation Domain (AAD) is absolutely essential for the activation of ATR, although the underlying mechanism for how this is achieved in unclear (Kumagai *et al.*, 2006, Mordes *et al.*, 2008). Therefore, the activation of ATR by TopBP1 is critical for proper ATR signaling in response to replication stress. TopBP1 is phosphorylated in response to replication fork stalling agents or ionising radiation, but the biological relevance of these modifications has not been ascertained yet (Yamane *et al.*, 2003). In keeping with its role as a mediator protein, TopBP1 has a number of other binding partners including, CtIP, BRCA1-associated C-terminal helicase 1 (BACH1) and the MRN complex (Duursma *et al.*, 2013, Gong *et al.*, 2010, Leung *et al.*, 2010, Ramirez-Lugo *et al.*, 2011, Yoo *et al.*, 2009). The significance of these multiple interactions is not well understood but it could influence the regulation of TopBP1-ATR signalling, depending on the type of genotoxic stress (Nam and Cortez, 2011).

1.6.3 53BP1

Originally identified as a p53 interacting protein in a yeast two-hybrid screen, 53BP1 has been characterised as an integral part of the DNA damage response to DSBs (Iwabuchi *et al.*, 1994). 53BP1 has several well-defined domains that are specific to its various functions. Similar to the classical mediator proteins, 53BP1 has C-terminal tandem BRCT domains. However, in addition it also possesses a tandem Tudor domain, which is necessary for interaction with methylated histones and a GAR (glycine/arginine-rich region) domain, possibly required for DNA binding (Alpha-Bazin *et al.*, 2005, Boisvert *et al.*, 2005c, Bork *et al.*, 1997, Charier *et al.*, 2004, Huyen *et al.*, 2004). Upstream of these domains lie a number of PIKKs consensus motifs, which are phosphorylated in a PIKK-dependent manner in response to IR (Jowsey *et al.*, 2007, Lee *et al.*, 2009, Lee *et al.*, 2010). The characterisation of these

phosphorylations is still not understood, however, work has shed light on the phosphorylation of serine 1219 which was found to be involved in DNA damage signaling and activation of the G2/M checkpoint following IR (Lee *et al.*, 2009). In addition phosphorylation of 53BP1 on serine 25 by ATM is required for an interaction with Pax2 transactivation domain-interacting protein (PTIP), and disruption of this interaction resulted in hypersensitivity to IR (Munoz *et al.*, 2007). Furthermore it has been shown RIF1, which promotes NHEJ, recruitment is also dependent on phosphorylated 53BP1 (Chapman *et al.*, 2013a, Di Virgilio *et al.*, 2013, Escribano-Diaz *et al.*, 2013, Feng *et al.*, 2013, Zimmermann *et al.*, 2013). Recent evidence has suggested the interaction of PTIP or RIF1 to distinct phosphorylated residues on 53BP1 occurs independently and serves as a mechanism by which either mutagenic DNA repair is suppressed or productive CSR is promoted (Callen *et al.*, 2013).

The ubiquitylation of H2A-type histones in response to DNA damage, is thought to promote relaxation of the chromatin structure allowing exposure of otherwise hidden epigenetic marks e.g. histone H3 methylated on lysine-79 or histone H4 methylated on lysine-20. Exposure of these methyl groups allows 53BP1 to bind to the chromatin surrounding the break. This is achieved through its Tudor domains, which were originally proposed to bind di-methylated K79 of histone H3. However, this has widely been disputed and rather it is thought that its Tudor domains preferentially bind to di-methylated K20 of histone H4 (Botuyan *et al.*, 2006, Huyen *et al.*, 2004). The recruitment of 53BP1 to damaged chromatin is also dependent on additional factors. For instance the histone acetyltransferase (HAT) activity of Tip60, that facilitates acetylation of histones possibly opening the chromatin further, is necessary for 53BP1 foci formation (Murr *et al.*, 2006). Moreover, 53BP1 has been reported to interact directly with RNF168 ubiquitylated H2A on K15 via its C-terminal ubiquitination-dependent recruitment (UDR) motif (Fradet-Turcotte *et al.*, 2013). The above examples suggest the

recruitment of 53BP1 to DNA DSBs is highly dependent on histone modifications instigated by damage signalling pathways. Once bound, 53BP1 serves to amplify ATM signalling and assist in the repair of the break. Interestingly, despite exhibiting defective repair of DSBs, cells lacking 53BP1 show only a mild ATM signalling deficits (Shibata *et al.*, 2010). This suggests that the role of 53BP1 in signal amplification maybe dispensable compared to its role in the repair of DSBs. Indeed, work has shown that following exposure to high doses of IR, ATM activity, substrate phosphorylation and cell cycle checkpoints are effectively normal in 53BP1 deficient cells (Noon and Goodarzi, 2011, Shibata *et al.*, 2010). However, accumulation of the MRN complex at later stages of the DDR signalling is reliant on 53BP1. In particular, 53BP1 contributes to this accumulation by interacting with Rad50 via its BRCT domains and this then allows further phosphorylated KAP1-dependent remodelling of heterochromatin proximal to the break (Lee *et al.*, 2010, Noon and Goodarzi, 2011, Noon *et al.*, 2010).

In summary, 53BP1 seems to regulate a number of pathways that promote genomic integrity. This is achieved through multiple roles including the recruitment of protein complexes, protection of DNA ends from re-section and a degree of chromatin remodelling to render the local environment surrounding the damage favourable for repair.

1.6.4 BRCA1

Of all the mediators of DNA damage, BRCA1 is perhaps the best studied, which is probably due to the fact that germline heterozygous mutations in the *BRCA1* gene account for a significant proportion of hereditary breast and ovarian cancers (Brody and Biesecker, 1998, Futreal *et al.*, 1994). Since the mapping and cloning of the BRCA1 protein 20 years ago, several hundred mutations have been identified, with the most common of these heavily

investigated with regards to breast and ovarian cancer predisposition (Caestecker and Van de Walle, 2013, Hall *et al.*, 1990, Miki *et al.*, 1994). BRCA1 plays a role in a number of cellular functions involving DNA DSBs including repair of the lesion and cell cycle checkpoint activation.

The varied roles of BRCA1 in the cell may be attributed to the number of interacting proteins that bind to its different domains. The N-terminus of BRCA1 houses a really interesting new gene (RING) domain and a nuclear export signal (NES), whereas the C-terminus contains two BRCT domains (Rodriguez and Henderson, 2000, Wu *et al.*, 1996). Other domains found between the N and C-terminus includes two nuclear localisation signals (NLS) and an S/TQ cluster domain (Koonin *et al.*, 1996, Thakur *et al.*, 1997). In addition to providing an interaction platform for a several protein complexes, BRCA1 functions with its binding partner BARD1 as a heterodimeric E3 ubiquitin ligase. Despite this, the identity of the *in vivo* targets for this activity has been a matter of considerable debate. To date, histone H2A and CtIP have been proposed as being substrates for the E3 ubiquitin ligase activity of the BRCA1/BARD1 complex, although the experimental evidence supporting these claims have been disputed (Yu *et al.*, 2006, Zhu *et al.*, 2011). The interaction between BRCA1 and BARD1 is mediated via the BRCA1 RING domain (Meza *et al.*, 1999). The formation of the BRCA1/BARD1 heterodimer acts to stabilise both proteins, so it could be suggested that the interaction is necessary for BRCA1, and by association BARD1, to carry out cellular functions (Joukov *et al.*, 2001). BARD1 enhances BRCA1 ubiquitin ligase activity once bound, and clinically the importance of this association as well as that of the BRCA1 ligase activity can be deduced from the observation that mutations in the BRCA1 RING domain are found frequently in patients with a predisposition for developing breast/ovarian cancer (Hashizume *et al.*, 2001, Xia *et al.*, 2003). Interestingly, BRCA1 has been found to be a part

of at least three different complexes, the assembly of which depends on the cellular response to the type of DNA damage present in the cell (Deng and Brodie, 2000, Greenberg *et al.*, 2006, Huen *et al.*, 2010, Wang *et al.*, 2000). BARD1 is present in each of these complexes, with the variation coming from distinct sub-units. The BRCA1-A complex refers to BRCA1 when in association with RAP80, Abraxas, BRCC4, BRCC5 and MERIT40 (Wang and Elledge, 2007, Wang *et al.*, 2007). Abraxas phosphorylation on serine 406 facilitates a specific interaction with the BRCT domains of BRCA1 (Kim *et al.*, 2007b, Liu *et al.*, 2007, Wang *et al.*, 2007). Abraxas then acts as a bridge between BRCA1 and RAP80, which acts to bring BRCA1 to the sites of damage (Huen *et al.*, 2010). The localisation of the BRCA1-A complex also relies on RNF8 and RNF168, whose E3 ligase activity promotes the creation of poly-ubiquitin chains on histones proximal to the DSB. It is thought that RAP80, along with the other members of the BRCA1-A complex have binding affinity for ubiquitin chains and, it this which helps to secure BRCA1 at the lesion (Kim *et al.*, 2007a, Sobhian *et al.*, 2007, van Attikum and Gasser, 2009, Wang and Elledge, 2007, Wang *et al.*, 2007, Yan *et al.*, 2007b). Once located at the break, one function of the BRCA1-A complex is to promote activation of G2/M checkpoint (Huen *et al*., 2010). This activity of BRCA1 is known to be partly through its ability to regulate Chk1 phosphorylation (Yarden *et al.*, 2002). However, it is clear that since cells deficient in other subunits of the BRCA1-A complex, such as RAP80 and Abraxas, only have partial defects in G2/M checkpoint activation, recruitment of BRCA1 to sites of DNA damage foci is not an absolute requirement for the checkpoint to be initiated and that perhaps its involvement in other damage responsive protein complexes also play a role in this (Huen *et al*., 2010).

The second complex, BRCA1-B (Wang and Elledge, 2007), contains BRCA1-interacting protein C-terminal helicase 1 (BACH1, also known as FANCJ or BRIP1) and TopBP1

(Cantor *et al.*, 2001, Greenberg *et al.*, 2006). Evidence has pointed to the involvement of the BRCA1-B complex in activation of the intra-S-phase checkpoint following the induction of DNA damage (Huen *et al.*, 2010, Xu *et al.*, 2001, Xu *et al.*, 2002b). In response to IR, BRCA1 has been demonstrated to be phosphorylated on multiple sites by ATM and this is indispensable for the intra-S-phase checkpoint. Although, it is likely that its phosphorylation on the same residues by ATR following the induction of damage by agents that perturb DNA synthesis is involved in its ability to activate the replication checkpoint (Cortez *et al.*, 1999, Kumaraswamy and Shiekhattar, 2007, Xu *et al.*, 2001, Xu *et al.*, 2002b). In addition to this, BRCA1 and BACH1 have been reported to interact throughout S-phase and importantly the association of these proteins with TopBP1 upon replication stress may play a role in facilitating the efficient activation of ATR (Greenberg *et al.*, 2006, Yu and Chen, 2004).

Despite the identification of numerous key regulators of the intra-S phase and replication checkpoint, the mechanisms underlying how the cell is able to suppress DNA synthesis is relatively unclear. It is known that DNA damaging agents such as IR are capable of suppressing the ability of the cell to fire dormant origins of replication (Merrick *et al.*, 2004). Whilst it is known that the CHK1-dependent inhibition of CDK activity plays a major role in activation of this checkpoint, it is thought that preventing the loading of the replication initiation factor CDC45 onto origins also contributes to this suppression of new origins of replication firing (Falck *et al.*, 2002). TopBP1 and BACH1 have both been reported to regulate the loading of CDC45, the former regulating loading in the absence of damage and the latter preventing loading in the presence of damage (Greenberg *et al.*, 2006, Schmidt *et al.*, 2008). Given that TopBP1 interacts with BRCA1/BACH1 in a damage dependent manner, it could be postulated that following treatment with IR, the interaction between

TopBP1 and BRCA1/BACH1 not only promotes CHK1 activation but also prevents loading of CDC45 (Greenberg *et al*., 2006).

The last of the three complexes BRCA1 is thought to form in response to DNA damage is the BRCA1-C complex ((Wang and Elledge, 2007)Wang *et al* 2007a). Here, BRCA1 associates with the MRN and CtIP, and this complex is central to HR-mediated repair during the S/G2 phase of the cell cycle. The phosphorylation of CtIP, possibly by ATM and CDKs, promotes its interaction with BRCA1 as well as enhancing the binding of BRCA1 to the MRN complex (Chen *et al.*, 2008, Yu and Chen, 2004). It is then thought that BRCA1 acts as a scaffold keeping MRN/CtIP at the break where 5' DNA-end resection can take place. The BRCA1/CtIP interaction, although active during HR, is not essential for this process (Reczek *et al.*, 2013). One study suggested that CtIP ubiquitylation by BRCA1/BARD1 is required to promote its retention on the DNA. However, a recent study has revealed that it is the dimerisation of CtIP that is essential for proper HR repair (Wang *et al.*, 2012, Yu *et al.*, 2006). The formation of the BRCA1-C complex is also necessary for activation of the ATR/ATRIP-dependent G2/M checkpoint in response to DSB, as insufficient resection of the DNA will prevent the loading of RPA and the subsequent activation of ATR/ATRIP. In addition to promoting resection during HR, BRCA1 is also required for the recruitment of RAD51 to the processed DNA ends although it is unclear as to whether there is a direct interaction between these two proteins (Chen *et al.*, 1998).

1.6.5 MDC1

MDC1, also known as NFBD1 (Nuclear Factor with BRCT domains), was first isolated nearly twenty years ago during analysis of large cDNAs originating from a human myeloid cell cDNA library (Nagase *et al.*, 1996). Since then, MDC1 has been the subject of many

studies focused on assessing its contribution to the DDR (Goldberg *et al.*, 2003, Lou *et al.*, 2003a, Lou *et al.*, 2003b, Peng and Chen, 2003, Shang *et al.*, 2003, Stewart *et al.*, 2003, Xu and Stern, 2003). The demonstration that MDC1 plays a key regulatory role in coordinating the DDR is highlighted by the observations showing that loss of MDC1 results in an increased sensitivity to a variety of different DNA damaging agents, intra-S and G2/M phase checkpoint defects, as well as impaired recruitment of DNA repair factors to sites of damage (Stewart *et al*., 2003, Goldberg *et al*., 2003).

1.6.5.1 Structure

MDC1 is a large protein spanning 2089 amino acids in length comprising an N-terminal FHA domain, a large central PST repeat domain and tandem BRCT (tBRCT) domains located at its C-terminus (Figure 1.13). Early studies demonstrated that MDC1 lacking the tBRCT domains failed to form IRIF and moreover, cells expressing this mutant protein exhibited reduced γH2AX foci (Shang *et al*., 2003). Further biochemical and cellular studies confirmed that the tBRCT domains interact directly with the phosphorylated C-terminus of γH2AX and it has become widely accepted that this is the primary function of this domain (Lee *et al*., 2005, Stucki *et al*., 2005, Lou *et al*., 2006). However, more recent evidence suggests that the tBRCT of MDC1 also serves as a phospho-binding platform for CDC27, a subunit of the APC/C as well as DNA topoisomerase IIα (Topo IIα) (Coster *et al.*, 2007, Luo *et al.*, 2009, Townsend *et al.*, 2009). The tBRCT domains of MDC1 have also been reported to interact with the V(D)J recombination recombinase RAG1 in a phospho-dependent manner (Coster *et al.*, 2012). The N-terminus of MDC1 harbours an FHA domain. Early studies into the role of this domain and how it contributes to MDC1 function have proven to be inconclusive. Firstly, CHK2 was proposed to interact with this region, however, the two proteins do not seem to co-

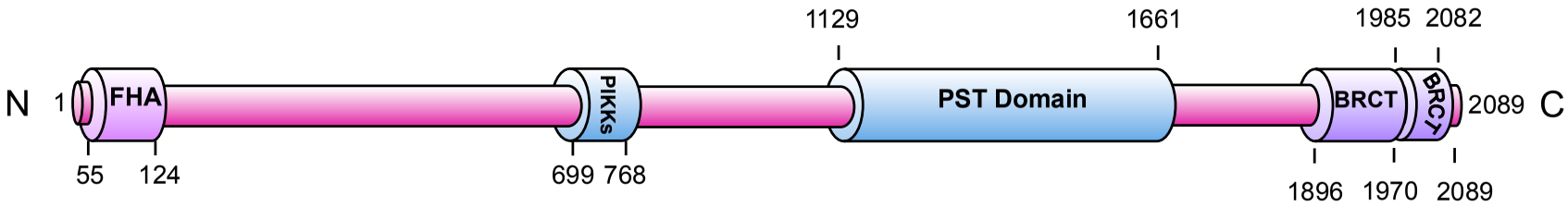


Figure 1.13 ***Schematic of human MDC1 showing known domains.*** *MDC1 is 2089 amino acids long. It possess a FHA domain at the N-terminus and a tandem BRCT domain at the C-terminus. Situated between these two phospho-peptide binding domains is a cluster of PIKKs residues and a unique PST repeat domain.*

localise in undamaged cells and more importantly, following exposure to DNA damaging agents, MDC1 forms IRIF but CHK2 remains dispersed in the nucleus (Lukas *et al.*, 2003).

The lack of co-localisation suggested that despite the fact that the two proteins have affinity for each other, the interaction may not be relevant within the cellular context or that if it does occur, it may be very transient (Jungmichel and Stucki, 2010). Another putative MDC1 FHA domain interacting factor has been suggested to be ATM (Lou *et al.*, 2006). The interaction was noticeably weak when ATM was incubated with the FHA domain of MDC1 *in vitro*, suggesting the interaction may not be direct (Lou *et al*., 2006). In addition to CHK2 and ATM, RAD51 has also been suggested to be a MDC1 FHA binding protein, albeit in a phospho-independent manner, and that this interaction is required for MDC1 to stimulate HR (Zhang *et al.*, 2005). Again though, due to conflicting evidence, this observation has not been substantiated (Bekker-Jensen *et al.*, 2006, Goldberg *et al.*, 2003). Recently, two studies have provided more convincing evidence that a major role for the FHA domain of MDC1 is to promote dimerisation. Both studies demonstrate the ATM-dependent phosphorylation of MDC1 on threonine-4 creates a binding site for the FHA domain of a different MDC1 molecule. It has been proposed that this damage-inducible dimerization of MDC1 is required to promote its efficient accumulation at sites of DNA damage. However, it is unclear to what impact loss of MDC1 dimerisation has on its ability to promote DSB repair or activate the intra-S and G2/M checkpoints (Jungmichel *et al.*, 2012, Liu *et al.*, 2012, Luo *et al.*, 2012).

Downstream of the FHA domain lies a group of PIKKs consensus sequences (Serine/Threonine-Glutamine). Subsets of these motifs have been demonstrated to be phosphorylated by ATM following IR (Kolas *et al*., 2007, Mailand *et al*., 2007). Phosphorylation of this cluster promotes the interaction with the FHA domain of RNF8, and this in turn facilitates RNF8 recruitment to damaged chromatin (Huen *et al*., 2007, Kolas *et*

al., 2007, Mailand *et al*., 2007). Disruption of this interaction negatively impacts on the DNA damage signalling cascade, which becomes evident from the loss of both BRCA1 and 53BP1 IRIF.

C-terminal to the PIKK motif clusters lies a proline-serine-threonine (PST)-rich repeat domain, the function of which has not yet been characterised. This region, consisting of 13 consecutive repeats, occupies almost a third of MDC1, and displays no known sequence homology with any other proteins (Jungmichel and Stucki 2010, Coster *et al*., 2012). The recruitment of both MDC1 and all downstream factors is independent of the PST repeat region (Lou *et al.*, 2004, Shang *et al.*, 2003, Xie *et al.*, 2007). Earlier work suggested that this region bound DNA-PK, and its deletion resulted in a partial NHEJ defect (Lou *et al*., 2004). However, this observation has not been reproduced by others. Moreover, it has been demonstrated that the recruitment of DNA-PK to sites of DNA damage occurs independently of MDC1 (Bekker-Jensen *et al.*, 2006, Gottlieb and Jackson, 1993). In addition, it was more recently shown that this PST repeat region is required to promote HR rather than NHEJ (Xie *et al*., 2007). Interestingly, this region is also important for mitotic progression in the absence of DNA damage as cells expressing a mutant form of MDC1 lacking this domain, displayed an increased accumulation of mitotic cells (Townsend *et al*., 2009).

1.6.5.2 Function

As discussed, MDC1 contains a number of structural and functional domains, which permit the protein to elicit its cellular activity. The importance of its biological function is highlighted from research that showed firstly, that loss of MDC1 in mice is coupled with increased tumour frequency, and secondly, that loss or reduction of MDC1 protein levels has been observed in various cancers (Bartkova *et al.*, 2007, Minter-Dykhouse *et al.*, 2008).

1.6.5.2.1 DNA double-strand break repair

The activation of γH2AX along regions of chromatin flanking the break initiates the signalling cascade necessary for repair. One way γH2AX achieves this is by binding directly to MDC1. The phosphorylated C-terminus of γH2AX contains the following binding sequence pS-X-X-Y/F-COOH (where X denotes any amino acid) (Stucki *et al*., 2005). A phospho-serine peptide library screen showed that the tBRCT domain of MDC1 had a strong selective affinity for this motif (Rodriguez *et al.*, 2003). In light of this, further investigation showed that the phosphorylated C-terminal tail of γH2AX provided a direct binding platform for the tBRCT of MDC1 (Lee *et al.*, 2005b, Lou *et al.*, 2006, Stucki *et al.*, 2005). Disruption of this interaction leads to abrogation of the recruitment of several DDR proteins into IRIF, such as phosphorylated ATM, MRN, 53BP1, BRCA1 and MDC1 itself, emphasising the importance of MDC1 for the re-localisation of a number of DSB repair proteins to DNA breaks (Lou *et al.*, 2006, Stucki and Jackson, 2006). Once bound, MDC1 creates a positive feedback loop propagating the damage signal along the chromatin by recruiting more phosphorylated ATM possibly via its FHA domain and thus, more γH2AX and subsequently more MDC1 and its interacting partners. In support of the requirement of MDC1 in signal propagation and amplification, is the observation that loss or disruption of MDC1 leads to reduced phosphorylation of H2AX and in the formation of smaller γH2AX foci (Lou *et al*., 2006, Stewart *et al*., 2003, Stucki *et al*., 2005). One explanation for the reduced H2AX phosphorylation observed in the absence of MDC1 could be attributed to the fact that MDC1 may be protecting γH2AX from phosphatases. Previous work showed that *in vitro*, a recombinant GST-fused tBRCT domain prevented phosphatases from dephosphorylating the C-terminus of γH2AX, and *in vivo*, overexpression of the tBRCT led to hyperphosphorylation of γH2AX (Stucki *et al*., 2005). In undamaged cells, H2AX is phosphorylated on tyrosine 142

located within a region that mediates binding to MDC1 tBRCT and this prevents the interaction with MDC1 (Cook *et al.*, 2009, Xiao *et al.*, 2009). Following exposure of cells to DNA damaging agents, this residue becomes dephosphorylated, allowing the association between H2AX and MDC1 and thus, providing another level of regulation of the DDR signalling cascade (Coster and Goldberg, 2010, Jungmichel and Stucki, 2010).

Once recruited to the break, MDC1 can then mediate the recruitment of its binding partners. Although the activation of ATM is not MDC1 dependent, accumulation of phosphorylated ATM appears to be. Cells depleted of MDC1 show a reduction in phosphorylated ATM recruitment and retention on the chromatin in response to DNA damage (Lou *et al*., 2006). The same study demonstrated that there is a direct interaction between ATM and the FHA domain of MDC1, and another study showed that this interaction was also dependent on the phosphorylation of ATM on serine 1981 (Lou *et al.*, 2006, So *et al.*, 2009). This phospho-dependent recruitment of ATM via MDC1 is one of the key components in the response to DSBs. The ability of MDC1 to retain phosphorylated ATM on the chromatin leads to an increase in phosphorylated H2AX, with further MDC1 re-localisation and therefore increased retention of phosphorylated-ATM.

In addition to binding phosphorylated ATM, MDC1 is also phosphorylated by the kinase in response to IR, on a cluster of PIKK motifs (Kolas *et al.*, 2007, Mailand *et al.*, 2007, Matsuoka *et al.*, 2007). It was shown that the phosphorylation of these sites on MDC1 facilitates the binding of the E3 ubiquitin RNF8, via a direct interaction of the FHA domain of RNF8 with the phosphorylated MDC1 motifs (Huen *et al*., 2007, Kolas *et al*., 2007, Mainland *et al*., 2007). The discovery of this interaction also revealed a function for MDC1 in promoting activation of the ubiquitin-dependent arm of DSB repair. Disruption of this interaction led to loss of 53BP1 and BRCA1 IRIF, whose localisation relies on the formation

of ubiquitin conjugates at the break region (Huen *et al*., 2007, Kolas *et al*., 2007, Mainland *et al*., 2007). MDC1 has also been shown to interact directly with 53BP1 (Eliezer *et al.*, 2009). Interestingly though, this interaction was seen to decrease following the generation of DSBs. It was suggested that a pool of MDC1 and 53BP1 interact in undamaged cells, and that following damage, the fraction of MDC1 bound to 53BP1 re-localises to the sites of DSBs where they disassociate and bind to different partners, MDC1 with γH2AX and 53BP1 with methylated histones (Eliezer *et al*., 2009).

In addition to its role in facilitating and amplifying DNA damage signalling at DSBs, MDC1 has also been implicated in the DSB repair process as its depletion by siRNA reduces the rates of both NHEJ and HR (Lou *et al.*, 2004, Zhang *et al.*, 2005). One possible way, by which MDC1 may influence NHEJ, is through interacting with DNA-PK_{CS}. DNA-PK was discovered as an MDC1 PST-repeat region binding protein, and it was suggested that this interaction was important for DNA-PK activation (Lou *et al*., 2004). The same study showed that NHEJ was defective in MDC1 depleted cells and that this defect was dependent on the PST region of MDC1. However, exactly how MDC1 influences DNA-PK is still unknown. Another indication that MDC1 has a role in NHEJ came from work examining the recruitment of DDR proteins at dysfunctional telomeres. Uncapped telomeres are recognised as DSBs and can be fused in a NHEJ dependent manner. Loss of MDC1 itself or of its ability to localise at telomeric regions resulted in a decrease in telomeric fusions (Dimitrova and de Lange, 2006).

A putative role of MDC1 in HR comes mainly from the work by Zhang, *et al* 2005 who showed direct interaction between MDC1 and RAD51, a protein essential for sister chromatid strand invasion during HR. Rather than regulating RAD51 localisation, MDC1 seems to regulate RAD51 stability based on the observation that upon MDC1 depletion, a marked

increase in RAD51 degradation is observed. Furthermore, under similar conditions RAD51 foci are also reduced (Goldberg *et al*., 2003, Zhang *et al*., 2005).

Following work that highlighted the contribution of ubiquitylation in DDR regulation, it was also revealed that MDC1 is targeted itself by ubiquitin and this was linked to its degradation (Zhang *et al.*, 2006). However, another study looking at the ubiquitylation of the tBRCT domain suggested this modification does not mark the protein for degradation (Strauss *et al.*, 2011). It was shown that the tBRCT undergoes ubiquitylation through K63 linkages, a modification primarily thought to regulate protein-protein interaction and localisation. Indeed, the ubiquitylation of MDC1 in this context fuels an interaction between MDC1 and the UIMs of RAP80. In particular, the interaction requires the poly-ubiquitylation of MDC1 on K1977, and the E2 conjugating enzyme UBC13 (Strauss and Goldberg, 2011). This study revealed that this modification of MDC1 was damage independent.

Ubiquitylation also regulates MDC1 turnover following completion of DNA repair. Earlier work indicated that the turnover of MDC1 foci depended on ubiquitin mediated degradation by the proteasome, as these foci persisted when the cells were treated with proteasomal inhibitors (Shi *et al.*, 2008). MDC1 has 103 lysines spanning its length and identifying which are important for its degradation remained elusive at the time of the above study. More recently, lysine 1840 of MDC1 has been reported to be sumoylated in response to DNA damage by PIAS4, and this modification was shown to target MDC1 for ubiquitylation and subsequent degradation by the E3 ubiquitin ligase RNF4 (Galanty *et al.*, 2012, Luo *et al.*, 2012, Yin *et al.*, 2012).

1.6.5.2.2 Cell cycle checkpoint control

MDC1 was suspected to have a role in cell cycle checkpoint control based mainly on the observation that proteins containing BRCT and FHA domains commonly participate in the regulation of this process (Bork *et al*., 1997). Indeed, cells depleted of MDC1 exhibit defective intra-S-phase and G2/M checkpoint activation following IR (Goldberg *et al.*, 2003, Lou *et al.*, 2003a, Lou *et al.*, 2003b, Stewart *et al.*, 2003). When activated, for example after IR, the intra-S-phase checkpoint acts to inhibit DNA synthesis. Two mechanisms exist to activate the intra-S-phase checkpoint in response to IR. The first involves CHK1/CHK2 phosphorylation by ATM and the other, NBS1 and SMC1 phosphorylation also by ATM (Lim *et al.*, 2000, Yazdi *et al.*, 2002). Cells harbouring a defect in the activation or maintenance of the intra-S-phase checkpoint display radioresistant DNA synthesis (RDS) (Falck *et al.*, 2001). Higher levels of RDS have been observed in cells treated with MDC1 siRNA, following IR, compared to controls, in several studies (Goldberg *et al.*, 2003, Lou *et al.*, 2003a, Lou *et al.*, 2003b, Stewart *et al.*, 2003). How MDC1 influences the intra-S-phase checkpoint is unclear as loss of MDC1 results in only a mild reduction in the phosphorylation of Chk1, which is required to initiate the checkpoint (Lou *et al.*, 2003b, Peng and Chen, 2003, Stewart *et al.*, 2003). A reduction in SMC1, but not NBS1, phosphorylation was also reported in cells depleted of MDC1 (Goldberg *et al*., 2003, Stewart *et al*., 2003). On the other hand, CDC25A degradation, which is also required for checkpoint activation remained unaffected (Goldberg *et al*., 2003). Therefore, the precise mechanism with which MDC1 influences the intra-S-phase checkpoint requires further investigation.

Similarly elusive is the mechanism by which MDC1 influences the G2/M checkpoint. An increase in the mitotic marker phospho-histone H3 was detected in MDC1 knockdown cells exposed to IR compared to controls (Stewart *et al*., 2003). This suggested that in the absence

of MDC1 and following IR, cells are unable to arrest the cell cycle at the G2/M transition. In support of this, a similar reduction upon MDC1 depletion was also observed in the levels of phosphorylated CHK1 on serine 345, another marker of the G2/M checkpoint activation (Stewart *et al*., 2003). Interestingly, a reduction in phospho-histone H3 was also observed in undamaged cells with low levels of MDC1, implying that MDC1 is also required for normal G2/M transition even in the absence of DNA damage (Bu *et al.*, 2010).

1.6.5.2.3 Mitosis

A major factor in the regulation of mitotic progression is the multi-subunit complex APC/C. In the absence of APC/C, cells are unable to separate sister chromatids in anaphase, and cannot exit from mitosis (Peters, 2006). Through its ubiquitin ligase activity, APC/C plays a pivotal role in the proteasome-mediated degradation of cell cycle regulators (Castro *et al.*, 2005, Peters, 2006, Wasch and Engelbert, 2005). MDC1 has been reported to interact with APC/C in a phosphorylation-dependent manner. In particular, the tBRCT domain of MDC1 interacts with the CDC27 subunit of the APC/C complex and this interaction is enhanced in response to IR (Coster *et al.*, 2007). Interestingly, the MDC1 binding region on CDC27 (APC3) is similar to that on γH2AX, and given that both interacting proteins associate with the MDC1 tBRCT domain, this could suggest that there may be some competition for binding. Alternatively, binding may occur in separate circumstances or there may be different MDC1 complexes in the cell (Coster and Goldberg, 2010, Coster *et al.*, 2007). A second study, expanded these observations, and showed that MDC1 also bound to CDC20, an activator protein of APC/C complex that is required for APC/C to control the metaphase-anaphase transition in mitosis (Townsend *et al*., 2009). The study of Townsend, *et al* 2009, showed that depletion of MDC1 by siRNA resulted in a mitotic block at metaphase, further

indicating that MDC1 has a role in mitosis in undamaged cells (Townsend *et al.,* 2009). From these observations it is likely that MDC1 has separable roles within the cell, one which is involved in controlling the DDR and the other is crucial for regulating the cell cycle in the absence of genotoxic stress.

1.6.5.3 Additional functions

Clearly MDC1 has been best characterised with respect to its function in promoting DNA repair and cell cycle checkpoint activation. Yet, a number of studies point towards the contribution of MDC1 in other cellular pathways.

Studies have suggested that MDC1 has anti-apoptotic potential, and one putative avenue through which this is achieved is via intervention of p53-dependent apoptosis. In one report, MDC1 was seen to protect cells from apoptotic death by blocking p53 activity (Nakanishi *et al.*, 2007). Upon DNA damage caused by ionising radiation p53 is phosphorylated on serine 15 by ATM, which results in p53 protein stabilisation and upregulation as well as an increase in its transcriptional activity that targets pro-apoptotic genes (Banin *et al.*, 1998, Canman *et al.*, 1998). Over expression of MDC1 was seen to reduce the damage-dependent phosphorylation of p53 on serine 15, suggesting an inhibitory role of the p53 stabilisation and upregulation (Nakanishi *et al.*, 2007). This inhibition was concluded to occur through the binding of p53 to the MDC1 tBRCT domain. Furthermore, a second study reported that MDC1 interacts with the p53 mediator Mouse double minute 2 homolog (MDM2) and increases its stability (Inoue *et al.*, 2008). MDM2 is an E3 ubiquitin ligase as well as a transcriptional target of p53, which once upregulated, targets p53 for poly-ubiquitylation and proteosomal degradation. Western blotting analysis revealed that the levels of both MDM2 and MDC1 decrease at later stages of the DDR signalling and that the two proteins interact *in vitro*. The tBRCT domain of MDC1 also mediates this interaction. The fact that both p53 and

MDM2 bind to the tBRCT of MDC1 suggests that MDC1 could be a platform for the MDM2-p53 interaction (Inoue *et al.*, 2008, Nakanishi *et al.*, 2007).

Previous work has identified a region of MDC1 that activated transcription of a chloramphenicol acetyl transferase reporter (Ozaki *et al.*, 2000). This led to the discovery of a number of genes that were down-regulated in MDC1 knockdown cells in the absence of DNA damage, and surprisingly, these were not DDR proteins (Wilson *et al.*, 2011). In addition, and due to MDC1 potentially regulating p53 activity, the same experiment was carried out in p53/MDC1 double knockdown cells, and similar results were achieved suggesting that the regulation was also p53 independent (Wilson *et al*., 2011). How MDC1 regulates the expression of these genes remains elusive.

Over the last decade MDC1 has been classified as a typical mediator of DNA damage signaling in the cell. The numerous domains spanning the length of the protein serve as protein interaction platforms, which are normally phosphorylation-dependent. The majority of studies assessing MDC1 function have focused on its role in the mammalian DNA damage response, where it serves as a molecular bridge recruiting repair proteins to the sites of damage, ensuring an intact cellular damage response. The importance of the role of MDC1 in the DDR is underlined by the defects in cell cycle checkpoint activation and lack of proper damage signalling observed upon reduction of its protein levels. While the mechanisms with which MDC1 recruits various repair factors to DNA breaks has been thoroughly studied, the role of MDC1 in undamaged cells remains unclear. To further complicate matters, a fraction of protein-protein interacting domains on MDC1 have multiple binding partners and these events can be either damage-dependent or independent. The ability of these domains to bind several different partners suggest that MDC1 exists in a number of diverse complexes present in damaged or undamaged cells that are capable of carrying out distinct tasks.

1.6.6 The MRN complex

Described as a sensor of DNA damage, the MRN complex can also be grouped with the mediator proteins, as one of its subunits, Nbs1, has protein-protein interaction domains that recruit repair factors to the sites of breaks. The diseases resulting from mutations in members of the complex highlight the significance of the MRN complex functions within the DDR. Mutations in *MRE11, NBS1* or *RAD50* cause the genetic syndromes ATLD, NBS and NBS-LD respectively, and have been discussed earlier in the text.

The MRN complex functions at the core of the cellular response to DSBs, and plays pivotal roles in sensing the lesion, initiating cell-cycle checkpoint activation and DNA damage signalling, as well as promoting or in the case of HR initiating DNA repair. In addition, the MRN complex is also required during telomere maintenance, DNA replication and meiosis. The complex was identified from genetic screens in *S. cerevisiae* looking for mutations in genes that resulted in sensitivity to DNA damaging agents or resulted in meiotic deficiencies, and as such were named: *mre11* (meiotic recombination 1), *rad50* (radiation sensitive 50) and *xrs2* (X-ray sensitive 2) (MRX) complex (Ajimura *et al.*, 1993, Game and Mortimer, 1974, Ivanov *et al.*, 1992). *MRE11* and *RAD50* are highly conserved and have homologues in a number of organisms, whereas *NBS1* is the orthologue of *xrs2* and seems to be unique to higher eukaryotes. The products of these genes were shown to form a complex in both yeast and mammals and displayed epistatic properties (Dolganov *et al.*, 1996, Ogawa *et al.*, 1995, Trujillo *et al.*, 1998, Usui *et al.*, 1998). It is the individual properties of each subunit that provide the opportunity for the complex to carry out numerous functions within the DDR and beyond. The association of the MRN components and formation of the complex is extremely important for its stabilisation and function. For instance, mutations in *MRE11* or siRNA

mediated *MRE11* knockdowns result in a reduction in the levels of both RAD50 and NBS1 (Stewart *et al.*, 1999, Zhong *et al.*, 2005).

1.6.6.1 MRE11

The structure of MRE11 is composed of an N-terminal phosphoesterase domain, within which lies the NBS1 interaction domain, and two C-terminal DNA-binding domains. MRE11 also interacts with RAD50, and this binding domain resides in between the two DNA-binding domains (Figure 1.14). MRE11 stably dimerises, and this dimerisation is required for MRN complex function. MRE11 specific functions include DNA binding, DNA-end synapsing and endo/exonuclease activity on various DNA substrates (de Jager *et al.*, 2001, Paull and Gellert, 1998, Williams *et al.*, 2008). Observations *in vitro* have shown MRE11 to possess ssDNA endonuclease activity, 3'-5' ssDNA exonuclease, dsDNA exonuclease and hairpin opening activity (Furuse *et al.*, 1998, Paull and Gellert, 1998, Trujillo and Sung, 2001, Trujillo *et al.*, 1998, Usui *et al.*, 1998). The nuclease activity of MRE11 is required to process DNA ends, leading the way for repair in a variety of pathways. In total, five motifs located in the N-terminal region of MRE11 are required for the nuclease activity (Moreau *et al.*, 1999, Williams *et al.*, 2008). From work in *S. pombe*, two specific residues were identified that could separate the exonuclease and endonuclease activity of MRE11 (Williams *et al*., 2008). Mutating histidine-68 resulted in a loss of its 3'-5' exonuclease activity whereas mutation of histidine-134 abrogated both its 3'-5' exonuclease activity and endonuclease activities (Williams *et al*., 2008). Disruption of the MRE11 nuclease activity had no effect on MRN checkpoint signaling, suggesting they act independently of each other. Despite this, the nuclease activity of MRE11 is essential for viability and DNA repair functions (Buis *et al.*, 2008). The nuclease activity of MRE11 and its DNA binding capabilities are, however, reliant

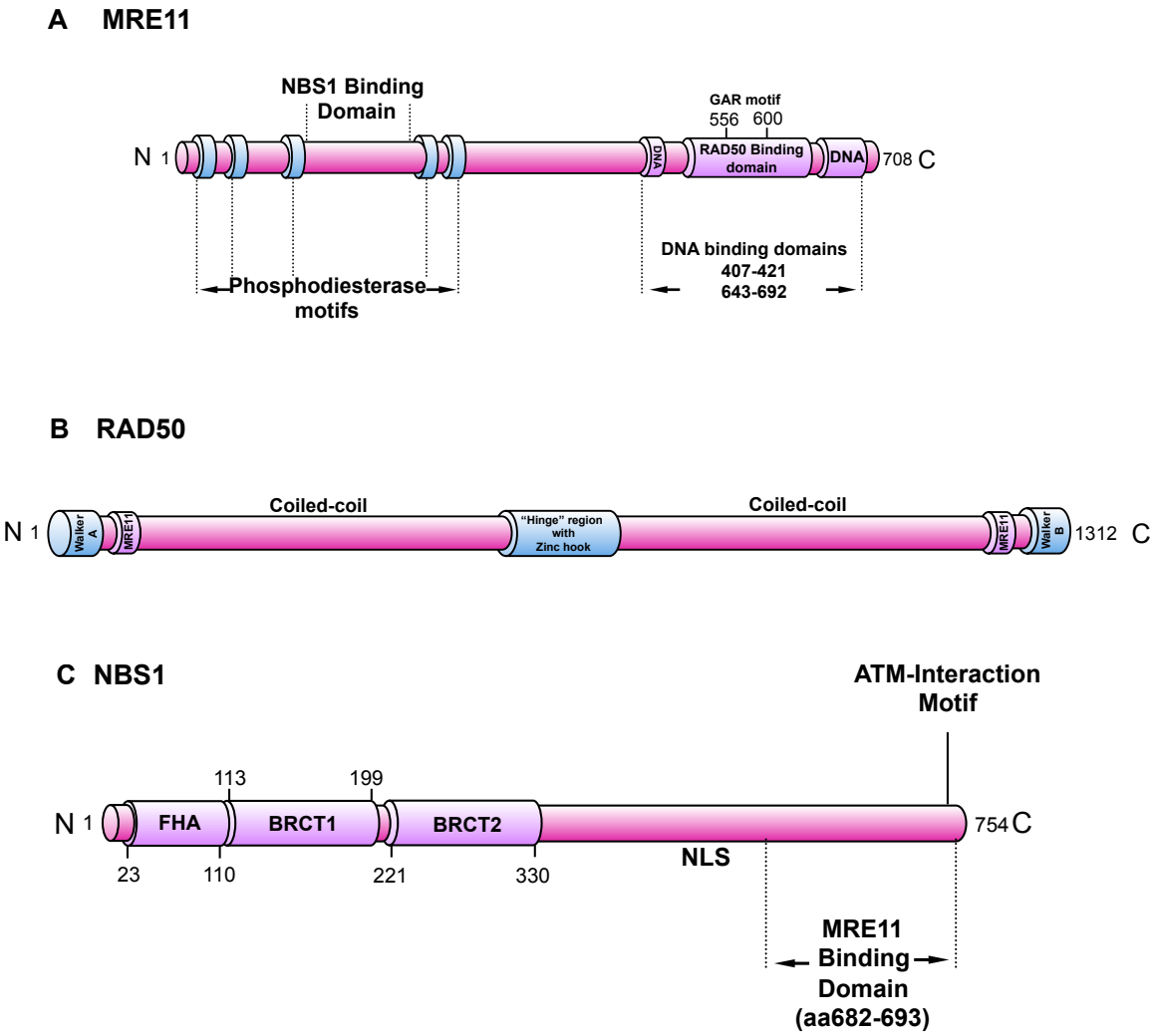


Figure 1.14 ***Schematics of the members of the human MRN complex. (A) MRE11.*** *MRE11 is 708 amino acids in length. Located at the N-terminus are five residues, named the phosphesterase domain which are required for MRE11 nuclease activity. Situated within this region is the NBS1 binding domain. At the C-terminus of MRE11 is the GAR motif and RAD50 binding domain, flanked by two DNA binding regions.* ***(B) RAD50.*** *1312 amino acids in length, RAD50 contains a Walker A domain and a Walker B domain at the N and C terminus respectively. Proximal to the Walker motifs are the MRE11 interacting domains. Located centrally is the "hinge" region which in the presence of ATP can fold back in on itself bringing the coiled-coiled regions together.* ***(C) NBS1.*** *NBS1 is 754 amino acids in length, and possess an FHA domain and two BRCT domains at the N-terminus. Upstream of these binding domains is the nuclear localisation signal (NLS). The region responsible for binding to MRE11 is located at the C-terminus, as is the region required for ATM activation (Adapted from Rupnik et al 2010).*

on the interaction with both RAD50 and NBS1. The MRE11 dimer forms the "core" complex of MRN, with each molecule of MRE11 binding one molecule of RAD50. A single molecule of NBS1 then binds to the complex via MRE11 (van der Linden *et al.*, 2009).

1.6.6.2 RAD50

Related to members of the structural maintenance of chromosome (SMC) family, with which it shares sequence and structural similarities, RAD50, like SMC proteins, can influence chromatid cohesion. In addition to this, RAD50 possesses other attributes necessary for the function of the MRN complex as a whole. The N and C termini of RAD50 contain a Walker A and B motif respectively, and in the presence of ATP they are required for binding and partial unwinding of the DNA (Chen *et al.*, 2005, de Jager *et al.*, 2002, Hopfner *et al.*, 2000, Raymond and Kleckner, 1993). Structural studies have shown that between the Walker A and B motifs is a coiled-coil domain separated by a central "hinge" region containing a zinc-hook, which has the ability to fold back on itself, bringing the two Walker motifs in close proximity to form a globular ATP-binding cassette (ABC)-type ATPase domain (Hopfner *et al.*, 2001, Hopfner *et al.*, 2000) (Figure 1.14). MRE11 has been shown to bind to RAD50 at the base of the globular domain (Hopfner *et al.*, 2001, Moreno-Herrero *et al.*, 2005).

Like MRE11, two molecules of RAD50 exist within the MRN complex bringing both ABC-ATPase domains from each molecule in close proximity with each other (Williams *et al.*, 2010). The binding of ATP to the RAD50 globular head, switches the conformation of the MRN complex from an 'open' to a 'closed' state. In the 'open' form the coiled-coil arms are not touching and the two Walker motifs of the same RAD50 molecule are distal from each other. The 'closed' state results in the coiled-coil arms coming together, bringing the Walker A and B motifs together, thus allowing the zinc-hooks from each molecule to interlock. In

addition to a requirement for ATP, this conformational change to the 'closed' form is also dependent on DNA binding, and once RAD50 and MRE11 are bound to NBS1, the conformation remains unchanged (Moreno-Herrero *et al*., 2005). RAD50 switches back to the 'open' conformation following hydrolysis and release of ATP (Hopfner *et al*., 2001, Williams *et al*., 2007). When present in the closed state, the coiled-coil arms protrude away from the globular ABC-ATPase domains (de Jager *et al*., 2001). Here, they can either link with the coiled coil arms of the second molecule of RAD50, bound to the same strand of DNA, as described above, or act as a bridge, tethering two separate DNA strands via dimerisation with a different MRN complex bound to a distal DNA molecule (Hopfner *et al*., 2002, Moreno-Herrero *et al*., 2005). This long-range tethering is thought to benefit homology-mediated repair, by creating a bridge between the sister chromatids. Evidently, the ATP-dependent conformational states of RAD50 are essential for the MRN complex function and furthermore, it was shown that ATP binding and hydrolysis by RAD50 also influences MRE11 nuclease activity in an NBS1-dependent manner, by regulating the switch between exo and endonuclease activities (Paull and Gellert, 1999, Trujillo and Sung, 2001, Williams *et al.*, 2010) (Figure 1.15).

1.6.6.3 NBS1

The NBS1 subunit is essential for the recruitment of both RAD50 and MRE11 to the nucleus. This recruitment is dependent both on the nuclear localisation signal of NBS1 as well as its C-terminus that interacts with MRE11 (Carney *et al.*, 1998, Cerosaletti *et al.*, 2000, Desai-Mehta *et al.*, 2001). The interaction of NBS1 with MRE11 and RAD50 stimulates the nuclease activity of MRE11 and the ATP-dependent unwinding of the DNA by RAD50 (Paull and Gellert 1999). Despite this stimulatory role, NBS1 lacks intrinsic enzymatic properties. However it is thought to act as a mediator/adaptor subunit within the MRN complex through

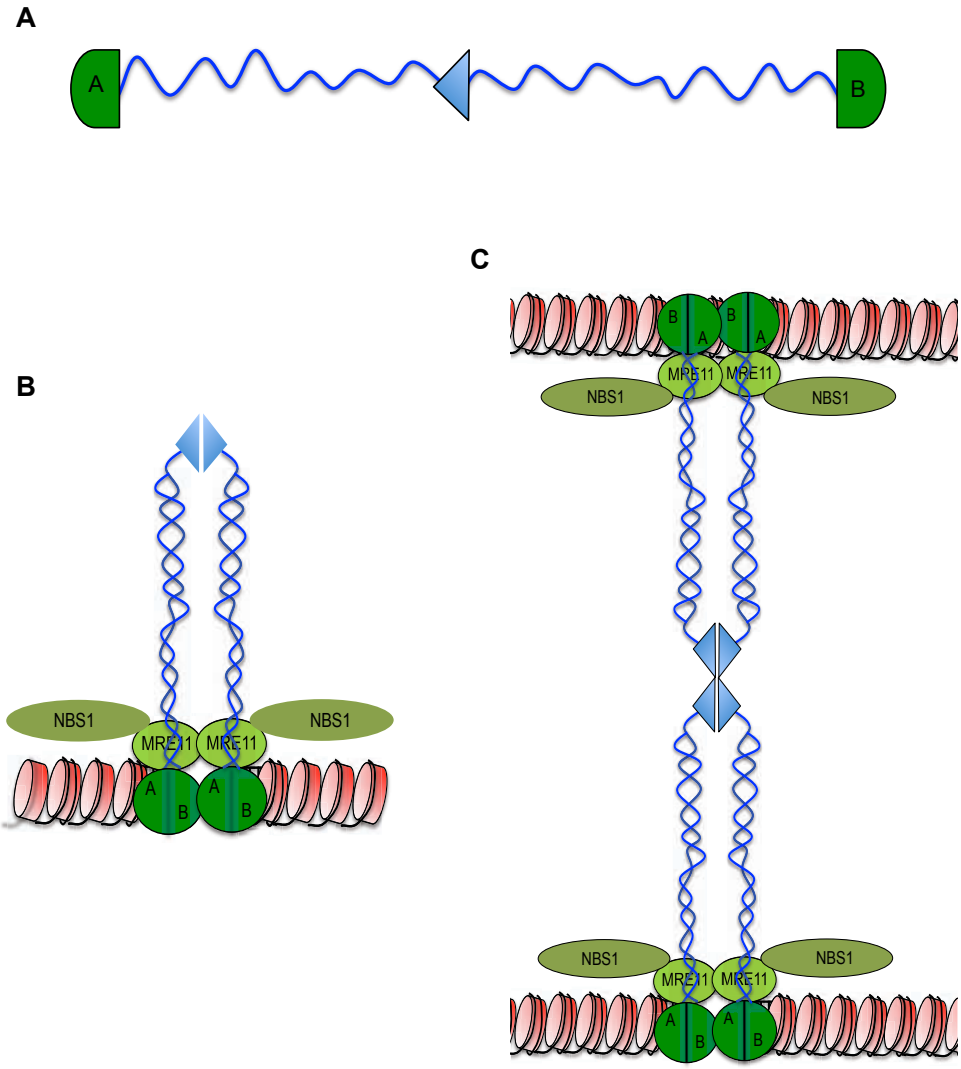


Figure 1.15 ***RAD50 and MRN configurations. (A) Schematic of RAD50 in the 'open conformation. (B) The MRN complex bound to chromatin.*** *A cartoon showing MRN bound to a DSB. Two RAD50 molecules are in the 'closed' conformation, where the coiled- coil domains have folded back on themselves, bringing the Walker A and B domains of each molecule into close proximity, forming a globular domain. The zinc hooks of the two molecules can then interlock. The MRE11 dimer resides at the globular domain bound to RAD50, where it can then interact with NBS1.* ***(C) The MRN bridging sister chromatids.*** *The coiled-coils protruding out from one MRN complex can link with zinc hooks of another, bound to a distal molecule of DNA. This conformation can be utilised when homology-mediated repair is required. (Adapted from Stracker & Petrini, 2011).*

its ability to promote the recruitment of other key DDR proteins to the site of a aDSB. This is mediated via its N-terminal FHA and tBRCT domains and the presence of these domains within NBS1 gives the MRN complex a role in DNA damage signaling (Lloyd *et al.*, 2009). Known damage response proteins that interact with these regions on NBS1 include ATR, TopBP1 and CtIP. The FHA/tBRCT domain is known to regulate the accumulation of γH2AX possibly through interacting with MDC1, although the mechanism by which this occurs remains elusive (Lloyd *et al.*, 2009, Williams *et al.*, 2009). NBS1 also contains an ATM interaction motif at the C-terminus, and this regulates accumulation of ATM at DSB (Falck *et al.*, 2005, You *et al.*, 2005). It should be noted that the interaction between NBS1 and ATM is not essential for the re-localisation and activation of the kinase, as disruption of this interaction can be compensated for by other mediator proteins (Difilippantonio *et al.*, 2007, Stracker *et al.*, 2007) (Figure 1.8).

1.6.6.4 Sensing DSBs and repair

It is widely accepted that the MRN complex is one of the earliest factors to arrive at the sites of DSBs and functions upstream of the ATM kinase. Once at the lesion, the MRN complex may help to prevent premature degradation of the free DNA ends and/or reduce the chance of the free ends migrating away from each other (Lamarche *et al.*, 2010). The MRN complex then initiates ATM-dependent signaling in part by assisting in the recruitment and activation of ATM via the C-terminus of NBS1, and therefore, propagating ATM autophosphorylation and in turn amplification of the cellular damage response to facilitate the recruitment of downstream DDR proteins.

In parallel to the sensing and signaling role of MRN, the complex also begins the processing of the DNA ends at the break, which can include unwinding, resection, removal of inhibitory complexes and the resolution of DNA structures such as hairpins (Rupnik *et al.*, 2010).

Strikingly, this arm of MRN function, which is facilitated through the MRE11 nuclease activity, is dispensable for ATM and checkpoint activation, but essential for the repair of DNA breaks (Buis *et al.*, 2008). MRN, and more specifically MRE11, has been shown to function in both HR and NHEJ repair pathways. Of the two, the role of MRE11 in HR has been better studied, where the nuclease activity is required to process the DNA ends to allow invasion of the sister chromatid. As described previously, HR is restricted to the S and G2 phases of cell cycle as in G1 there is a lack of a sister chromatid. It is thought that MRE11, in conjunction with CtIP, can promote resection of the DNA ends, which are then further processed by a second set of nucleases, namely EXO1 and DNA2. The precise mechanism of resection by MRE11 during HR is still under discussion, due to the fact that MRE11 possess 3'-5' but not 5'-3' exonuclease activity that is normally required for resection. One possible strategy proposed by Garcia *et al* (2011), involves MRE11/CtIP creating multiple nicks near the break. MRE11 then resects the DNA toward the break, with EXO1 performing long-range resection away from the break (Garcia *et al.*, 2011). In support of this hypothesis, MRX in yeast is only capable of resecting several hundred nucleotides, insufficient for complete HR and therefore requiring further processing by nucleases such as EXO1 (Mimitou and Symington, 2008, Zhu *et al.*, 2008). Although not possessing nuclease activity itself, CtIP has been shown to enhance the nuclease activity of MRE11 (Sartori *et al.*, 2007, You *et al.*, 2009). Resection of the DNA relies also on BRCA1, which forms a complex with the MRN complex as well as CtIP and potentially functions to anchor MRN and CtIP at the lesion (Chen *et al.*, 2008, Sartori *et al.*, 2007, Yun and Hiom, 2009). The formation of this complex is also dependent on CDK activity, with work showing that both CtIP and NBS1 are phosphorylated by CDK kinases and that these modifications are essential for resection and therefore, HR (Falck *et al.*, 2012, Huertas and Jackson, 2009).

Evidence has implicated a role for MRN in both NHEJ and Alt-EJ as siRNA knockdown of *MRE11* in both *Xrcc4-/- and Xrcc4+/+* backgrounds resulted in end joining defects (Rass *et al.*, 2009, Xie *et al.*, 2009). The role of MRN during NHEJ may be a structural one, as a nuclease deficient *Mre11* mutant expressed in *S.cerevisiae* exhibited no defects in end-joining (Zhang and Paull, 2005). Another possibility is that MRN along with CtIP may serve to remove unwanted DNA structures and inhibitory proteins, "preparing" the ends for other processing enzymes (Rass *et al*., 2009). The potential role of MRN during Alt-EJ is a little clearer, and would appear that the MRE11 nuclease activity is required in the absence of classical NHEJ nucleases to resect the DNA needed for homology searching (Xie *et al*., 2009). More recently, MRN has been shown to interact with DNA ligase IIIα/XRCC1, a participant of Alt-EJ, where it functions in a stimulatory capacity aiding the joining of the DNA molecules, possibly via end-bridging followed by resection (Della-Maria *et al*., 2011).

NHEJ seems to be the major pathway for DSB repair throughout the cell cycle (Fattah *et al.*, 2010). Furthermore, factors such as the absence of a sister chromatid as well as the low levels of CtIP during G1 reduces the likelihood of HR repair happening. NHEJ repair is initiated by the KU70/KU80 heterodimer, which like the members of the MRN complex is in high abundance throughout cell cycle. Even in S/G2 phases where repair can occur via HR, a number of reports have demonstrated NHEJ still repairs the vast majority of the DSBs in the later phases of cell cycle (Beucher *et al.*, 2009, Karanam *et al.*, 2012). The question, therefore, arises as to what determines which repair pathway is utilised to repair DSB in S/G2? Very little is understood about how this is regulated but it could be that the MRN and the KU complexes compete at the site of breaks to determine the repair process or that the cell defaults to using NHEJ but if this fails then MRN-dependent repair processes take over (Lamarche *et al*., 2010).

1.6.6.5 MRN and cell cycle checkpoint control

Indications that the MRN complex contributes to cell cycle checkpoints arose from the observation that genetic syndromes caused by hypomorphic mutations in any gene of the complex displayed defects in the G1, intra-S and G2/M cellular checkpoints. Following exposure to IR or treatment with radiomimetic drugs, cells from NBS patients display defects in cell cycle checkpoint activation (Jongmans *et al.*, 1997, Yamazaki *et al.*, 1998). Elevated levels of RDS in these cells point to an inability to activate the intra-S-phase checkpoint, resulting in the continuation of DNA synthesis following damage. Exactly how NBS1 influences intra-S-phase checkpoint control is still unclear. A number of mechanisms exist to regulate the intra-S-phase checkpoint and all rely on ATM following IR (Falck *et al.*, 2002). Two of these mechanisms are dependent on NBS1 and may shed light on the proteins function in regulating the checkpoint. The first involves SMC1, whose phosphorylation on serine 957 and 966 is required for checkpoint activation and suppression of RDS, and this is dependent on the phosphorylation of NBS1 on serine 287 and 343 by ATM (Kim *et al.*, 2002, Yazdi *et al.*, 2002). The second may involve the ATM-dependent phosphorylation of FANCD2 on serine 222 that is also dependent on ATM mediated phosphorylation of NBS1 at serine 343 (Nakanishi *et al.*, 2002, Taniguchi *et al.*, 2002). In addition to these findings, a role for MRN in regulating the intra-S-phase checkpoint downstream of the Nbs1 dependent phosphorylation of SMC1 has also been reported. MRN was shown to associate with RPA through MRE11 in a cell cycle-dependent manner more preferably at the G1-S phase transition and to relocalise to replication origins during S-phase in both undamaged and irradiated cells (Olson *et al.*, 2007b). Interestingly, in response to IR, ATM phosphorylates MRN bound to RPA and this prevents DNA replication initiation, suggesting that

phosphorylated MRN somehow interferes with RPA function at replication origins (Olson *et al*., 2007).

The role of MRN in the G1/S checkpoint regulation has presented some controversy. Again based on work using patient NBS deficient cells, checkpoint abnormalities were observed at low doses of IR but not at high doses, suggesting that MRN may in some way contribute to the activation of this checkpoint (Zhao *et al.*, 2000). However, other studies have failed to report such defects (Antoccia *et al.*, 1999, Jongmans *et al.*, 1997). What is consistent in NBS deficient cells is a reduction in p53 stabilisation following IR and transcriptional upregulation of its effector protein $p21^{waf1}$, which are required for G1 cell cycle arrest (Antoccia *et al.*, 1999, Girard *et al.*, 2002, Jongmans *et al.*, 1997, Matsuura *et al.*, 1998, Yamazaki *et al.*, 1998). More recently, problems in G1 checkpoint activation were also reported in a patient harbouring mutations in RAD50 (Waltes *et al.*, 2009).

The MRN complex has also been found to be required for ATM-dependent CHK2 phosphorylation following IR. Cells derived from NBS patients display reduced CHK2 activation and as consequence, an inability to prevent transit into mitosis. The ATM phosphorylation of NBS1 on serine 343 has been found to be required for G2/M checkpoint activation, as NBS deficient cells expressing NBS1 mutated at this site failed to activate the checkpoint in response to IR (Buscemi *et al.*, 2001). Although more recently from experiments in *Nbs1* knockout mice observations showed that the phosphorylation on serine 343 and serine 278, both targeted by ATM in the response to DNA damage, were not required for the G2/M checkpoint or indeed the S-phase checkpoint (Difilippantonio *et al.*, 2007). However the same study did show that activation of both these checkpoints was dependent on the FHA domain of NBS1. In conclusion, it appears that the MRN complex, possibly but not exclusively through NBS1, can regulate cellular checkpoint activation.

1.6.6.6 Additional functions

The MRN complex is involved in telomere homeostasis. Dysfunctional telomeres that arise in the absence of components of the Shelterin complex become recognised as DNA DSB, in an ATM-dependent manner (Takai *et al.*, 2003). MRN is localised to these regions independently of its nuclease activity, suggesting that the complex may be required for sensing and signalling of DNA breaks rather than DNA end-processing (Denchi and de Lange, 2007). Studies in yeast described a second role for MRN at telomeres, based on the observation that disruption of members of the MRX complex resulted in shortened telomeres in *S.cerevisiae* (Chamankhah and Xiao, 1999, Kironmai and Muniyappa, 1997). In yeast telomeres are synthesised during S-phase by telomerase, which prevents telomeric erosion. Further work led to the speculation that MRX may resect telomeres, which are then bound by Cdc13, a telomeric DNA binding protein in yeast, that then recruits telomerase (Tsukamoto *et al.*, 2001). An indication that this may also be the case in higher eukaryotes arose from the observation that NBS1 deficient cells displayed shortened telomeres, and that expression of both NBS1 and telomerase in these cells resulted in longer telomeres than expression of telomerase alone (Ranganathan *et al.*, 2001). Again how MRN functions within the telomere micro-environment in vertebrates requires further investigation.

The MRE11 nuclease activity is utilised during meiotic recombination. Meiotic recombination occurs prior to the first meiotic division and ensures that the correct chromosomes are physically paired through connection of homologous chromosome arms and cohesion between sister chromatids (Kleckner, 2006, Neale and Keeney, 2006, Petronczki *et al.*, 2003). Essentially, the cell creates DNA DSB breaks and then uses repair to lock chromosomes together. These scheduled DSB breaks are created by the topoisomerase II-like enzyme SPO11, which functions as a dimer and once the break has been created one molecule

remains covalently linked on each side of the break. In order for repair to take place, the SPO11 molecules must be removed from either side of the break. Work in yeast has provided evidence that the nuclease activity of Mre11, along with Sae2 (CtIP in humans), is needed for the removal of SPO11 (Lengsfeld *et al.*, 2007, Neale *et al.*, 2005). Upon removal of SPO11, repair via HR, the repair mechanism in meiosis due to NHEJ being suppressed, can initiate (Goedecke *et al.*, 1999). In mammalian cells MRE11 is likely to cooperate with CtIP, the orthologue of Sae2, during meiotic recombination to carry out similar functions (Sartori *et al.*, 2007).

MRN also has roles during immune system development. Based on studies from mice models, the MRN complex, although not essential, may play a regulatory role in V(D)J recombination. In a *Nbs1* hypomorphic mutants, the joining but not the ligation step of coding ends via Alt-EJ during V(D)J was impaired (Deriano *et al.*, 2009). This finding suggested the MRN complex deals with V(D)J recombination intermediates by stabilising break ends and preventing unwanted joining reactions. Moreover, MRN involvement in V(D)J recombination seems to require NBS1 but not the MRE11 nuclease activity (Deriano *et al*., 2009).

The role of MRN in CSR appears to be more involved. One role is the processing of DNA ends. However, only a subset of programmed DNA breaks generated during CSR, those that are repaired using NHEJ, require the nuclease activity of MRE11 (Dinkelmann *et al.*, 2009). The same study also reported that a nuclease-deficient *MRE11* mutant displayed only a mild defect in CSR compared to complete MRN loss, adding support to the notion that the nucleolytic function of MRN in CSR is not essential for repair. A second possible role of MRN that may support the severe CSR defect noted with complete MRN loss may be the requirement for RAD50 to bridge distal DNA ends for ligation following resection essential for microhomology searching (Dinklemann *et al*., 2009). In contrast to the first proposed role

of MRN in CSR, resection over longer regions of DNA is active when the generated DSB are repaired through the Alt-EJ machinery, and therefore, MRN may function in two separate pathways that can be utilised by the cell to repair breaks of this type.

The individual properties of each subunit allow the MRN complex to participate within different cellular processes. Reduced levels of any of the members results in the destabilisation of the complex, reiterating the epistatic nature of MRN, and explaining the similar phenotypes seen in patients harbouring mutations in *MRE11, NBS1* or *RAD50*. A role of MRN during DNA replication is beginning to surface, with many of the functions of the complex required in DNA repair overlapping. For instance, evidence has shown the MRN complex, and specifically the nuclease activity of MRE11, is required for replication fork restart following collapse or stalling of the fork (Bryant *et al.*, 2009). Just exactly how the MRN complex is regulated to carry out its functions over a wide subset of cellular pathways is still to be revealed.

1.7 Post-translational modifications and the DNA DSB damage response

As previously discussed, the cellular response to DNA DSB is a tightly regulated orchestrated series of events, involving a large number of different proteins. The recruitment of these proteins to damage sites or their activation relies on various post-translational modifications (PTM). These modifications can act either to promote the activity of DDR proteins or suppress activity once the lesions are repaired. From work dissecting repair pathways a multitude of PTMs have been uncovered that are required to control the sensing of damaged DNA, signalling of the damage response and ultimately initiate cell cycle checkpoints.

1.7.1 Ubiquitylation in response to DNA DSBs

Studies have revealed ubiquitin to be highly important in the response to DSBs. In support of this, disruption of these ubiquitin-dependent mechanisms required for an intact DDR, increases cancer risk (Scully and Livingston, 2000, Wang and Elledge, 2007). The ubiquitin machinery involves three enzymatic steps (Hershko *et al.*, 2000). Firstly, in an ATP-dependent manner the E1 enzyme activates ubiquitin, which is then transferred to an E2 conjugating enzyme that along with an E3 ligase attaches the ubiquitin to the substrate. Two families of E3 ligases exist. The larger of the two are the really interesting new gene (RING) E3 ligases, which number more than 600 in mammalian cells (Li *et al.*, 2008). The second family, and the smaller of the two is the homolgous to E6-AP carboxy terminus (HECT) E3 ligases. The RING E3 ligases facilitate the transfer of the ubiquitin molecule from the E2 conjugating enzyme to the substrate (Zheng *et al.*, 2000). Whereas the HECT domain E3 ligases are thought to possess catalytic activity and this is required for attachment of ubiquitin to the substrate (Scheffner and Kumar, 1995).

Ubiquitin can either be bound to the substrate as a single molecule, or form chains. Ubiquitin itself contains several lysines and in theory any one of them could be a targeted for chain formation. The generation of ubiquitin chains and how each ubiquitin molecule is linked dictates the outcome of the substrate. For example it is accepted that ubiquitin chains formed through K48 ubiquitin linkage usually direct the substrate for degradation by the proteosome, whereas those chains linked via K63 are associated with signalling mechanisms (Glickman and Ciechanover, 2002, Hicke, 2001, Messick and Greenberg, 2009, Thrower *et al.*, 2000). Current understanding denotes, in addition to K48 and K63 linkage, ubiquitin chains can also form through linkages at K6, K11, K27, K29 and K33, although the functional consequences of attaching these atypical ubiquitin chains to a substrate is unclear (Kim *et al.*, 2007c). It has

been recently demonstrated that the ubiquitin chains catalysed by the APC/C linked through K11 may be involved in tagged proteins for proteasome-dependent degradation (Dimova *et al.*, 2012).

Within the DDR, a role for ubiquitin was first identified in yeast with the discovery that RAD6, a protein involved in postreplication repair, was an E2 ubiquitin-conjugating enzyme (Jentsch *et al.*, 1987). Subsequent investigations revealed a major role for ubiquitin in mammalian DNA repair (Bekker-Jensen *et al.*, 2010, Doil *et al.*, 2009, Galanty *et al.*, 2012, Hashizume *et al.*, 2001, Huen *et al.*, 2007, Kolas *et al.*, 2007, Mailand *et al.*, 2007, Stewart, 2009, Wang and Elledge, 2007). While certainly ubiquitin-mediated degradation of DDR proteins occurs, surprisingly, most of the ubiquitin conjugates identified during repair seem to be associated with protein-protein interactions. Several E3 ubiquitin ligases that modulate the cellular response to DNA DSBs have been identified, including BRCA1, RNF8, RNF168, HERC2 and RNF4. BRCA1, along with its binding partner BARD1, was the first to be identified (Table 1.1) (Hashizume *et al.*, 2001, Morris and Solomon, 2004). The importance of the BRCA1 ubiquitin ligase activity is highlighted by the number of clinical mutations reported in the BRCA1 RING domain necessary for the catalysis of ubiquitin conjugation to the substrate (Yu *et al.*, 2006). The ubiquitylation of histones H2A and H2AX has been attributed to RNF8, an E3 ligase that is recruited to DNA breaks by interacting with MDC1. The ubiquitylation of histones by RNF8 causes chromatin relaxation around the break that allows the recruitment of further DDR proteins such as 53BP1 and BRCA1 to DNA breaks. The E3 ubiquitin ligase HERC2 is also required in this process, by recruiting the E2-conjugating enzyme UBC13 (Bekker-Jensen *et al.*, 2010). The RNF8-dependent ubiquitylation of histones is further amplified by a third E3 ubiquitin ligase, RNF168 in

E3 ligase	Target
BMI1	γH2AX
BRCA1	Unknown
HERC2	Promotes chromatin ubiqutitylation
RNF8	H2A/H2AX
RNF168	H2A/H2AX
TRIP12	RNF168?
UBR5	RNF168? Additional DDR E3s

Table 1.1 **E3 ligases and substrates involved in the cellular response to DNA double-strand breaks.**

DUB	Substrate or Interaction partner
USP3	H2A
USP10	p53
USP11	H2A, BRCA2
USP16	H2A
USP28	53BP1, TopBP1, MDC1.
USP44	H2A
BRCC36	BRCA1, BRCA2, RAD51, RAP80
POH1/PSMD14	K63 ubiquitin chains
OTUB1	UBC13

Table 1.2 **DUBs and target proteins involved in the cellular response to DNA double-strand breaks.**

collaboration with other ubiquitin ligases such as TRIP12 and UBR5 (Table 1.1) (Gudjonsson *et al*., 2012). How TRIP12 and UBR5 achieve this is unclear. An additional study showed B lymphoma Mo-MLV insertion region 1 homolog (BMI1) and RING2, both members of the polycomb group proteins involved in maintaining gene repression patterns, are also required for mono-ubiquitylating γH2AX (Ismail *et al.*, 2010). The recruitment of these proteins and ubiquitylation is independent of RNF8, however depletion of BMI1 does have an impact on the formation of both 53BP1 and BRCA1 foci (Ismail *et al*., 2010). It is commonly thought that the ubiquitylation of histones after DNA damage results in relaxation of the local chromatin structure, allowing visualisation of di-methylated H4K20 by the Tudor domain of 53BP1 to promote its recruitment (Botuyan *et al.*, 2006). However, recent evidence suggests that the Tudor domains of JMJD2A/B preferentially bind this methylated residue on histone 4 in the absence of damage. Following exposure to IR, RNF8 and RNF168, as well as ubiquitylating H2A, are thought to catalyse the ubiquitin-dependent degradation of JMJD2A/B, allowing 53BP1 access to the methylated histone (Mallette *et al.*, 2012). Lastly, the ubiquitin chains formed on damaged chromatin can be recognised by proteins containing ubiquitin interacting motifs (UIM) and motif interacting with ubiquitin (MIU) domains. For example, the recruitment of RAP80, a BRCA1 associated protein, to the sites of DNA breaks is thought to be mediated through its tandem UIMs that recognise poly-ubiquitylated histones (Kim *et al.*, 2007a, Sobhian *et al.*, 2007, Wang *et al.*, 2007).

Deubiquitylation at sites of DSB repair plays an equally important role in the regulation of these events (Table 1.2). Many of the deubiquitylation enzymes (DUBs) identified so far active in various DNA DSB repair pathways, function to down-regulate the RNF8/RNF168-dependent DDR. BRCC36, a member of the BRCA1-RAP80 complex, has affinity for K63-linked ubiquitin chains, and is thought to remove such chains generated by RNF8 and UBC13

(Cooper *et al.*, 2009b, Shao *et al.*, 2009a). Similarly, a proteasome-associated DUB, POH1/PSMD1, which also exhibits a strong selectivity for K63-linked ubiquitin, was shown to have a negative impact on the RNF8 pathway (Butler *et al.*, 2012, Cooper *et al.*, 2009a). This is an example of the tight interplay involved in the ubiquitin regulated DDR. Several other DUBs are involved in this regulation, for example USP3, USP16 and OTUB1, which counteract the products of RNF8/RNF168 ubiquitin ligase activities. Both USP3 and USP16 have also been shown to act on ubiquitylated H2A (Joo *et al.*, 2007, Nicassio *et al.*, 2007). Interestingly, OTUB1 negatively regulates the activity of the E2 enzyme, UBC13, by disrupting its ability to transfer ubiquitin to an RNF168 bound substrate (Juang *et al.*, 2012, Wiener *et al.*, 2012). More recently, in a DUB overexpression screen USP44 was shown to antagonise the RNF8/RNF168 pathway through de-ubiquitylation of H2A (Mosbech *et al.*, 2013). Whereas the majority of DUBs identified so far in this pathway act on the ubiquitin chains formed on the histone H2A, the DUB, USP28 was shown to be involved in stabilising the mediators TopBP1, 53BP1 and MDC1 (Zhang *et al.*, 2006).

1.7.2 SUMOylation in response to DNA DSBs

SUMOylation involves the conjugating of a ubiquitin-like molecule, SUMO (small ubiquitin-related modifier) to target substrates, which can target substrates for degradation, alter their cellular localisation or promote protein-protein interactions. Mammalian cells possess 4 *SUMO* isoforms, *SUMO-1*, and the highly related *SUMO-2* and *SUMO-3*. A fourth gene encoding *SUMO-4* occurs in the human genome but it is unclear whether its product can be conjugated to proteins (Zlatanou and Stewart, 2010). Differing by only three amino acids, SUMO-2 and -3 are able to form poly-SUMO chains whereas only a single molecule of SUMO-1 can be attached to a substrate (Morris, 2010). Targeting of SUMO to substrates is mediated by a single E1 SUMO activating enzyme, Uba2/Sae2, a single SUMO conjugating

enzyme E2, UBC9 and a handful of E3s and like ubiquitin this is usually coordinated onto a lysine residue on the substrate (Desterro *et al.*, 1999, Hochstrasser, 2001, Jackson, 2001, Kagey *et al.*, 2003, Okuma *et al.*, 1999, Pichler *et al.*, 2002). SUMOylation is reversible and SUMO molecules are removed by the peptidases SENP1-SENP3 and SENP5-SENP7 (Yeh *et al.*, 2000).

SUMOylation was recently identified as an additional modification in the cellular response to DSBs with the observation that SUMO1-3, UBC9 and the E3 ligases PIAS1/4 and MMS21 localised to sites of laser-induced DSB (Galanty *et al.*, 2009, Morris *et al.*, 2009). More specifically inactivation of PIAS4 abrogated RNF168 accumulation, while depletion of PIAS1 affected RAP80 and BRCA1 recruitment. Unsurprisingly, depletion of PIAS1 and 4 also showed a negative affect on the HR and NHEJ repair pathways (Galanty *et al*., 2009, Morris *et al*., 2009). RNF168 and HERC2 were found to be SUMOylated in a PIAS4-dependent manner, as ablation of PIAS4 led to reduced levels of RNF168, impacting on HERC2 binding to RNF8 (Danielsen *et al.*, 2012).

It has been demonstrated that the E3 ubiquitin ligase activity of BRCA1 is enhanced by SUMOylation following the induction of DSBs, possibly by regulating its interaction with its E2 or recruiting proteins containing SUMO interacting motifs (SIMS) that are BRCA1 ubiquitin-targeted proteins (Morris *et al*., 2009). For this reason, BRCA1 is termed a SUMO-regulated ubiquitin ligase (SRUbL). Therefore, the role of SUMO maybe to enhance the ubiquitin ligase activity of E3s during the DNA DSB repair pathway. This may also be true of the role SUMO plays in regulating RNF168 and HERC2 activities. Further cross talk between ubiquitylation and SUMOylation is evident in a group of proteins termed SUMO-targeted ubiquitin E3 ligases (STUbLs). In particular, SUMOylation of a protein is recognised by the SIM of the STUbL, resulting in the ubiquitylation of the target protein and its subsequent

degradation. The mammalian E3 ubiquitin ligase RNF4 is one such ligase that is recruited to DNA DSBs and facilitates DSB repair (Galanty *et al.*, 2012, Lallemand-Breitenbach *et al.*, 2008, Tatham *et al.*, 2008, Weisshaar *et al.*, 2008). RNF4 is shown to be recruited to a number of SUMOylated repair proteins via its tandem SIM domains, including RPA1 and MDC1, regulating their turnover during the repair process (Galanty *et al*., 2012). It has also been reported that MDC1 was SUMOylated on lysine 1840 by PIAS4 in a damage-dependent manner and was then targeted by RNF4, leading to its ubiquitylation and proteasome-mediated degradation (Luo *et al.*, 2012).

In summary, SUMOlyation appears to aid both the recruitment and retention of repair proteins within the topography of the DDR landscape. Beyond this, recent evidence has revealed a role for SUMOylation in regulating E3 ubiquitin ligase activity, as well as providing a means of interaction between E3s and SUMOlyated substrates, resulting in degradation of the substrate.

1.7.3 Methylation and acetylation in response to DNA DSBs

Unlike the previously described PTMs, methylation, especially that of histones seems to occur constitutively (Huyen *et al.*, 2004, Sanders *et al.*, 2004). It is thought that in the absence of damage methylated histones are hidden due to the chromatin conformation, and upon the formation of DSBs the chromatin conformation changes in order to expose the methylated histones (Huyen *et al.*, 2004, Sanders *et al.*, 2004). For example, the ubiquitylation of histones in response to DSBs results in the relaxation of the chromatin surrounding the break, revealing their methylated residues that are recognised by the Tudor domains of 53BP1 (Botuyan *et al.*, 2006).

More recently, studies have shown that arginine methylation of individual DDR proteins is required for their recruitment. In mammalian cells, there are 9 protein arginine N-

methyltransferases (PRMTs) that catalyse this PMT (Bedford and Clarke, 2009). In the context of the damage response, PRMT1 seems to be the main PRMT and has an affinity for glycine-arginine-rich (GAR) motifs present in some DDR proteins (Boisvert *et al.*, 2005b, Boisvert *et al.*, 2005c, Dery *et al.*, 2008, Tang *et al.*, 2000).

53BP1 contains a GAR domain that was shown to be methylated via PRMT1 irrespective of damage (Boisvert *et al.*, 2005c). The same study also showed that the methylation of 53BP1 on this domain promoted its association with both single and double-stranded DNA. Although mutation of the GAR domain did not disrupt 53BP1 localisation to sites of DNA damage, the authors hypothesised that methylation of its GAR domain served to strengthen the interaction between the Tudor domains of 53BP1 and the exposed methylated histones following DNA damage (Boisvert *et al.*, 2005c). MRE11 also possesses a GAR domain that is methylated by PRMT1 (Boisvert *et al.*, 2005a). More importantly, disruption of the MRE11 GAR domain impairs its exonuclease activity and its ability to activate the intra-S-phase checkpoint (Boisvert *et al.*, 2005a). Another study showed that in the absence of damage, MRE11 co-localises with PRMT1 in PML bodies and that its methylation is required for its re-localisation to sites of DNA damage (Boisvert *et al.*, 2005b). Finally, a more recent study supported these findings by demonstrating that methylation of MRE11 was required both for nuclease activity and chromatin retention (Dery *et al*., 2008). Like 53BP1, the methylation of MRE11 is thought to be constitutive, as no increase in methylation was observed in response to DNA damage (Dery *et al*., 2008).

Acetylation and deacetylation, often associated with transcription, have also been shown to be involved in regulating the DDR. The acetyltransferase Tip60 is recruited to sites of DNA damage where it functions in two ways. Firstly, Tip60 acetylates ATM on lysine 3016, a modification required for its autophosphorylation and subsequent activation following the

induction of DSBs (Sun *et al.*, 2005, Sun *et al.*, 2007). This observation implies that the acetylation of ATM is possibly the earliest PTM to occur during the DSB signaling cascade. Tip60, which is ubiquitously expressed also targets histones H2A and H4, and it is thought that the acetylation of these proteins promotes chromatin relaxation proximal to the break increasing its accessibility for the recruitment of repair factors such as 53BP1 and BRCA1 (Murr *et al.*, 2006). Furthermore, the acetylation of H4 on lysine 16 (H4K16) by the MOF (males absent on the first) acetyltransferase, has been shown to promote MDC1 foci, possibly by stimulating an interaction between H4 and H2AX which in turn might regulate the binding between MDC1 and γH2AX (Li *et al.*, 2010, Sharma *et al.*, 2010). Interestingly it has recently been shown that acetylation and deacetylation by Tip60 and HDACs respectively determines 53BP1 and BRCA1 association at the chromatin (Tang *et al.*, 2013).

Deacetylation observed at DSBs is carried out by members of the histone deacetylase (HDAC) family, SIRT1 and SIRT6 (Kao *et al.*, 2003, Murr *et al.*, 2006, Oberdoerffer *et al.*, 2008). For instance, SIRT1 was shown to directly interact with Nbs1 and that deacetylation of Nbs1 was necessary for its re-localisation to DSBs as well as for its ability to promote checkpoint activation (Oberdoerffer *et al.*, 2008, Yuan and Seto, 2007). HDACs also play a role in the repair of the breaks during HR and NHEJ. CtIP is deacetylated by SIRT6, an event required for resection, which is essential for HR mediated repair (Kaidi *et al.*, 2010). In addition, DSB repair by NHEJ requires the deacetylation of histone H3K56 mediated by HDAC1 and 2, which is thought to promote chromatin compaction (Miller *et al.*, 2010). The same study suggested that during HR, acetylation functions to relax the chromatin in order to promote this type of repair. In contrast, during NHEJ a more compact and therefore, deacetylated chromatin seems to be beneficial (Miller *et al.*, 2010).

1.7.4 Phosphorylation in response to DNA DSBs

Of all the protein modifications that occur during DNA DSBs repair and checkpoint signaling phosphorylation has been the most studied. This comes as no surprise considering the vast number of phosphorylated targets within the DDR that have been identified by stable isotope labeling by amino acids in cell culture (SILAC)-based mass spectrometry screens (Bennetzen *et al.*, 2010, Matsuoka *et al.*, 2007, Stokes *et al.*, 2007). In light of the requirement for phosphorylation during the DDR this type of modification has been extensively described in previous sections. For this reason only the salient phosphorylation events will be covered here.

The serine/theronine PIKKs kinases, ATM, ATR and DNA-PK are extremely important in the repair of damaged DNA. Activation of these kinases is responsible for the phosphorylation of numerous downstream targets, either by the kinases themselves or as a result of their activation. This group of kinases display levels of redundancy, in particularly ATM and ATR, and are able to phosphorylate the same downstream targets. ATM, primarily required for the repair of DNA DSBs is activated by autophosphorylation on a number of residues following IR, and once this occurs it can phosphorylate downstream substrates such as H2AX, MDC1 and Nbs1. Phosphorylation of these targets facilitates the recruitment and protein-protein interactions essential for a proper damage signaling. Exactly how activation of ATM occurs in response to DNA DSBs is still under discussion. However, it is clear from studies, where these sites have been modified and the subsequent defects seen in the ATM-dependent DDR that these autophosphorylation targets are essential (Kozlov *et al.*, 2006, Kracker and Durandy, 2011). Activated ATM has also been shown to facilitate chromatin decondensation, which is vital for the repair of these lesions. The KRAB-associated protein (KAP1) is phosphorylated in an ATM-dependent manner on serine 824, which leads to the spreading of

KAP1 along the chromatin (Li *et al.*, 2007a, White *et al.*, 2012). Loss of this phosphorylation was shown to reduce the amount of damage induced chromatin relaxation near the lesion and caused sensitisation to DNA damaging agents (Ziv *et al.*, 2006).

Once these early phosphorylation events have occurred, further phosphorylation-mediated mechanisms can then control the next steps of the signalling cascade. The phosphorylation of proteins by the PIKKs creates docking sites for repair proteins harbouring phospho-peptide binding domains. This ensures that only when proteins become phosphorylated can downstream regulatory proteins be recruited ensuring the temporal and spatial coordination of signalling events are tightly regulated. This is exemplified by MDC1, which only following the interaction with γH2AX can it then be phosphorylated by ATM, and this then allows the recruitment RNF8 through its FHA domain. Repair of the breaks themselves is also dependent on phosphorylation. For instance phosphorylation of BRCA2 by CDKs causes its disassociation with Rad51, allowing for the formation of the nucleoprotein filament and therefore repair via HR (Esashi *et al.*, 2005).

Whilst repair of the DNA DSBs is occurring, cell cycle checkpoints are activated to allow time for this to happen. The control of cell cycle progression during damage signalling and repair involves phosphorylation and dephosphorylation of substrates. These PMTs are coordinated by ATM and ATR and aided by the Chk1 and Chk2 kinases, which upon activation, phosphorylate downstream substrates such as members of the Cdc25 family and p53, thereby instigating cell cycle arrest (Falck *et al.*, 2001, Hirao *et al.*, 2000, Sanchez *et al.*, 1997). In one example upon ATR recruitment, following exposure of ssDNA possibly through resection, Chk1 is phosphorylated on serine 345. Chk1 is thought to be recruited to the chromatin in response to damage, then following its phosphorylation it disassociates from

the break into the nucleus, where it can then phosphorylate the Ccdc25 phosphatases and inhibit CDK cell cycle progression (Smits, 2006).

Like all the PMTs occurring in the response to DNA DSBs, phosphorylation of substrates is balanced by the removal of the modification. Equally important as the phosphorylation of substrates, dephosphorylation of substrates has been observed in response to damage (Bennetzen *et al.*, 2010, Bensimon *et al.*, 2010). The activation of ATM kinase activity by autophosphorylation is prevented in undamaged cells by the Repo-Man (Recruits PP1 onto mitotic chromatin at anaphase)-PP1γ complex, which contains the PP1 phosphatase. Following DNA DSB induction, PP1 no longer associates with ATM, leading to the kinases activation and initiation of the DDR (Peng *et al.*, 2010). ATM autophosphorylation is also regulated by the PP2A phosphatase, as is the ATR, DNA-PKs and furthermore Chk1 and Chk2 kinases (Douglas *et al.*, 2001, Dozier *et al.*, 2004, Freeman *et al.*, 2010, Goodarzi *et al.*, 2006, Leung-Pineda *et al.*, 2006). The wild-type p53-inducible phosphatase 1 (Wip1), also involved in dephosphorylating ATM, has been shown to dephosphorylate γH2AX (Cha *et al.*, 2010, Shreeram *et al.*, 2006). The dephosphorylation of γH2AX is thought to revert the modified histone back to H2AX once repair has been completed. The dephosphorylation of these kinases acts to downregulate the signalling pathway once repair has been carried out, returning cell cycle progression back to normal. The ability for the cell to "turn off" these pathways is equally important as "turning them on", as in the case of the DDR pathway persistence of signalling can be just as harmful as unrepaired DNA DSBs.

1.8 Casein Kinase 2 (CK2)

As previously highlighted, phosphorylation is an extensively studied modification in the cellular DDR cascade. In addition to the kinases described so far, several reports have also

indicated a significant contribution in the phosphorylation events of this pathway by another kinase, namely, Casein Kinase (CK2) (Iles *et al.*, 2007, Loizou *et al.*, 2004, Yata *et al.*, 2012).

Discovered nearly 60 years ago from studies using casein as a substrate, the actual physiological roles of CK2 were not been investigated until sometime later (Burnett and Kennedy, 1954). Since, CK2 has proven to be a promiscuous kinase with the number of its identified substrates reaching over 300 (Meggio and Pinna, 2003). This figure is likely to be a conservative estimate as a recent analysis of 10,899 phosphorylation sites present in the cellular proteome indicated that 2275 conformed to a typical CK2 phosphorylation consensus motif (Salvi *et al.*, 2009). CK2 preferentially targets serine/threonine residues surrounded by acidic amino acids: S/T-X-X-D/E, where X denotes any amino acid (Pinna, 1990). Ubiquitously expressed in numerous tissues and cell types, CK2, unlike many other kinases, is constitutively active. For this reason it has been observed to play a role in a wide range of cellular pathways including cell survival, apoptosis, gene expression to name but a few (Izeradjene *et al.*, 2004, Lin *et al.*, 1992, Lorenz *et al.*, 1994, Pepperkok *et al.*, 1994, Yamane and Kinsella, 2005a). Interestingly, fluctuations in CK2 levels have been noted in several human diseases, and more specifically an elevation in the levels of CK2 has been reported in a number of cancers (Landesman-Bollag *et al.*, 2001, Wang *et al.*, 2006). Unsurprisingly, this observation has encouraged the development of CK2 inhibitors as potential anti-cancer treatments.

1.8.1 Structure and activity

CK2 often exists as a tetrameric complex, comprising of two catalytic subunits and two regulatory subunits. The catalytic subunits in human cells have two isoforms, named CK2α and CK2α′ (Litchfield *et al.*, 2001). A third isoform has also been identified termed CK2α′′

although this only makes up a small fraction of the cellular pools of CK2 catalytic subunits (Shi *et al.*, 2001). In contrast, a single regulatory subunit, CK2β, exists in humans, and forms dimers in the CK2 tetrameric complex (Boldyreff *et al.*, 1996, Chantalat *et al.*, 1999, Gietz *et al.*, 1995, Graham and Litchfield, 2000). CK2 complexes can contain combinations of the two catalytic subunits: CK2α/CK2α, CK2α′/CK2α′ or CK2α/CK2α′(Gietz *et al*., 1995). CK2α and CK2α′ both exhibit approximately 90% homology in their catalytic domains, and subsequently, display similar traits regarding their enzymatic activity, at least *in vitro* (Bodenbach *et al.*, 1994, Litchfield *et al.*, 1990). The third catalytic subunit, CK2α′′ is closely related to CK2α however, little is known about this subunit (Shi *et al*., 2001). The C-terminal domains of CK2α and CK2α′ differ in amino acid sequence, suggesting existence of some non-redundant functions. For instance, CK2α is phosphorylated at sites located in its C-terminal domain, and this phosphorylation was shown to be cell cycle-dependent (Bosc *et al.*, 1995, Litchfield *et al.*, 1992). Thus, it could be that CK2α and CK2α′ are regulated differentially during cell cycle (Litchfield, 2003). Interestingly, knockout mouse models have demonstrated that the loss of either CK2α or CK2α′ does not result in lethality, supporting the notion that these subunits can compensate for the loss of each other. However, male CK2α knockout mice did display fertility defects indicating that is not the case for all CK2-dependent functions (Xu *et al.*, 1999).

The regulatory subunit, CK2β, is highly conserved between species (Heller-Harrison *et al.*, 1989, Jakobi *et al.*, 1989). From structural studies a dimer of CK2β was shown to sit at the centre of the tetrameric CK2 complex, with the two catalytic subunits attaching separately to the dimer (Boldyreff *et al.*, 1996, Chantalat *et al.*, 1999, Gietz *et al.*, 1995, Graham and Litchfield, 2000, Niefind *et al.*, 2009). A number of studies have shown that CK2β, within the CK2 tetrameric enzyme, serves to promote the catalytic activity, the stability of the complex

and the regulation of substrate specificity (Marin *et al.*, 1999, Meggio *et al.*, 1992). The CK2β subunit has also been reported to have higher expression levels than the catalytic subunits (Luscher and Litchfield, 1994). In light of this observation CK2β, via immunofluorescence experiments, has been reported to localise in areas where the α catalytic units are not present (Krek *et al.*, 1992). Indeed, it is accepted that a population of CK2β exists independently of the CK2 complex and has separate functions. To highlight this, the kinase Chk1 has been to shown to interact with CK2β independently of the catalytic subunits (Guerra *et al.*, 2003). Moreover, the same study noted that CK2β in complex with CK2α/α´ was unable to bind to Chk1, and conversely CK2β in complex with Chk1 could not form a complex with the catalytic subunits. The interaction between CK2β and Chk1 was seen to enhance the kinase activity of Chk1 (Guerra *et al*., 2003).

Clearly, a kinase containing subunits that function independently of each other in addition to functioning within a complex, broadens the number of potential functional interactions as well as substrates of CK2. For this reason, and in the context of this study, only substrates targeted by the holoenzyme will be focused on, and more specifically, a subset of those involved in regulation of the cell cycle, apoptosis and the DNA damage response.

1.8.2 CK2 and cell cycle

The involvement of CK2 in cell cycle regulation has been known for some time. Several studies have demonstrated that siRNA depletion or inhibition of CK2 halts cell cycle progression at the G0/G1, G1/S and G2/M borders in mammalian cells (Lorenz *et al*., 1994, Pepperkok *et al*., 1994). Furthermore, CK2 is itself also phosphorylated in a cell cycle-dependent manner. Cdk1, the primary mitotic cyclin-dependent kinase, has been shown to phosphorylate both CK2β and CK2α (Bosc *et al.*, 1995, Litchfield *et al.*, 1991, Litchfield *et*

al., 1992). However, the CDK-dependent phosphorylation of either of the subunits did not affect the overall activity of the holoenzyme, and therefore, the physiological reasoning behind these events is unknown. One possibility is that the CDK-dependent phosphorylation of CK2 may aid in the recruitment or the selection of substrates. Equally, it could function to direct CK2 away from substrates not required for cell cycle progression. Below are two examples of how CK2 influences cell cycle progression.

At the onset of mitosis, the Wee1 family, which prevents entry into mitosis by suppressing the CDKs, is degraded (Watanabe *et al.*, 2004). Phosphorylation of Wee1 by CDKs, CK2 and Plk1 on serines 123, 121 and 53 respectively, mark it for proteasomal degradation via ubiquitylation by the E3 ligase SCFβ^{-TrCP} (van Vugt *et al.*, 2004, Watanabe *et al.*, 2005, Watanabe *et al.*, 2004). The phosphorylation of all three residues is essential for its interaction with SCFβ-^{TrCP}, and thus for the subsequent degradation of Wee1, which stimulates progression of the cell into mitosis (Watanabe *et al*., 2005).

In addition to a requirement of CK2 for cell cycle progression, the kinase can also function to suppress cell cycle in the presence of DNA damage. It has been shown that the tumour suppressor p53 is phosphorylated by CK2 on serine 392 in response to UV irradiation and this enhanced both its DNA binding as well as its transcriptional activity (Kapoor and Lozano, 1998, Keller and Lu, 2002). Elevating the transcriptional activity of p53 results in the up-regulation of p53 target genes which function to stall the cell cycle, the best example of this being the CDK inhibitor p21$^{WAF1/CIP1}$ (Sherr and McCormick, 2002). Moreover, the CK2 kinase activity towards p53 is increased by a UV-dependent interaction between CK2 and the facilitating chromatin-mediated transcription (FACT) complex, containing hSPT16/CDC68 and structure specific recognition protein 1 (SSRP1) (Keller and Lu, 2002, Keller *et al.*, 2001).

1.8.3 CK2 and apoptosis

Many studies have pointed towards a role for CK2 in apoptosis. This can either be through receptor-mediated apoptosis or apoptosis that is a result of excessive DNA damage (Izeradjene *et al.*, 2004, Yamane and Kinsella, 2005a). Similarly treating cells with CK2 inhibitors is sufficient to induce cell death (Ruzzene *et al.*, 2002, Wang *et al.*, 2005a). From the literature it becomes apparent that CK2 is a pro-survival enzyme, preventing apoptosis by phosphorylating a number of proteins involved in anti-apoptotic pathways. In many examples, phosphorylation by CK2 of proteins involved in apoptosis prevents caspase-mediated degradation (Desagher *et al.*, 2001, Krippner-Heidenreich *et al.*, 2001). The caspases are a group of proteases that in response to pro-apoptotic signals function to cleave a group of proteins culminating in the breakdown of the cell. Caspase-inhibiting proteins that prevent caspase activity contain a caspase recruitment domain (CARD) (Hofmann *et al.*, 1997, Koseki *et al.*, 1998, Roy *et al.*, 1997). One such protein is "Apoptosis Repressor with a CARD" (ARC) (Koseki *et al*., 1998). ARC requires phosphorylation on theronine 149 to carry out its caspase inhibiting function, and this phosphorylation is facilitated by CK2 (Li *et al.*, 2002d). Specifically, the phosphorylation allows ARC to localize to the mitochondria, where it can then prevent caspase 8 activation, thereby inhibiting apoptosis (Li *et al*., 2002c). On the other hand CK2-dependent phosphorylation can also regulate caspase activation. Pro-caspase 2 is phosphorylated by CK2 on serine 157, and this modification acts by repressing its pro-apoptotic activity (Shin *et al.*, 2005).

1.8.4 CK2 and the DNA damage response

In light of the ubiquitous nature of CK2, it comes as no surprise that the kinase is involved in DNA damage signaling and repair. Often, CK2-dependent phosphorylation of DNA repair

proteins creates a phospho-motif capable of mediating an interaction with FHA and BRCT binding domain containing proteins.

The first study to reveal a role for CK2 during DNA repair, reported the phosphorylation of XRCC1 by CK2 (Loizou *et al.*, 2004). XRCC1 is required for single-strand DNA break repair (SSBR) as well as DSB during Alt-EJ, where it acts as a scaffold recruiting a number of repair factors. Phosphorylation by CK2 on a cluster of consensus motifs on XRCC1 promotes its interaction with the FHA domain of PNKP. As a consequence, either mutation of the CK2 phosphorylation sites on XRCC1 or treatment of cells with a CK2 inhibitor impairs SSBR (Loizou *et al*., 2004). A similar CK2-dependent phosphorylation motif was also identified in the NHEJ protein XRCC4. This modification promotes an interaction between XRCC4 and the FHA domain of PNKP (Koch *et al.*, 2004). Again, disruption of this interaction impaired DNA repair, although more specifically DNA end joining by DNA ligase-IV. It could be speculated that the two interactions represent a method by which constitutive CK2-dependent phosphorylation ensures a pool of XRCC1/PNKP and XRCC4/PNKP exists in the absence of damage, already primed and ready to respond immediately following the induction of genotoxic stress.

Interestingly, the CK2-mediated phosphorylation of XRCC1 and XRCC4 was also found to be important for the binding of Aprataxin, the product of the gene mutated in the autosomal recessive disorder Ataxia-oculomotor 1 (AOA1). This interaction was required for Aprataxin to repair abortive DNA ligation intermediates following exposure to IR (Ahel *et al.*, 2006, Date *et al.*, 2004). Lastly, to complicate matters, a third FHA domain containing protein termed Appratаxin –and PNKP-like factor (APLF) was also reported to bind to the same CK2 phosphorylated motifs on XRCC1 and XRCC4 (Iles *et al.*, 2007). Like the previous studies this interaction appears to promote NHEJ and stimulate the ligation of IR induced DNA DSBs

(Grundy *et al.*, 2013). Taken together the above studies demonstrate that CK2-dependent phosphorylation of XRCC1 or XRCC4 is essential for establishing separate DNA end-processing repair complexes.

Another example of CK2 mediated recruitment of proteins in response to DNA damage involves TopBP1 and the 9-1-1 complex. Briefly, the BRCT1 domain of TopBP1 was shown to be the binding site for RAD9 phosphorylated on serine 387 by CK2, and that this interaction is necessary for the association of TopBP1 with the 9-1-1 complex at dsDNA/ssDNA junctions (Rappas *et al.*, 2010). The biological importance of this interaction is emphasized by the observation that mutation of this phosphorylation site in RAD9 results in an increased cellular sensitivity to UV radiation and to methyl methane sulphonate (MMS) (Takeishi *et al.*, 2010).

Modification of a substrate by CK2 can also be dependent on the phosphorylation of adjacent residues by other kinases. Examples of these CK2 "phosphorylation cascades" can be found within the cellular response to DNA damage. Plk1 phosphorylates the HR protein RAD51 on serine 14 in a cell cycle- and DNA damage-dependent manner. However, this phosphorylation primes RAD51 for phosphorylation by CK2 on threonine 13 (Yata *et al.*, 2012). The phosphorylation of RAD51 on threonine 13 facilitates an association with NBS1, via its FHA domain. This study also showed that mutation of theronine 13 to an alanine prevented IR-induced RAD51 foci, suggesting that this series of events promotes the chromatin retention of RAD51 through its interaction with NBS1 and thus, HR (Yata *et al.*, 2012). Moreover, an interplay between CK2 and another kinase was reported for structure of maintenance of chromosome 3 (SMC3), a member of the cohesin complex. The cohesin complex is essential for the cohesion of sister chromatids during S and G2 phase and for DNA repair (Strom *et al.*, 2004, Uhlmann and Nasmyth, 1998). SMC3 is also constitutively phosphorylated by CK2 on

serine 1067, and this modification is required to promote its phosphorylation by ATM on a neighbouring serine residue (Ser-1083) and the subsequent activation of the intra-S phase checkpoint. As such, a Radio-resistant DNA Synthesis (RDS) phenotype was observed in cells expressing SMC3 harbouring a mutation on either serine 1067 or 1083 (Luo *et al.*, 2008). Furthermore, a similar S-phase checkpoint defect was also seen in cells lacking CK2. Therefore, it appears that can CK2 participate in DNA damage checkpoint activation through its ability to prime key effector proteins for phosphorylation by ATM and ATR. In support of this, it has also been documented for the transcription factor cyclic AMP response element-binding protein (CREB), whose target genes include a number of DNA damage repair enzymes and cell cycle regulators (Impey *et al.*, 2004, Mayr and Montminy, 2001). Work by Shanware, *et al*, (2007) showed that basal phosphorylation of CREB by CK2 on serine 111, which occurs in the absence of DNA damage, is necessary for several other sites (serine 108, serine 114, serine 117 and serine 121) to be phosphorylated, the latter of which is targeted by ATM following the induction of DNA damage (Shanware *et al.*, 2007). Therefore, it would appear that constitutive phosphorylation of proteins by CK2 provides the cell with a mechanism with which to control both the order and timing of subsequent stimulus-induced PTMs.

Lastly, as previously mentioned, chromatin relaxation at DNA breaks is necessary for the recruitment of DDR proteins as well as for amplification of the damage response signal. The heterochromatin protein 1 (HP1-β) is essential for chromatin compaction and is recruited to DNA breaks through an interaction with H3K9me (Ayoub *et al.*, 2008). Following IR, HP1-β is phosphorylated by CK2 on theronine 51 increasing its mobilisation (Ayoub *et al.*, 2008). In the presence of a CK2 inhibitor, HP1-β mobilisation was suppressed and a reduction in γH2AX foci was also observed (Ayoub *et al*., 2008). This suggests that the increased

mobilisation of HP1-β following CK2 phosphorylation after IR may relax the chromatin, which could then allow for the correct recruitment of DDR proteins.

1.9 Aims and objectives

The past decade has revealed an emerging role of CK2 in the DDR. In a number of examples the phosphorylation of substrates by CK2 permits interactions with proteins via specifc phosphopeptide binding domains namely FHA and BRCT domains. The publication of a number of largescale phosphoproteomic screens has aided in the identification of as yet uncharacterised putative phospho-residues on a wealth of proteins, including many involved in the repair of DNA breaks. Studying this data we noted a cluster of putative CK2 repeat motifs located on the N-terminus of MDC1. MDC1 is classed as a mediator protein, where upon recruitment to sites of DNA DSBs it functions as a scaffold to recruit other repair proteins to the lesions, promoting checkpoint activation and repair of the lesion. Both XRCC1 and XRCC4, required for the repair of DNA breaks by Alt-EJ and NHEJ respectively, are also classed as scaffold proteins, and both are phosphorylated by CK2 on several residues. Phosphorylation of these residues permits the binding to a number of interacting partners with FHA domains (Iles *et al.*, 2007, Koch *et al.*, 2004, Loizou *et al.*, 2004). The discovery of the CK2 motif cluster on MDC1, and comparisons to XRCC1 and XRCC4 function led to the hypothesis that these residues may be phosphorylated by CK2 and permit binding, and therefore the recruitment of other DNA DSB repair factors, in a mechanism that echoes that seen with XRCC1/XRCC4. Therefore the aims and objectives of this study were:

i) *Verify the putative CK2 sites of MDC1, using in vitro and in vivo techniques;*

ii) *Identify potential binding partners of MDC1, whose interaction may be mediated by these motifs;*

iii) *Investigate other CK2 substrates involved in the DDR.*

CHAPTER 2

Chapter 2 Materials and Methods

2.1 Molecular biology techniques

2.1.1 Bacterial strains

Strain	Genotype
DH5α (Invitrogen)	F- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rk-, mk+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1
Stbl2 (Invitrogen)	F- *mcr*A Δ(*mcr*BC-hsdRMS-*mrr*) *rec*A1 *end*A1*lon gyr*A96 *thi sup*E44 *rel*A1 λ- Δ(*lac-pro*AB)
BL21 (Stratagene)	E. coli B F– dcm ompT hsdS(rB– mB–) gal
XL1-Blue supercompetent cells (Agilent)	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB lacIZΔM15 Tn10 (Tet)]

2.1.2 Media

Luria Broth (LB): LB was purchased from Sigma and was made up to a concentration of 25 g per litre, pH 7.3-7.5, and sterilised prior to use via autoclaving.

LB-agar plates: Prior to sterilisation LB was supplemented with 1.2% agar (Sigma) per 100 mls. Once sterilised, the LB-agar was melted, allowed to cool, and the relevant antibiotic added.

2.1.3 Antibiotics

Ampicillin (Sigma) was added to LB and LB-agar at a concentration of 50 µg/ml.

Kanamycin (Roche) was added to LB and LB-agar at a concentration between 25-50 µg/ml.

2.1.4 Primer design

All primers were purchased from Sigma at a concentration of 0.5 µmol and pre-purified via desalting. Primer lengths varied according to the method used, but were generally 25-30 base pairs (bp) in length, with an A/T and C/G content of 50%. On arrival primers were kept at a stock concentration of 1 µg/µl at -20 °C.

2.1.5 Enzymes

Taq polymerases were either obtained from Stratagene or Roche. ABI PRISM® BigDye Terminator v 3.0 Cycle Sequencing Ready Reaction kit with ampli-Taq DNA Polymerase was purchased from Applied Biosystems (ABI), Perkin Elmer.

All restriction enzymes were either purchased from New England Biolabs (NEB) or Roche.

2.1.6 Transformation of bacteria

Transformation of plasmids into DH5α was carried out as follows: cells were thawed on ice, then between 20-50 ng of DNA were added and gently mixed. Next, cells were incubated on ice for 30 minutes, heat shocked at 42 °C for 30 seconds followed by further incubation on ice for 2 minutes. After addition of 400 µl of SOC media, (Invitrogen) cells were incubated at 37 °C for 1 hour at 200-220 rpm in a shaking incubator. Between 20-50 µl of the reaction was then spread on to pre-prepared LB-agar plates containing the relevant antibiotic and incubated over night at 37 °C.

For Stbl2 competent cells, 50 µl of cells were aliquoted into a pre-chilled 14 ml falcon tube and between 20-50 ng of DNA added, which was then gently mixed by swirling the tube. The cells were incubated on ice for 30 minutes, heat shocked for 25 seconds at 42 °C then placed on ice for a further 2 minutes. Next, 450 µl of SOC media (Invitrogen) was added to the cells which were then incubated for 1.5 hours at 30 °C shaking at 200-220 rpm. Following this, 20-50 µl of the reaction was spread on LB-agar plates and then incubated at 37 °C overnight.

When using BL21 competent cells, 100 µl of thawed cells were added to a pre-chilled 14 ml falcon tube followed by addition of 50 ng of DNA. Cells were then gently mixed by pipetting, incubated on ice for 30 minutes, heat shocked for 20 seconds at 42 °C and incubated on ice for 2 minutes. SOC media was pre-heated to 37 °C, then 900 µl was added to the cells which were then incubated at 37 °C for 1 hour shaking at 200-220 rpm. After this last step 20-50 µl of the reaction was plated out on LB-agar plates supplemented with the relevant antibiotic, and incubated overnight at 37 °C.

Gene	Vector	Source
MDC1 (aa1-381) WT	pGEX4T-1	PCR from cDNA
MDC1 (aa341-752) WT	pGEX4T-1	PCR from cDNA
MDC1 (aa141-621) WT	pGEX5X-3	Digested from pEGFP-C2 MDC1
MDC1 (aa141-621) CK2 S>A 1	pGEX5X-3	
MDC1 (aa141-621) CK2 S>A 2	pGEX5X-3	
MDC1 (aa141-621) CK2 S/T>A 3	pGEX5X-3	
MDC1 (aa141-621) CK2 S/T>A 4	pGEX5X-3	
MDC1 (aa141-621) CK2 S/T>A 5	pGEX5X-3	
MDC1 (aa141-621) CK2 S/T>A 6	pGEX5X-3	
MDC1 (aa141-621) CK2 S/T>A 7	pGEX5X-3	
MDC1 (aa141-621) CK2 S/T>A 8	pGEX5X-3	
MDC1 (aa141-621) CK2 S>A 9	pGEX5X-3	
MDC1 (aa141-621) CK2 S>A 10	pGEX5X-3	
MDC1 WT FL	pEGFP-C2	
MDC1 ΔCK2	pEGFP-C2	Dr G Stewart
MDC1 CK2 S>A 3-8	pEGFP-C2	
MDC1 CK2 S>A 1+3-8	pEGFP-C2	
hMRE11 (aa360-708) WT	pGEX4T-1	Xiaohua Wu (TRSI, California, USA)
hMRE11 (aa360-708) 1A	pGEX4T-1	
hMRE11 (aa360-708) 2A	pGEX4T-1	
hMRE11 (aa360-708) 3A	pGEX4T-1	
hMRE11 (aa360-708) 4A	pGEX4T-1	
hMRE11 WT	pCLNC	Dr M Weitzman
hMRE11 1A	pCLNC	
hMRE11 2A	pCLNC	
hMRE11 3A	pCLNC	
hMRE11 4A	pCLNC	
MRE11 WT	pCDNA5 FRT	Digested from pCLNC MRE11 using AscI and NotI
hMRE11 1A	pCDNA5 FRT	
hMRE11 2A	pCDNA5 FRT	
hMRE11 3A	pCDNA5 FRT	
hMRE11 4A	pCDNA5 FRT	

Table 2.1 ***DNA constructs used in the study***

2.1.7 DNA constructs

A list of all DNA constructs used in this study can be found in Table 2.1.

2.1.8 Small-scale preparation of DNA constructs

Individual colonies were picked from positive LB-agar plates and cultured in 150 µl of LB media containing antibiotics in a 96 well plate, for approximately 3 hours or until turbid. Next 10 µl of this culture was used to inoculate 5 mls of LB media which was then shaken at 200-220 rpm at 37 °C overnight. Following this, 3 ml of the culture was pelleted by centrifugation at 12000 g for 1 minute. All small-scale preparation of plasmid DNA was performed using the Sigma GenElute™ Plasmid Miniprep Kit as per instructions supplied by the manufacturer.

The DNA was then stored at -20 °C.

2.1.9 Large-scale preparation of DNA constructs

Briefly, 10 µl of culture from the previously described 96 well plate was used to inoculate 10 ml of LB media supplemented with antibiotic, and incubated at 37 °C overnight shaking at 200-220 rpm. The following day 250 µl of the overnight culture was used to inoculate 200 ml of LB media plus antibiotic, which was further incubated overnight at 37 °C shaking at 200-220 rpm. The bacteria were then pelleted at 4000 g for 10 minutes. All maxipreps were carried out using the Invitrogen PureLink™ HiPure Plasmid Filter Purification Kit. Next, the eluted DNA was split into two falcon tubes and 5.25 ml of isopropanol added, and mixed. The DNA was precipitated by centrifugation at 5000 g for 1 hour. The isopropanol was carefully removed and the pellet resuspended in 5 ml of 70% ethanol and combined with the second pellet from the same sample. The DNA was then spun for a further hour at 5000 g, the ethanol

removed and the pellet air-dried. The pellets were then rehydrated using between 300-500 µl of DNase-free water and stored at -20 °C.

2.1.10 Measuring DNA concentrations

All DNA concentrations were measured using the Nanodrop 1000 (Thermo Scientific) spectrophotometer. Briefly, 1 µl of DNA was loaded on to the pedestal, after blanking and calibrating the apparatus with distilled water according to the manufacturers instructions, and the concentration in ng/µl and $^{260/280}$ ratio noted.

2.1.11 Cloning

2.1.11.1 PCR of a gene sequence from plasmid DNA constructs

Primers were designed to the 5' and 3' regions of the gene of interest. For sub-cloning purposes the primers were engineered to also have unique 5' restriction sites that were present in the target vectors multiple cloning site but did not appear within the gene of interest. Primers for cloning were generally 25-30 bp long. A list of primers used for sub-cloning can be found in Table 2.2. 10-20 ng of plasmid DNA was mixed with 250 ng of forward and reverse cloning primers, 5 µl of 10x buffer, 1.5 µl of dNTPS (10 mM), 1 µl of *pfuUltra* DNA polymerase (Stratagene) and injection water, to a final volume of 50 µl. The PCR reaction was then mixed by pipetting, placed in a thermal cycler (Applied Biosystem Veriti 96 Well Thermal Cycler) and subjected to the following conditions: 94 °C for 3 minutes, proceeded by 37 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds, 68 °C for 1 minute/Kb followed by 68 °C for 10 minutes.

Gene	Sequence	Restriction Site
MDC1 Fragment 1 Forward (aa1-381)	5'CATGCAGTCGACTGATGGAGGACACCCA GGCTATTGAC 3'	Sal1 GTCGAC
MDC1 Fragment 1 Reverse (aa1-381)	5'ATCAGCGCGGCCGCCACATCTGTATCACT ACCAGCCTG 3'	Not1 GCGGCCGC
MDC1 Fragment 2 Forward (aa341-725)	5'GCATCGGTCGACTGACCCCAGTTGTCATT CCTATGAAG 3'	Sal1 GTCGAC
MDC1 Fragment 2 Reverse (aa341-725)	5'CGTATCGCGGCCGCCAACATGAAGGCCT GGGTAGGTTC 3'	Not1 GCGGCCGC
MRE11 FRT Forward	5'5'TCATGAGGCGCGCCAATGAGTACTGCA GATGCACTTGAT 3'	Asc1 GGCGCGCC
MRE11 FRT Reverse	5'CAAACCGCGGCCGCTTATCTTCTATTTTT CTTCTTAAGA 3'	Not1 GCGGCCGC

Table 2.*2* ***Primers used for subcloning genes***

2.1.11.2 Visualising PCR products via agarose gel electrophoresis

Purified PCR products were visualised on 0.8% agarose gel. Firstly, 100 ml of 1X TBE (880 mM tris, 890 mM orthoboric acid, 20 mM Na_2EDTA) buffer containing 0.8 g of agarose (Sigma) was heated, and 1 µl of ethidium bromide added to a final concentration of 1 µg/ml. The solution was poured into a gel caster and allowed to cool. Next 1 µl of sample was mixed with 2 µl of loading buffer (30% v/v of glycerol, 0.25% w/v of bromophenol blue in injection quality water) and 7 µl of 0.25x TBE buffer and loaded along with a DNA marker (Roche). The agarose gel was placed in 1X TBE buffer and ran at 120 V for 45 minutes depending on the product size. DNA was visualised using the G:BOX gel doc system (Syngene).

2.1.11.3 Gel extraction

DNA generated from PCR reactions was exercised and purified from agarose gels using the QIAquick Gel Extraction kit (Qiagen) as per the manufactures instructions.

2.1.11.4 Restriction digest and ligation

Restriction digests were either carried out on PCR products harbouring engineered restriction sites or plasmid DNA. Reactions were usually performed at 37 °C for approximately 1 hour and contained 100 ng to 3 µg of PCR or plasmid DNA, 5 µl of enzyme-specific 10x buffer and 10 units of the restriction enzyme. If two enzymes were required for the reaction and the buffers for a double digest were not compatible, a sequential digest was performed, which involved passing the first reaction through a PCR clean up column (Roche) before adding the second buffer and enzyme. Plasmid DNA was treated with alkaline phosphatase (1 unit/µl) to prevent religation, which was added to the final ligation reaction.

Pre-digested PCR product and vector DNA were ligated overnight at 11-14 °C. The total reaction volume was 20 µl comprising of 2 µl 10x buffer, 50 ng of vector, the relevant amount of PCR insert, 1 µl of T4 ligase (Invitrogen) and injection water. The following day, 3 µl of the ligation was transformed into competent cell lines as described above.

2.1.11.5 Screening of colonies via bacterial PCR

Colonies were picked and cultured in a 96 well plate as mentioned. After 3 hours, 1 µl of the culture was taken and added to 2.5 µl of 5x buffer+Mg^{2+}, 0.75 µl of dNTPS (10 mM), 1 µl of forward and reverse primers (250 ng) and 0.5 µl of Taq polymerase (Roche). Injection water was added to a final volume of 25 µl. The PCR conditions were as follows: 1 cycle of 94 °C for 4 minutes, 37 cycles of 94 °C for 15 seconds, 60 °C for 30 seconds, 68 °C for 3 minutes, then 1 cycle of 68 °C for 10 minutes. Positive colonies were screened using agarose gel electrophoresis.

2.1.11.6 Sequencing

Sequencing was required to verify the ligated PCR product was in the correct reading frame and that no mutations had occurred during the PCR reaction. A kit from ABI was used for all reactions. Firstly 200-500 ng of purified PCR product was added to 3.5 µl of 2.5x sequencing buffer, 125 ng of primer (Table 2.3), 1 µl of terminator ready reaction mix (Big Dye™ terminator V3.0) and made up to a final volume of 20 µl with injection grade water. Samples were then placed in a thermal cycler and subjected to the following parameters: 95 °C for 10 seconds, 50 °C for 5 seconds and 60 °C for 4 minutes, the conditions were repeated over 30 cycles. Following this step the PCR products were precipitated. This was achieved by adding 2 µl of 3 M sodium acetate, pH5.2, and 50 µl of 100% ethanol to the reaction and incubating at room temperature for 15 minutes. Samples were then spun at 15000 g for 20 minutes, the

Primer	Sequence
pGEX4T1/pGEX5X-3 Forward	5' GGGCTGGCAAGCCACGTTTGGTG 3'
pGEX4T1/pGEX5X-3 Reverse	5' CCGGGAGCTGCATGTGTCAGAGG 3'
pEGFP-C3 Forward	5' CCTGAGCAAAGACCCCAACGAGAAG 3'
pEGFP-C3 Reverse	5' CCCCCTGAACCTGAAACATAAAATG 3'
pCLNC Forward	5' CTCCATAGAAGACACCGGGACCGA 3'
pCLNC Reverse	5' TTGATCTGAACTTCTCTATTCTCA 3'
pCDNA3 Forward	5' GGCAAATGGGCGGTAGGCGTG 3'
pCDNA3 Reverse	5' TAGAAGGCACAGTCGAGG 3'
MDC1 1B Forward	5' ACAACATGCAGAGATTGAA 3'
MDC1 2 Forward	5' TTCATCGACAGCGACACTGATGCG 3'
MDC1 2B Forward	5' AACAGCGATACAGATGACGAGG 3'
MDC1 1 Reverse	5' AGGGCCCCGGGAGACAAAGGGCAGGAGG 3'
MDC1 1C Reverse	5' TACCATACGCCTTTCAGAAAG 3'
MDC1 2A Reverse	5' CACATCTGTATCACTACCAGCCTG 3'
MDC1 2B Reverse	5' CTGCAGAAGGACCACACGTTG 3'
MDC1 2D Reverse	5' TGTGTTGATGTCCACTGTGGTG 3'
MRE11 19235 Forward	5' AGTATTCATGGCAATCATGACG 3'
MRE11 19237 Forward	5' TCCTCTTCACACAGTGCGGCAG 3'
MRE11 21464 Forward	5' CCAGAGAAGCCTCTTGTACGACTG 3'
MRE11 19239 Forward	5' GAACGTCATATTGATGCCCTCG 3'
MRE11 21584 Forward	5' AGGAGGGTCTCAAAGAGGAAG 3'
MRE11 22219 Forward	5' CTACCCGTAGCAGGAACTCAAAGAC 3'
MRE11 19236 Reverse	5' TCTATCTTCTCCACAGACATTG 3'
MRE11 19238 Reverse	5' AAACAGAAGCTTTGTATGGCTTG 3'
MRE11 19240 Reverse	5' GCCCTGGTCATAGCCTCACGGAC 3'
MRE11 19242 Reverse	5' TCTTCCACATCTGATTCATCTAC 3'

Table 2.3 ***Sequencing primers used in the study***

supernatant was removed and 250 µl of 70% ethanol added, briefly vortexed and then spun again at 15000 g for 5 minutes. Following removal of the supernatant, samples were either dried in an air cabinet or using a vacuum centrifuge. Before loading, 10 µl of formamide was added to the sample, which was then vortexed, heated for 5 minutes at 95 °C and then quenched on ice. The reaction was then loaded onto the ABI Prism 337 Automated DNA sequencer (Perkin Elmer, Applied Biosystems). Sequences were analysed using a combination of FinchTV (http://www.geospiza.com/Products/finchtv.shtml) and the NCBI nucleotide–nucleotide BLAST analysis (http://www.ncbi.nlm/nih.org).

2.1.12 Mutagenesis

All mutagenesis reactions were carried out using a Stratagene QuickChange® Site-Directed Mutagenesis Kit (Stratagene). This contained the DNA polymerase, dNTPs and the competent cells required.

2.1.12.1 Primer design

Table 2.4 lists all primers used for mutagenesis in these studies. Primers were designed to contain either a single point mutation, a number of mutations or deletion of an entire region of the gene of interest. The primers were constructed in such a way that both the forward and reverse contained the mutation and annealed to the same region on the opposite strands of the sequence of interest. Primers were between 25-45 bp in length, the mutation(s) being located in the middle of the primer with approximately 10-15 bases of sequence either side, had a minimum G/C content of 40% and a melting temperature of ≥78 °C. The following calculation was used to determine the melting temperature:

$$T_m = 81.5 + 0.41(\%GC) - 675/N - \%\ mismatch$$

Where *N* is primer length in bases, and values for %G/C and % mismatch are whole numbers.

Primer	Sequence
MDC1 S/T>A 1 Forward	5' CCGCCTTTTGCCTTCAAT TTGAACGCTGACGCAGATGTGGAAG 3'
MDC1 S/T>A 1 Reverse	5' CTTCCACATCTGCGTCAGCG TTTCAAATTGAAGGCAAAAGGCGG 3'
MDC1 S/T>A 2 Forward	5' CCTCCTGGAGAGGACGC TGACGCAGATGTGGATGATGAC 3'
MDC1 S/T>A 2 Reverse	5' GTCATCATCCACATCTG CGTCAGCGTCCTCTCCAGGAGG 3'
MDC1 S/T>A 3 Forward	5' GGCTTCATCGACGCCGA CGCTGATGCGGAAGAAGAG 3'
MDC1 S/T>A 3 Reverse	5' CTCTTCTTCCGCATCAG CGTCGGCGTCGATGAAGCC 3'
MDC1 S/T>A 4 Forward	5' AGCCAGGCTGGTGCTGA TGCAGATGTGGAAGAAGGC 3'
MDC1 S/T>A 4 Reverse	5' GCCTTCTTCCACATCTGC ATCAGCACCAGCCTGGCT 3'
MDC1 S/T>A 5 Forward	5' GCTTCCATGGTTATCAAC GCCGATGCAGATGACGAGGAAGAAG 3'
MDC1 S/T>A 5 Reverse	5' CTTCTTCCTCGTCATCTG CATCGGCGTTGATAACCATGGAAGC 3'
MDC1 S/T>A 6 Forward	5' ACCACTGAGAGAGACGC TGACGCAGACGTGGAGGAG 3'
MDC1 S/T>A 6 Reverse	5' CTCCTCCACGTCTGCGTCA GCGTCTCTCTCAGTGGT 3'
MDC1 S/T>A 7 Forward	5' TTGGCTGAGGACGCGGAG GAGGAAGTAGATTTTC 3'
MDC1 S/T>A 7 Reverse	5' GAAAATCTACTTCCTC CTCCGCGTCCTCAGCCAA 3'
MDC1 S/T>A 8 Forward	5' TCTGTGATAGTTCCAG AGGCTGATGAAGAGGGGCATTCC 3'
MDC1 S/T>A 8 Reverse	5' GGAATGCCCCTCTTC ATCAGCCTCTGGAACTATCACAGA 3'
MDC1 S/T>A 9 Forward	5' AGAAGCCAAGCCTCCGCC ACAGTGGACATCAAC 3'
MDC1 S/T>A 9 Reverse	5' GTTGATGTCCACTGTG GCGGAGGCTTGGCTTCT 3'
MDC1 S/T>A 10 Forward	5' GGGGACTGTGAAGCA GATGCAGAGGAGGGCACC 3'
MDC1 S/T>A 10 Reverse	5' GGTGCCCTCCTCTGC ATCTGCTTCACAGTCCCC 3'
MRE11 S649A Forward	5' GTGATTGAGGTAGA TGAAGCAGATGTGGAAGAAGACA 3'
MRE11 S649A Reverse	5' TGTCTTCTTCCACATC TGCTTCATCTACCTCAATCAC 3'
MRE11 S688/689A Forward	5'TCGAAAGGGGGTTGATTTTGAAGCA GCTGAGGATGATGATGATGATCCT 3'
MRE11 S688/689A Reverse	5'AGGATCATCATCATCATCCTCAGCTG CTTCAAAATCAACCCCTTTCGA 3'
MRE11 S541A Forward	5' GAGTCTGCTTCTGCCTTTGCTGCTGATG ACCTTATGAGT 3'

Table 2.4 ***Mutagenesis primers used in the study***

2.1.12.2 PCR

Briefly, 5-50 ng of template DNA was mixed with 5 µl of 10x reaction buffer, 1 µl of dNTP mix, 125 ng of forward and reverse primer, 1 µl of *PfuUltra* HF DNA polymerase (2.5 U/µl) and made up to 50 µl with injection water. Reactions were then placed in a thermal cycler and subjected to the following conditions, 95 °C for 30 seconds, 95 °C for 30 seconds, 55 °C for 1 minute then 68 °C for 1 minute/per Kb of plasmid length. The last three cycling parameters were dependent on the type of mutation required.

2.1.12.3 Digestion and transformation

Following PCR, 1 µl of *Dpn1* was added to the reaction and mixed by pipetting followed by incubation at 37 °C for approximately 3 hours. For transformation, XL1 Blue Supercompetent Cells were thawed on ice and 50 µl aliquotted into a 14 ml falcon tube, 1 µl of the digested mutagenesis reaction was added to the cells, mixed by gentle swirling and then allowed to incubate on ice for 30 minutes. Reactions were then heat-shocked at 42 °C for 45 seconds and left on ice for a further 2 minutes. After the addition of 500 µl of SOC media the cells were incubated at 37 °C for 1 hour shaking at 200-220 rpm. The entire transformation reaction was then plated on two LB-agar plates and left overnight at 37 °C. Clones were then picked and screened as described above.

2.1.13 GST purification

GST plasmids containing sequences of interest were transformed into BL21 competent cells as described above. Colonies were picked and cultured in 150 µl of LB media for 3 hours. Next 10 µl of the culture was used to inoculate 10 ml of LB media which was grown overnight at 37 °C shaking at 200-220 rpm. This was used to inoculate 1L of LB media which was then grown for 3 hours at 37 °C shaking at 200-220 rpm or until an OD of between 0.3-

0.4 was reached. The culture was then induced with 1 mM of IPTG overnight at room temperature shaking at 200-220 rpm. Cultures were then pelleted at 8,000 g for 15 minutes, and then lysed by sonication (5x for 30 seconds on ice) in PBS containing 1% Triton X-100 supplemented with Complete Protease Inhibitor Cocktail (Roche). If the protein induced was insoluble then pellets where lysed in SSTE buffer (1.5% Sarcosyl, 50 mM Tris, 120 mM NaCl, 1 mM EDTA). The bacterial lysate was then spun at 48,000 g for 10 minutes, the supernatant transferred to a fresh tube and spun again for a further 20 minutes at 48,000 g . The supernatant was then filtered through a 0.45 μM syringe filter. For samples that were insoluble 1/5th the supernantant volume of 20% trition was added to re-anneal proteins. 4 ml of glutathione sepharose beads were pre-washed three times in PBS containing 1% Triton X-100 and twice in PBS containing 1 mM EDTA. The washed beads were resuspended in PBS containing 1 mM EDTA to give 8 mls of 50% beads slurry of which 4 mls was then added to the lysate. The lysate was then rotated at 4 °C for 2 hours. The lysate was removed and the beads washed three times in PBS containing 1 mM EDTA and 1% Triton X-100 and twice in PBS containing 1 mM EDTA. Following this, 4 ml of elution buffer (25 mM glutathione pH8.0, 50 mM Tris, pH8.0) was added to the beads, which were then rotated at 4 °C for 1 hour. The beads were pelleted, the eluent removed and a further 4 ml of elution buffer added to the beads, which were again rotated at 4 °C for 1 hour. The beads were again pelleted and the eluent removed and combined with the first elution. This was then added to dialysis tubing (MWCO 12-14 Daltons) which had been boiled in 2 mM EDTA, 3% Sodium Bicarbonate for 1 minute, and dialysed in 5 litres of dialysis buffer (50 mM Tris pH7.5, 10%v/v glycerol and 1 mM DTT) overnight at 4 °C. The following day the protein concentration was determined using Bradford reagent (Biorad) as described.

2.1.14 Generation of cDNA for sequencing

2.1.14.1 RNA extraction and purification

Cells were harvested and pelleted at 4 °C for 5 minutes at 600 g. RNA extraction was carried out using the Qiagen RNeasy® Mini Kit. Firstly pelletes were disrupted by flicking, and then lysed in RLT buffer by passing the lysate a minimum of five times through a 20-gauge needle. One volume of 70% ethanol was then added to the lysed and pellet, which was then transferred to a RNeasy spin column and centrifuged for 15 seconds at 8, 000 g. The through flow was discarded and 700 µl of buffer RW1 was added to the column, which was again spun at the above conditions. Next 500 µl of buffer RPE, which previous to use had been diluted with 4 volumes of ethanol, was added to the column following the discard of the through flow, followed by further centrifugation. Again the through flow was discarded and a further 500 µl of buffer RPE added to the column which was then spun for 2 minutes at 8, 000 g. The column was then placed in a fresh RNase free collection tube and 30 µl of RNase free water added and the tube span at maximum speed for 1 minute. This step was repeated to ensure maximum yield of RNA from the prep. The concentration of RNA extracted, in ng/µl, was then determined using a Nanodrop 1000 (Thermo Scientific) spectrophotometer as described. RNA samples were then stored at -80 ºC for further use.

2.1.14.2 Production of cDNA

cDNA was generated using the Invitrogen SuperScript® VILO™ cDNA Synthesis Kit. Briefly up to 2.5 µg of RNA was added to 4 µl of 5X VILO™ Reaction Mix, 2 µl 10X SuperScript® Enzyme Mix and made up to a volume of 20 µl of DEPC-treated water in an RNase-free tube. The contents were mixed and then incubated at 25 ºC for 10 minutes, 42 ºC for 60 minutes and followed by termination of the reaction at 85 ºC for 5 minutes. The cDNA was either

stored at -20 ºC or used in a PCR with primers specific to the gene or interest, followed by sequencing of the PCR product.

2.2 Tissue culture techniques

2.2.1 Cell lines

Cells used in this study are listed in Table 2.5. The HCT116 Flp-In/T-Rex cell line was received as a kind gift from Dr Daniel Durocher, Toronto, Canada. The U2OS Flip-In/T-Rex were a gift from Jakob Nilsson's laboratory at the Novo Nordisk Foundation Centre for Protein Research, University Copenhagen.

2.2.2 Tissue culture media and solutions

Cells were either cultured in Dulbecco modified eagle medium (DMEM) or McCoy's 5A medium supplemented with 10% foetal calf serum (FCS), 30,000 units penicillin and 3,000 mg streptomycin (pen/strep). When necessary cells were cultured in the presence of combinations of selective antibiotics (Geneticin (Invitrogen), Puromycin (Clontech), Blasticidin (Invitrogen), Hygromycin (Roche)). All media and reagents were obtained from Invitrogen. Media was stored at 4 °C for up to 3 months.

2.2.3 Maintenance of cells

Cells were incubated at 37 °C in humidified incubators supplied with 5% CO_2. Cells were allowed to reach confluency before passage, at which stage all media was removed, and the

Name	Type	ATCC number
HeLa	Human cervical carcinoma cell line.	
ATLD2	Human skin fibroblasts hypomorphic mutation in MRE11.	
HCT116	Human colon carcinoma missense mutation in MRE11.	CCL-247™
HT29	Human colon adenocarcinoma.	HTB-38™
HCT116 Flp-In™ T-rex	Human colon carcinoma missense mutation in MRE11. Containing an integrated FRT site.	
U2OS Flp-In™ T-rex	Human osteosarcoma cell line. Containing an integrated FRT site.	

Table 2.5 ***Human cells lines used in the study***

Target Protein	Name	Sense Sequence
Non-silencing	**Control siRNA**	**CGUACGCGGAAUACUUCGAdTdT**
CK2α	CK2α siRNA	GAUGACUACCAGCUGGUUCdTdT
CK2α'	CK2α' siRNA	CAGUCUGAGGAGCCGCGAGdTdT
MRE11	MRE11 5'UTR	GAACCUGGUCCCAGAGGAGdTdT

Table 2.6 ***siRNAs used in the study***

	6%	10%
H2O	27.4 ml	22 ml
1 M Tris/1 M Bicine	4 ml	4 ml
Acrylamide 30%	8 ml	13.4 ml
SDS 10%	400 µl	400 µl
TEMED	80 µl	80 µl

Table 2.7 ***SDS gel components***

cells were washed with warmed, sterile PBS. To detach the cells the PBS was removed, 1.5 ml of 1x trypsin (Invitrogen) added and the cells incubated at 37 °C for a maximum of 5 minutes. Tapping the flask dislodged any cells still adhering to the flask. Fresh warmed media was added to halt the trypsinisation and the appropriate amount of cells transferred to a new flask containing fresh media. Cells were routinely checked for infection by Mycoplasma using the MycoAlert™ detection kit (Lonza) as per manufacturers instructions.

2.2.4 Cryopreservation of cell lines

Cells were washed and trypsinised as above. Upon the addition of media, the cells were transferred to a sterile 15 ml falcon tube and centrifuged at 596 g for 5 minutes. The media was removed and the cell pellet resuspended in 1 ml of 50% FCS, 40% media, containing no FCS or antibiotics, and 10% DMSO (Sigma). The cells were transferred to a 1.5 ml cryovial and cooled slowly at -80 °C in isopropanol for at least 24 hours. The frozen aliquots were then placed in liquid nitrogen for long-term storage at -180 °C. When required, cells were thawed rapidly at 37 °C, added to fresh media supplemented with FCS and antibiotics, pelleted by centrifugation, resuspended in fresh media and plated out.

2.2.5 Transient transfection of DNA constructs into cell lines

Firstly, $3x10^5$ cells were plated per well of a 6-well plate in media lacking antibiotics a day prior to the transfection. The following day 0.8 µg plasmid DNA and 6 µl of Lipofectamine (Invitrogen) was added to 50 µl of Opti-MEM I Reduced Serum Medium (Invitrogen) separately, and allowed to incubate at RT for 5 minutes. The DNA/Opti-MEM mixture was then added to the Lipofectamine/Opti-MEM, mixed gently and incubated for 20 minutes at RT. The DNA/Lipofectamine mixture was then added to the cells which were slowly shaken to evenly disperse the transfection cocktail and left for 4 hours at 37 °C. The media was then

removed from the cells and replaced with fresh media lacking antibiotics. Western blotting or immunofluorescence between 24-72 hours later monitored exogenous expression of the gene of interest.

2.2.6 Generation of stable cell lines via retroviral transfection

On the first day, 293T cells were seeded at a density of approximately $1x10^7$ on a 10 cm dish in the relevant media lacking antibiotics but supplemented with 10% FCS. Cells were then left overnight at 37 °C. The following day the cells were transfected with Lipofectamine 2000 (Invitrogen) using a ratio of 3 µl of reagent per 1 µg of DNA. The DNA ratio added was, 4 µg retroviral backbone, 6 µg GAG/Pol (psPax2) plasmid and 2 µg VSV-G (pMD2.G) plasmid.

Briefly 36 µl of the Lipofectamine reagent was added to 1.5 ml Opti-MEM and mixed by gentle pipetting, followed by incubation at RT for 5minutes. The DNA was added to 1.5 ml of OPTI-MEM, mixed by gentle pipetting and then combined with the Lipofectamine/OPTI-MEM mix. The transfection mixture was then incubated at RT for 20 minutes. This was then added drop wise onto the cells in 5 ml of complete media lacking antibiotics. The cells were then rocked gently to ensure even distribution of the transfection mixture and left overnight at 37 °C.

The next day the media was removed from the cells and replaced with 5 ml of fresh media lacking antibiotics. On day three the viral supernatant was harvested, filtered through a 0.45 µM syringe filter to remove any contaminating cells and was stored at 4 °C overnight. Fresh media was added to the cells. Additionally on the third day, the target cells were plated out to 50% confluency on 6 cm dishes. The following day, the second lot of viral supernatant was harvested and combined with the previous days supernatant, which was then aliquoted and stored at -80 °C. On the same day polybrene was added to final concentration of 4 µg/ml to 2

ml of viral supernatant per 6 cm dish and mixed gently by pipetting. The media from the target cells was removed and replaced with the retroviral supernatant/polybrene mixture. The cells were then left overnight at 37 °C. The virus was removed on day 5 and the previous days steps repeated with fresh virus. On day 6 the virus was removed again and the dishes washed once with complete media, followed by the addition of fresh media. Finally on day seven the media was removed and replaced with media containing the selective antibiotic. These were then left to allow outgrowth of antibiotic resistant cells. Expression of the virally-encoded protein was determined by Western blotting. Cells were cloned to allow for isolation of a single population of cells containing equal levels of expressed protein.

2.2.7 Generation of stable Flp-In/T-Rex cell lines

Host Flp-In/T-Rex cells containing an integrated FRT recognition site were plated out at a density of $3x10^5$ in DMEN media, supplemented with zeocin (15.5 μg/ml), blasticidin (5 μg/ml) and 10% FCS. The cells were left overnight at 37 °C. The following day the host Flp-In cell lines were transfected with a modified pcDNA5 FRT TO vector containing a pcDNA3 flag-tagged polylinker, (received from the Durocher Laboratory, University of Toronto, Canada) in addition to the gene of interest, and a pOG44 vector. Again a ratio of 3:1, 36 μl of Lipofectamine 2000 to 12 μg of DNA, was used for each transfection. However the 12 μg of DNA comprised of both the pcDNA5 FRT TO vector, containing the gene of interest and the pOG44 vector. For the purpose of this transfection protocol, 2 μg of pcDNA5 FRT vector was mixed with 10 μg of pOG44 vector. The DNA and Lipofectamine were added to 1.5 ml of Opti-MEM and the separate reactions incubated for 5 minutes. Following this the DNA/Opti-MEM mix was mixed with the Lipofectamine/ Opti-MEM mix and incubated for a further 20 minutes. During this incubation the media was removed from the cell lines and 5 ml of

DMEN added, supplemented with 10% FCS, but no antibiotic. The Lipofectamine/DNA reaction was then spotted on to the cells and the cells then left for 48 hours. After 48 hours the media was removed and replaced with DMEN containing hygromycin (250 μg/ml), blasticidin (5 μg/ml) and 10% FCS. The cells were then left in the selection media to allow outgrowth of those non-resistant cells. Once the cells had reached confluency they were split, allowed to grow again, then a proportion taken to test induction of the gene of interest by addition of doyxcycline (1 μg/ml), and the remainder frozen down for future experiments.

2.2.8 Cell irradiation

Cell lines were mock-treated or treated with ionising γ-rays from a ^{137}Cs source at a dose rate of approximately 1 Gy per 20 seconds and then incubated at 37 °C.

2.2.9 Treatment of cells with DNA damaging agents

For cell lines treated with camptothecin (CPT), growth media was completely removed and replaced with media supplemented with 100 nM camptothecin. Cells were then incubated at 37 °C and harvested at relevant timepoints.

2.2.10 Transfection of cell lines with siRNA

The required number cells, dependent on type, were plated per 60 mm dish, in media lacking antibiotics the day before the transfection. The following day media was removed, the cells washed once in 2 mls Opti-MEM warmed to 37 °C and then 1.6 ml of Opti-MEM was added to each dish. Next two mixtures were made, one containing 10 μl siRNA (stock 20 μM) and 360 μl of Opti-MEM per dish, and the other containing 8 μl oligofectamine (Invitrogen) and 22 μl of Opti-MEM per dish. These two volumes were left at room temperature for 5 minutes, and then mixed, adding the oligofectamine/Opti-MEM mixture to the siRNA/Opti-MEM

mixture, and left for a further 20 minutes at room temperature. Next, 400 µl of the siRNA/oligfectamine mixture was then drop-pipetted on to the cells. The dish was then rocked gently and incubated for 4-6 hours. Then 2 ml of media containing 20% FCS without antibiotics was added to each dish. Cells treated with siRNA were generally left for 72 hours to achieve sufficient depletion of target genes. Depletion of the target gene was assayed by Western blotting. All siRNA used in the study is listed in Table 2.6.

2.2.11 Cell extraction and protein purification

Cells were trypsinised as above, transferred to a 30 ml universal and spun at 596 g for 5 minutes to pellet. The media was then removed, the pellet washed in 10 ml of sterile PBS and spun for a further 5 minutes at 596 g. All the PBS was removed and the pellet re-suspended in approximately 50-200 µl of lysis buffer (8 M urea, 50 mM Tris, 150 mM β-mercaptoethanol, pH7.5). Cells were sonicated twice for 10 seconds and then spun at 25 000 g for 20 minutes at 4 °C. The supernatant was then transferred to a pre-chilled 1.5 ml tube, flash frozen in liquid nitrogen and stored at -80 °C.

2.2.12 G2/M checkpoint assay and cell cycle analysis

Cells were irradiated at indicated doses and harvested after 1 hr. During harvesting the media and wash PBS were kept until the cells were pelleted. Once trypsinised, washed and pelleted, the cells were resuspended in 3 ml of PBS. Cells were then fixed in 7 ml of ice-cold ethanol, which was added while vortexing, as this prevented the cells from clumping. The fixed cells could then be stored at -20 °C overnight or longer.

When required, the cells were pelleted at 596 g for 5 minutes and washed twice in PBS. The cells where then permeabilised in ice-cold PBS containing 0.25% Triton X-100 for 15 minutes. Following this, cells were washed in 10 mls of PBS with 1% BSA, then incubated with an anti-phospho-histone H3 (Ser 10) antibody (NEB) in 100 µl PBS/BSA for 1.5 hours at room temperature. The samples were then washed twice with 1ml PBS containing 1% w/v BSA, then resuspended in 100 µl of PBS/BSA containing fluorescein isothiocyante (FITC) conjugated anti-rabbit antibody (Invitrogen), and left in the dark at RT for 30 minutes. Cells were again washed in 1 ml PBS/BSA then 1 ml of PBS only, followed by re-suspension in 1 ml PBS containing propidium iodide (PI) (25 µg/ml) and 0.1 mg/ml of RNAse A, and incubated in the dark at RT. Fluoresence was then measured using a Coulter XL-MCL flow cytometer (Beckman-Coulter). For measuring cell cycle only, cells were harvested as described above, permeablised, washed in PBS and then PI and RNAse added to the sample.

2.3 Protein biochemistry techniques

2.3.1 Protein determination

The BioRad Bradford Reagent was used to measure the protein concentration of cell lysates. Briefly, a stock of BSA 1 mg/ml was diluted to give standards ranging from 0-500 µg/ml and 10 µl aliquotted into a well of a 96 well plate in triplicate. Cell lysates were usually diluted 1:10 in sterile water and 10 µl aliquotted into a well of a 96 well plate in triplicate. The protein determination reagent was diluted 1:5 with sterile water and 200 µl of this was added to both the standards and samples. Samples were then read in a plate reader using a wavelength of 595 nm (BioRad). Protein concentration was then calculated using Microsoft Excel.

2.3.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels were prepared with the percentage of acrylamide varying depending on the size of the protein being studied (Table 2.7). Generally, proteins over 50 kDa were run on a 6% Acrylamide gel. Proteins less than 50 kDa were run on a 10% Acrylamide gel. The Gel apparatus was purchased from GE Healthcare and assembled according to manufacturers instructions, with gels run in buffer containing 100 mM Tris/bicine (1 M Tris/1 M Bicine) and 1% SDS. Typically, 50 μg of cell lystate or purified protein was loaded on each gel. These were prepared by the addition of equal volumes of 2x Laemmli buffer and boiling for 5 minutes followed by centrifugation at 15,000 g for 1 minute. Molecular weight markers (GE Healthcare) were also loaded on gels for identification of protein size. Gels were run at 0.02 mA for approximately 5 hours. However, this varied depending on protein size.

2.3.3 Visualisation of proteins

Following separation of proteins, gels were placed in coomassie blue solution (0.1% Coomassie Brilliant Blue R-250 (Sigma), 40% methanol, 10% glacial acetic acid and water) to stain for 10 minutes at room temperature with agitation. Gels were then placed in a destaining solution (10% glacial acetic acid, 20% methanol and water) overnight at room temperature again with agitation. Destained gels were then dried in a vacuum gel dryer at 80 °C for 2 hours.

2.3.4 In-gel digestion of protein for analysis by mass spectrometry

Proteins were separated on pre-cast Tris/Glycine gels using the NuPAGE Novex system (Invitrogen) and stained with colloidal coomassie (0.08% Coomassie Brilliant Blue, 1.6%

orthophosphoric acid, 8% ammonium sulphate, 20% methanol) for 6 hours, then destained in 1% acetic acid in distilled water until the background was clear. Using a sterile scalpel blade, bands were excised form the gel and placed in a sterile 1.5 ml tube and washed twice with 100 µl of 50% acetonitrile/50 mM ammonium bicarbonate for 45 minutes at 37 °C with agitation. The gel slices were then dried in a DNA-Mini vacuum centrifuge for 30 minutes. Next, 50 µl of 50 mM DTT, diluted in 10% acetonitrile/50 mM ammonium bicarbonate, was added and the gel fragment incubated at 56 °C for 1 hour. The supernatant was removed and 50 µl of 10% acetonitrile/50mM ammonium bicarbonate containing 100 mM iodoacetamide added. The gel pieces were allowed to incubate at room temperature in the dark for 30 minutes. The samples were then washed 3 times with 10% acetonitrile in 40 mM ammonium bicarbonate for 15 minutes at room temperature with agitation. The gel slice was then dried in a DNA-Mini vacuum centrifuge for 30 minutes. To digest any fixed proteins the gel slice was re-hydrated in 20 µl of 12.5 µg/ml Promega seq. grade modified trypsin in 10% acetonitrile/40 mM ammonium bicarbonate for 1 hour at room temperature. To cover the gel slice, 20 µl of 10% acetonitrile/40 mM ammonium bicarbonate was added to the tube and then left to incubate overnight at 37 °C. The following day the supernatant was collected and transferred to a new 1.5 ml tube. 30 µl of 3% formic acid in water was then added to each gel slice and incubated at 37 °C for 1 hour. The supernatant was then removed and added to the previous supernatant. A further 30 µl of 3% formic acid was added to the gel slice with incubation at 37 °C for 30 minutes and then the supernatant collected again. The collected supernatant could then be stored at -20 °C until mass spectrometric analysis.

2.3.5 In vitro kinase assay

Purified GST fusion proteins were incubated with 5 µl of 5x kinase buffer (100 mM Tris, 250 mM KCl, 50 mM $MgCl_2$, 1 mM ATP pH7.5), 1 µl recombinant CK2 (500,000 U/ml) (NEB),

10 µCi ATP (α-^{32}P) (Perkin Elmer), 1 µg GST fusion protein, made up to a final volume of 25 µl with nuclease free water and incubated for 10 minutes at 30 °C. For mass spectrometry the radio-labelled ATP was omitted from the reaction. The reaction was then stopped with 8.3µl of 4x Laemmli buffer, boiled for 5 minutes then span at 16,000 g for 1 minute. The reaction was then run on an SDS-PAGE gel as described above. Gels were then stained, destained and dried as previously stated. The gels were then exposed to autoradiography film (GE Healthcare) for detection of radio-labelled proteins.

2.3.6 GST pull-down

Firstly, purified GST fusion proteins were incubated with 100 Units of recombinant CK2 (NEB). Following this, 5 µg of the purified protein was mixed with 200 µg of cell extract and incubated at 4 °C for 30 minutes to allow binding. Next 20 µl of Glutathione-Sepherase beads were added and the reaction incubated for a further 60 minutes at 4 °C. The beads were then washed three times with wash buffer (50 mM Tris, pH 7.5, 120 mM NaCl, 1 mM DTT, and 0.2% NP-40). Laemmli buffer was then added, and the samples boiled for 5 minutes and then run on an SDS-PAGE gel.

2.4 Immunochemistry techniques

2.4.1 Antibodies

All antibodies, dilutions and origins are listed in Table 2.8.

2.4.2 Western blotting

Following separation on SDS-PAGE, proteins were transferred onto nitrocellulose membrane (Thermo) using a Hoeffer Scientific Transfer system. In brief, gels along with the

nitrocellulose membrane were soaked in transfer buffer (20% Methanol, 50 mM Tris, 190 mM glycine), sandwiched between two pre-soaked pieces of Whatman 3 mm blotting paper and two pre-soaked sponges and then placed in a cassette. The cassette was then placed into a transfer tank and electro-transfer carried out at 200 mA for up to 18 hours at RT depending on protein size. The nitrocellulose was then stained with Ponceau S solution (Sigma) for 1-2 minutes and then washed in distilled water until the excess Ponceau was removed and protein bands became visible. The residual Ponceau was completely removed by washing in either PBS-T (1x PBS, 0.1% Tween) or TBS-T (200 mM Tris and NaCl, 0.1% Tween pH7.6) depending on the primary antibody. The membrane was then placed in 5% milk powder (Marvel) in PBS-T/ TBS-T for 2 hours, with agitation to block non-specific binding.

Following a brief wash, in either PBS-T or TBS-T, the blots were then incubated with primary antibodies diluted in 5% milk at the indicated dilution, amount of time and temperature, with agitation (Table 2.7). Next the blots were washed three times for 10 minutes in either PBS-T or TBS-T before incubation with the relevant horseradish peroxidase-conjugated secondary antibody (Dako) diluted in 5% milk/PBS-T/TBS-T for 1 hour at room temperature with agitation. After another three 10 minute washes in PBS-T/TBS-T, the blots were incubated for 1 minute with enhanced chemiluminescence (ECL) reagents (GE Healthcare) and proteins detected using GE Healthcare Hyperfilm.

2.4.3 Immunoprecipitation

Following trypsinisation, cells were washed in PBS, resuspended in 0.5 ml NETN buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1% NP40, 90 U/ml Benzonase (Novagen), 2 mM $MgCl_2$, supplemented with protease inhibitors (Roche)), transferred to a thick walled 1.5 ml centrifuge tube and rotated for 30 minutes at 4 °C. The Lysed cells were then spun at 84,000 g

for 30 minutes at 4 °C. Next, 25 µl pre–washed protein G or A beads (dependant on antibody subclass) and 10 µg of non-specific species-matched antibody were added to the supernatant to aid removal of any non-specific immunoglobulin/protein A/G-binding proteins, and rotated at 4 ºC for 1 hour. Samples were then spun at full speed in a microcentrifuge at 4 ºC for 5 minutes, and the supernatant transferred to a new tube. Next, 5-10 µg of immunoprecipitating antibody was then added followed by a 2-3 hour incubation at 4 °C with rotation. Lystates were then spun for 15 minutes at 84,000 g, the supernatant removed, 30 µl of pre-washed protein A/G beads added and rotated for 1-2 hours at 4 °C. Lastly, samples were spun at 833 g for 20 seconds and the supernatant removed. The immunoprecipitated proteins bound to the beads were washed in NETN (0.5% NP-40, without Benzonase) by inversion 4 times. Following the last wash, all the supernatant was removed with a syringe needle and 25 µl of Laemmli Sample Buffer added to elute the protein complexes. Samples were then boiled and either loaded onto a SDS-PAGE gel for separation and Western blotting or stored at -80ºC.

2.4.4 Immunofluoresence

Cells were trypsinised washed and seeded onto poly-L-lysine coated coverslips or 12-well glass slides and left overnight at 37 °C in the appropriate growth media. The following day, media was removed and cells fixed in either ice-cold methanol for 10 minutes, or permeablised, first in extraction buffer (10 mM pipes pH 6.8, 300 mM sucrose, 20 mM NaCl, 3 mM $MgCl_2$, 0.5% TX-100) and then fixed in 3.6% paraformaldahyde in PBS. Coverslips and slides were washed once in PBS at RT and then blocked in 10% filtered FCS/PBS for 1 hour at room temperature or overnight at 4 °C. Coverslips and slides were then washed twice with PBS. Primary antibodies were made up in 2% FCS/PBS to the relevant dilution, spotted onto the coverslips or wells on the glass slide and then incubated for 1 hour at room temperature (for antibody dilutions see Table 2.7). This was followed by three washes in PBS

and an hour incubation at RT in the dark with the secondary antibodies (Alexa-Fluor, Invitrogen) diluted to the appropriate concentration in 2% FCS/PBS (Table 2.7). The slides or coverslips were then washed a further three times in PBS. Slides and coverslips were then mounted in Vectashield Mounting Medium (Vector Laboratories) containing 4', 6-diamidino-2-phenylindole (DAPI) and the edges sealed with nail polish. Slides and coverslips were stored at 4 °C in the dark. Cells were viewed using a Nikon Eclipse E600 microscope using the Velocity imaging software.

Antibody	Antigen	Species	Application	Dilution	Source
KIAA0170	MDC1	Rabbit	IF	1:500	G.Stewart
BRAC1	BRAC1	Mouse	IF	1:500	Santa Cruz
NBS1	NBS1	Rabbit	IF	1:100	Merck
Phospho-H2AX Ser 139	Phospho-H2AX Ser 139	Mouse	IF WB	1:1000 1:1000	Millipore
H2A	H2A	Rabbit	WB	1:5000	Upstate
MRE11	MRE11	Rabbit	WB	1:1000	Abcam
NBS1	NBS1	Rabbit	WB	1:10000	Genetex
Phospho-NBS1 Ser 343	Phospho-NBS1 Ser 343	Rabbit	WB	1:500	Signalway
RAD50	RAD50	Mouse	WB	1:3000	Abcam
53BP1	53BP1	Rabbit	WB IF	1:1000 1:300	Novus Biologicals
Phospho-53BP1 Ser1778	Phospho-53BP1 Ser 1778	Rabbit	WB	1:1000	Cell Signalling
11G12	ATM	Mouse	WB	1:500	M.Taylor
Phospho-ATM Ser 1981	Phospho-ATM Ser 1981	Mouse	WB IF	1:1000	Upstate
SMC1	SMC1	Rabbit	WB	1:1000	Bethyl
Phospho-SMC1 Ser 966	Phospho-SMC1 Ser 966	Rabbit	WB	1:1000	Bethyl
CHK1	CHK1	Mouse	WB	1:1000	Santa Cruz
Phospho-CHK1 Ser 345	Phospho-CHK1 Ser 345	Rabbit	WB	1:1000	Cell Signaling
CHK2	CHK2	Rabbit	WB	1:10000	S.Elledge
Phospho-Chk2 Thr 68	Phospho-Chk2 Thr 68	Rabbit	WB	1:500	R and D Systems
RPA2	RPA2	Mouse	WB	1:1000	Merck

Table 2.8 ***Antibodies used in the study***

Antibody	Antigen	Species	Application	Dilution	Source
Phospho-RPA2 Ser 4/8	Phospho-RPA2 Ser 4/8	Rabbit	WB	1:100	Cell Signaling
CK2α	CK2α	Rabbit	WB	1:3000	Bethyl
CK2α'	CK2α'	Rabbit	WB	1:5000	Bethyl
βActin	βActin	Mouse	WB	1:25000	Sigma-Aldrich
Phospho-MRE11 Ser 676	Phospho-Mre11 Ser 676	Rabbit	WB	1:500	Cell Signaling
Phospho-MRE11 Ser 688	Phospho-Mre11 Ser 688	Rabbit	WB	1:250	Invitrogen
Methylated-MRE11 Arg587	Methylated-Mre11 Arg587	Rabbit	WB	1:1000	Stephane Richard Quebec, Canada
Phospho-histone H3 Ser 10	Phospho-histone H3 Ser 10	Rabbit	FACS	1:100	Cell Signaling
Anti-Mouse HRP	Mouse IgG	Goat	WB	1:1000	DAKO
Anti-Rabbit HRP	Rabbit IgG	Swine	WB	1:3000	DAKO
Alexa fluor® 488 anti-Rabbit	Rabbit IgG	Donkey	FACS	1:50	Invitrogen
Alexa fluor® 546 anti-Rabbit	Rabbit IgG	Goat	IF	1:1000	Invitrogen
Alexa fluor® 488 anti-Mouse	Mouse IgG	Goat	IF	1:1000	Invitrogen

Table 2.8 ***Antibodies used in the study***

CHAPTER 3

Chapter 3 Constitutive phosphorylation of MDC1 by CK2 is required for retention of the MRN complex at the sites of DNA double-strand breaks.

3.1 Introduction

MDC1 was first shown to have involvement early in the DNA damage response after it was seen to colocalise with γH2AX following IR (Shang *et al*., 2003). Following this, further insight into the role that MDC1 undertakes in the DDR has come to light. MDC1 is now classed as a member of the mediator/adapter protein family, which also includes 53BP1, BRCA1 and TopBP1 (Schultz *et al.*, 2000, Venkitaraman, 2001, Wang *et al.*, 2002, Yamane *et al.*, 2002). This particular group of proteins is characterised by the presence of BRCT domains and/or FHA domains, which act as phospho-peptide binding platforms that mediate the interaction with other DDR proteins.

MDC1 localises at the sites of damaged chromatin via its ability to bind to γH2AX through its C-terminal BRCT domains. Once localised to the sites of DNA breaks, MDC1 functions as a scaffold allowing further recruitment of several DNA damage response proteins, including the MRN complex, RNF8 and 53BP1 (Eliezer *et al.*, 2009, Goldberg *et al.*, 2003, Kolas *et al.*, 2007, Mailand *et al.*, 2007, Stewart *et al.*, 2003). Therefore, MDC1 functions to amplify the intracellular signal generated following the induction of DNA damage and potentiate its repair by facilitating the recruitment of various DNA repair proteins. Due to the early involvement of MDC1 in the DDR, loss of the protein is likely to have a detrimental affect on the cells ability to maintain the integrity of its genome. For instance, loss or lack of MDC1 results in a failure to efficiently recruit the MRN complex, BRCA1 and 53BP1 to the sites of DNA

damage which, as a consequence, disrupts the cells ability to activate both the intra-S phase and G2/M checkpoints as well as promote DNA repair (Goldberg *et al*., 2003, Stewart *et al*., 2003). Consequently loss of MDC1 is associated with genomic instability and cancer predisposition (Minter-Dykhouse *et al*., 2008).

In addition to binding proteins via its phospho-protein binding domains, MDC1 itself is also phosphorylated in response to DNA damage. A TQXF domain is targeted by ATM following treatment with IR leading to the recruitment of RNF8 (Jungmichel and Stucki 2010, Kolas *et al*., 2007, Mailand *et al*., 2007). Moreover, following the publication of various large-scale phosphoproteomic screens, it became apparent that MDC1 is a highly phosphorylated protein even in the absence of DNA damage (Beausoleil *et al.*, 2004, Dephoure *et al.*, 2008, Matsuoka *et al.*, 2007, Olsen *et al.*, 2006). Interestingly, a number of the MDC1 phosphorylation sites identified in these screens clustered in a conserved N-terminal region consisting of 6 repeated motifs bearing the consensus sequence for the serine/threonine kinase, Casein Kinase 2 (or CK2) (Beausoleil *et al.*, 2004, Dephoure *et al.*, 2008, Olsen *et al.*, 2006).

Recently evidence has started to emerge for CK2 playing a critical part in regulating the DNA damage response. For instance, phosphorylation of the SSB repair protein XRCC1 by CK2 facilitates an interaction with the FHA domain containing DNA repair proteins PNK and APTX (Loizou *et al*., 2004). Of note, not unlike MDC1, XRCC1 is also classified as a mediator/adaptor protein and is thought to provide a scaffold function for the binding of SSBR factors to the sites of DNA damage. In this respect, its phosphorylation by CK2 provides the specificity with which it can interact both PNKP and APTX. Based on this, it is therefore conceivable that the CK2 sites identified on MDC1 also mediate the interaction with one or more binding proteins that contain phospho-peptide binding domains that are critical

for its role in DNA repair of cell cycle checkpoint activation. Therefore the aims of this study were to:

i) *Validate the phosphorylation of MDC1 by CK2*

ii) *Investigate the potential role of CK2-dependent phosphorylation in regulating the function of MDC1 during the cellular DNA damage response*

3.2 Results

3.2.1 Identification of potential phosphorylation motifs on MDC1

Large-scale screens using mass spectrometry on protein sequences has aided the identification of novel target motifs for protein modification. Results from a number of published phospho-proteomic screens identified a number of phosphorylated residues on MDC1 that lie within motifs consistent with those targeted by the protein kinase CK2 (Table 3.1). Typically CK2 target motifs consist of either a serine (S) or threonine (T) residue followed by either an aspartic acid or a glutamic acid (E) residue located at the n+3 position relative to the phosphorylated Ser or Thr (S/T-X-X-D/E). Interestingly, 12 of the 16 potential CK2 phosphorylation sites clustered within an N-terminal domain of MDC1 that consisted of a repetitive amino acid motif, S-D-T-D-X-E-E termed SDTD motifs (Figure 3.1.A).

An indication of whether regions of proteins have significant biological function can be obtained by comparing the sequences of various species to see whether there is any evidence

Phosphorylation Site	Peptide Φ	Reference	Designation δ
S168	LLLAEDS*EEEVDF	Olsen, *et al*. 2006. Dephoure, *et al*. 2008. Beausoleil, *et al*. 2004.	7
S196	SVIVPES*DEEGHS	http://phospho.elm.eu.org/	8
S218 T220	FAFNLNS*DTDVEE FNLNSDT*DVEEGQ	http://phospho.elm.eu.org/	1
S299 T301	QPPGEDS*DTDVDD GEDSDT*DVDDDS	Dephoure, *et al*. 2008. Dephoure, *et al*. 2008.	2
S329 T331	PFGFIDS*DTDAEE GFIDSDT*DAEEER	Beausoleil, et al. 2004, Olsen, *et al*. 2006. Dephoure, et al. 2008, Olsen, *et al*, 2006. Dephoure, *et al*. 2008.	3
S376 T378	QESQAGS*DTDVEE SQAGSDT*DVEEGK	Matsuko, *et al*. 2007 Matsuko, *et al*. 2007	4
S402 T404	ASMVINS*DTDDEE MVINSDT*DDEEEV	Olsen, *et al*, 2006. Dephoure, et al. 2008. Olsen, *et al*, 2006. Dephoure, *et al*. 2008.	5
S453 T455	TTTERDS*DTDVEE TERDSDT*DVEEEE	Dephoure, *et al*. 2008. Beausoleil, *et al*. 2004, Dephoure, *et al*. 2008.	6
T516	RSQAST*TVDINT	Matsuoka, *et al*. 2007	9
T579	LHGDCET*DAEEGT	http://phospho.elm.eu.org/	10

Table 3.1 ***Summary of potential CK2 phosphorylation sites in MDC1: Φ*** *peptide detected/used for in silico analysis.* ***δ*** *number designated in present study.*

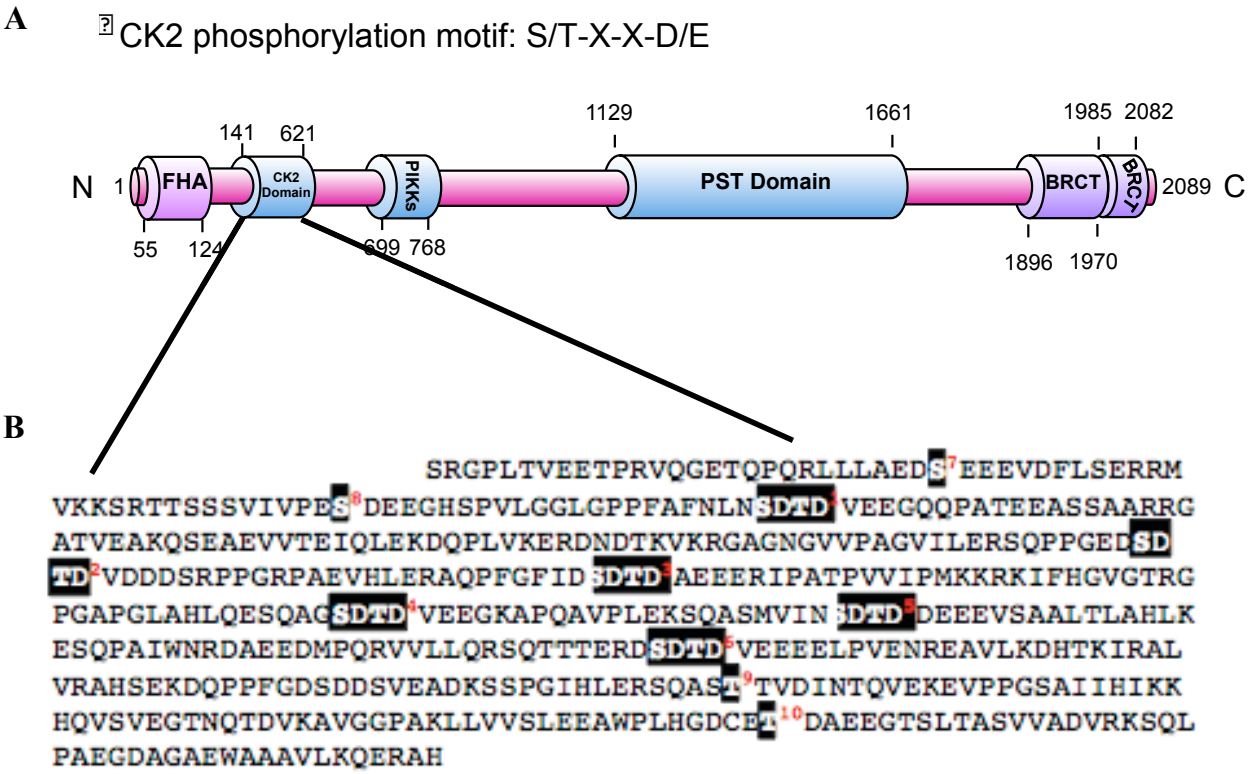


Figure 3.1 ***Location and amino acid sequence of the CK2 phosphorylation domain of MDC1. (A) Schematic of human the MDC1 protein showing known domains including the putative N-terminal CK2 phosphorylation domain. (B) Amino acid sequence of the MDC1 putative CK2 phosphorylation domain.*** *Potential CK2 phosphorylation sites are highlighted and numbered 1-10, as in Table 3.1.*

of evolutionary conservation. Using an internet based multiple sequence alignment programme, Clustal omega (http://www.ebi.ac.uk/Tools/msa/clustalo/), the region of human MDC1 (*H.sapiens*) containing the cluster of CK2 SDTD motifs was aligned with MDC1 of mouse (*M.musculus*), rat (*R.norvegicus*), Dog (*C.lupus*), Swine (*S.scrofa*) and chimpanzee (*P.troglodytes*). Strikingly, it was apparent that the majority of the 16 potential CK2 target sites identified either showed complete or partial homology in all vertebrate orthologues studied (Figure 3.2). Of particular interest was the complete conservation of the 6 SDTD repeat sequences between many of the species, suggesting that these motifs may have some functional importance.

3.2.2 A N-terminal region of MDC1 is phosphorylated by CK2 in vitro and in vivo

In order to investigate whether the identified potential sites on MDC1 were phosphorylated by CK2, two overlapping GST-fusion proteins were generated spanning this N-terminal region. Initially the fragments were amplified from full length MDC1 by PCR. Fragment 1 encompassed amino acids 1-381 and fragment 2 contained amino acids 341-725 (Figure 3.3.A). The PCR products were then cloned into the pGEX 4T-1 vector, sequenced and transformed into BL21 competent bacteria. Recombinant protein was purified from bacterial extract using GST-coupled agarose beads. These two purified proteins where then used as substrates in a CK2 *in vitro* kinase assay. Each fragment, or GST alone (used as a negative control), was incubated with recombinant CK2, in the presence of $\gamma(^{32}P)$-ATP. The kinase reaction was then separated by SDS-PAGE and the gel stained with Coomassie blue to visualise the proteins. Radio-labelled fragments were visualised by exposing the dried gels to autoradiography film.

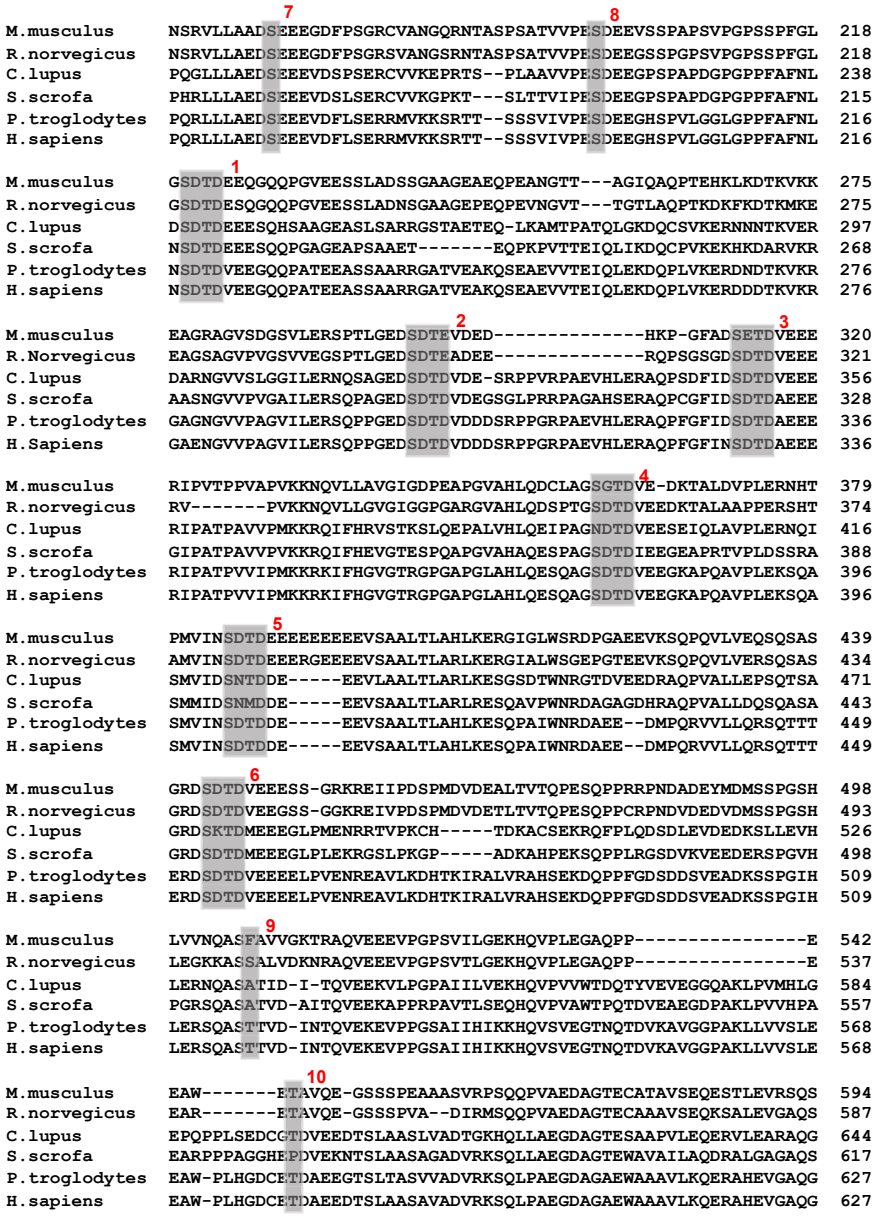


Figure 3.2 ***Amino acid alignment of MDC1 N-terminal CK2 motifs across species.*** *The region of human (H.sapiens) MDC1 containing a putative CK2 phosphorylation motifs was aligned with several other vertebrate species, Mouse (M.musculus), Rat (R.norvegicus), Dog (C.lupus), Swine (S.scrofa) and Chimpanzee (P.troglodytes), to identify any evolutionary conservation, using the internet based multiple sequence alignment programme, Clustal omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Shaded areas represent conserved potential target sites for CK2 phosphorylation, and the number designation for this study in red.*

Figure 3.3C clearly shows that CK2 phosphorylated both of the MDC1 fragments *in vitro*. In order to investigate which of the motifs were potentially phosphorylated by CK2, a plasmid expressing a GST-MDC1 fusion protein encompassing just the CK2 motifs was constructed, (aa141-621), and subjected to numerous rounds of site-directed mutagenesis to sequentially mutate each of the potential target CK2 motifs (Figure 3.4.A). In each case the serine and/or threonine of each motif was mutated to an alanine. In total 10 different constructs were created, transformed into BL21 competent bacterial cells and the GST-tagged fusion protein purified with GST-coupled agarose beads. The first six GST proteins comprised of the six SDTD motifs found in MDC1 mutated sequentially. The additional four GST proteins contained each of the single potential CK2 phosphorylation sites mutated in sequence on a background of the 6 mutant SDTD motifs. The purified GST-MDC1 fusion proteins were then used in an *in vitro* kinase assay again using recombinant CK2 and radiolabelled ATP. Sequentially mutating each of the potential CK2 sites resulted in a progressive loss of the phosphorylation, cumulating in a complete loss of phosphorylation when all 10 of the sites were mutated (Figure 3.4.B). The most significant loss in phosphorylation was observed after mutating sites 1-6 (The SDTD motifs). This suggests that the vast majority of the phosphorylation sites targeted by CK2 reside within the cluster of SDTD motifs.

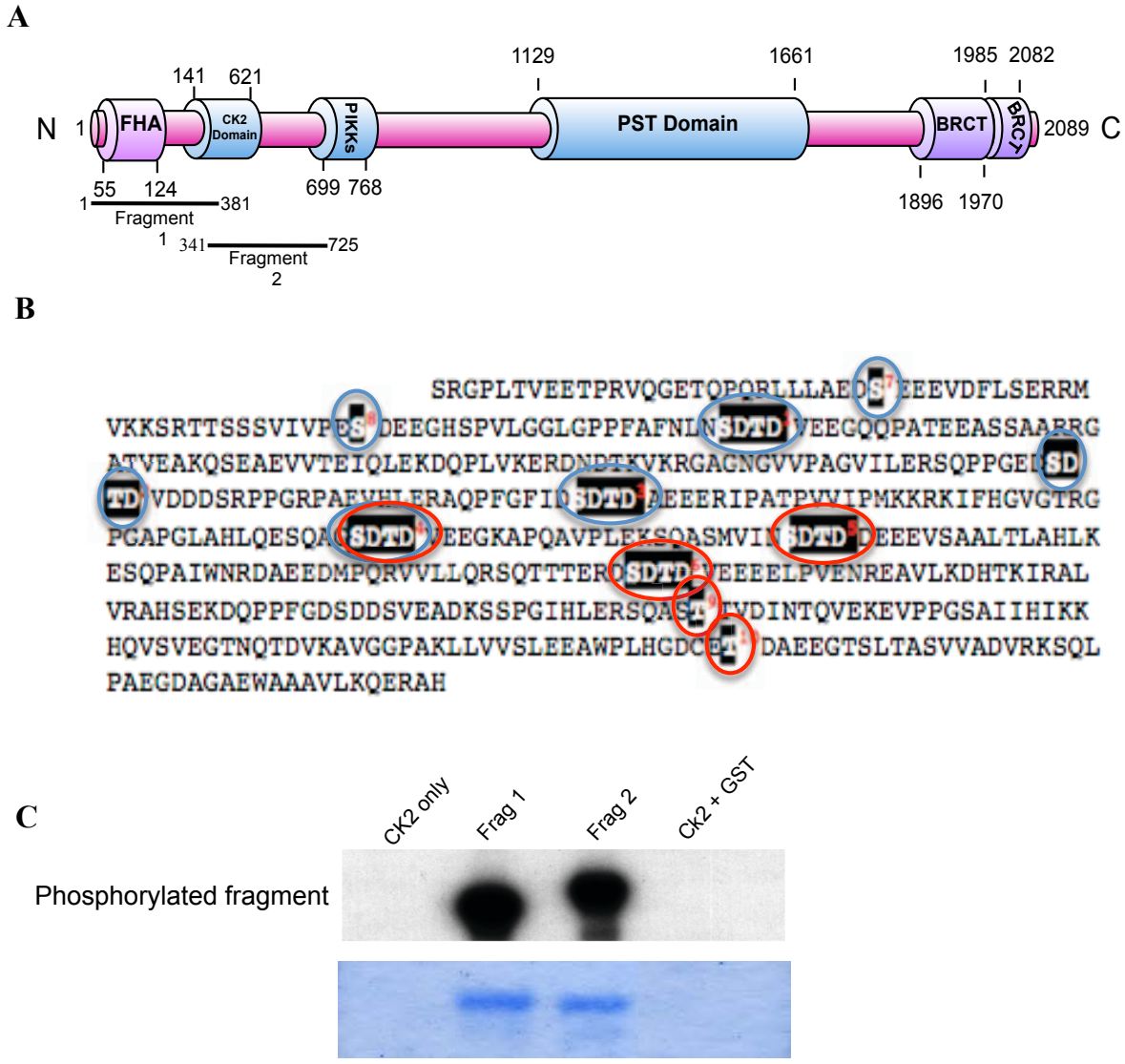
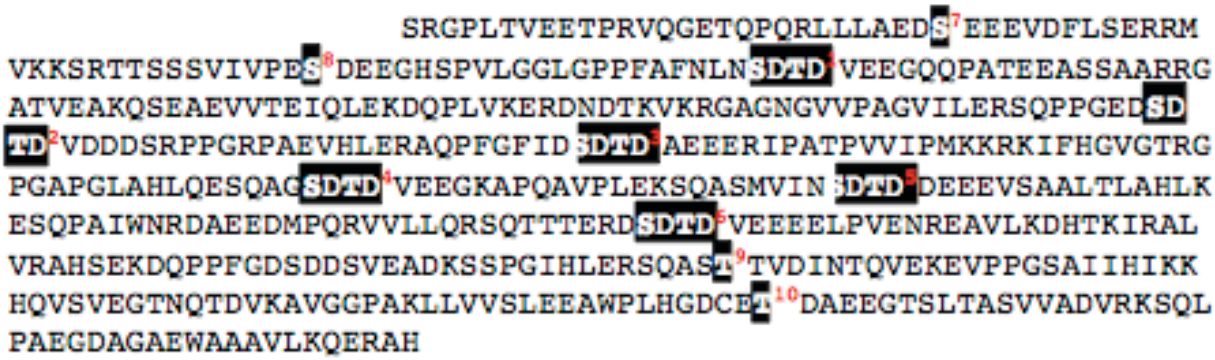


Figure 3.3 ***The N-terminal region of MDC1 is phosphorylated by CK2 in vitro.*** ***(A) A schematic of MDC1 depicting the location of the GST fragments 1 (aa1-381) and 2 (aa341-725)***. ***(B) Peptide sequence of the MDC1 putative CK2 phosphorylation domain***. *The potential CK2 phosphorylation sites in fragment 1 are ringed in blue whereas the potential CK2 phosphorylation sites present in fragment 2 are ringed in red.* ***(C)*** *N-terminal fragments of MDC1 were expressed as GST fusion proteins and purified using GST-coupled agrose beads. 1 µg of GST-MDC1 fragment 1 and 2 was incubated with recombinant CK2 and radio-labelled ATP (γ-^{32}P) for 10 minutes at 30°C. Proteins were separated by SDS-PAGE, stained with Coomassie blue and dried. Radio-labelled proteins were detected via autoradiography.*

A



B

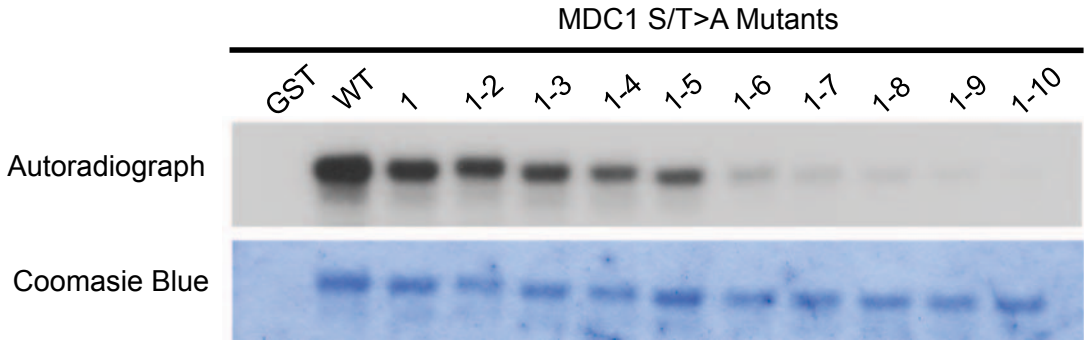


Figure 3.4 ***Mapping of the CK2 phosphorylation sites on MDC1 by in vitro kinase assay. (A) Amino acid sequence of the MDC1 CK2 phosphorylation region****. Potential CK2 phosphorylation sites are highlighted and numbered 1-10. (**B**) GST-fusion proteins were generated, where all of the possible targeted serine or threonine residues were mutated to alanine. 1 µg of each of the GST proteins was incubated with recombinant CK2 and radio-labelled ATP (γ-^{32}P) for 10 minutes at 30 °C. Proteins were fractionated by SDS-PAGE, the gels were then stained with Coomassie blue and dried. Radio-labelled proteins were detected by autoradiography.*

3.2.3 Identification of MDC1 CK2 target sites by mass spectrometry analysis

Since analysis of the phospho-proteomic data indicated that both the serine and the threonine residues of the SDTD motifs were phosphorylated *in vivo* either singly or in combination, it was necessary to determine whether CK2 preferentially targeted either one of the residues in the SDTD motifs for phosphorylation, or could phosphorylate both. Therefore, to determine which of the identified CK2 phosphorylation sites on MDC1 were phosphorylated, the WT MDC1 GST-fragment encompassing all the CK2 phosphorylation motifs (aa141-621) was subjected to an *in vitro* kinase assay with recombinant CK2, separated on a pre-cast Tris/Glycine gel and then stained with colloidal blue. The phosphorylated GST-MDC1 band was excised from the gel and then treated with trypsin to digest the GST-fusion protein into peptides. The resulting peptides were subjected to mass spectrometry analysis to determine which of the serine/threonine residues were phosphorylated by CK2 *in vitro*.

Table 3.2 lists the identified peptides that were phosphorylated by CK2. Of the 16 potential phosphorylation sites originally identified at the beginning of the study, the mass spectrometry analysis identified 11 phosphorylated residues, of which 10 were located within the SDTD motifs (Figure 3.4.B). Furthermore, the mass spectrometric analysis demonstrated that both the serine and threonine residues of the SDTD motifs could be phosphorylated in combination. Taken together, this analysis highlighted the 6 clustered SDTD motifs of MDC1 as a target for CK2-mediated phosphorylation.

MDC1 Peptide	CK2 Phosphorylation Site	Corresponding Designation
DSD*TDVEEEELPVENR	T455	6
AQPFGFIDSD*TDAEEER	T331	3
LLLAED*SEEEVDFLSE	S168	7
SQASMVIN*SDTDDEEEVSAALTLAHLK	S402	5
SQASMVINSD*TDDEEEVSAALTLAHLK	T404	5
SQTTTERD*SDTDVEEEELPVENR	S453	6
SQTTTERD*SD*TDVEEEELPVENR	S453 T455	6
SQPPGED*SDTDVDDDSRPPGRPAEVHLER	S299	2
SQPPGED*SD*TDVDDDSRPPGRPAEVHLER	S299 T301	2
AQPFGFID*SD*TDAEEERIPATPVVIPMK	S329 T331	3
GPGAPGLAHLQESQAG*SD*TDVEEGKAPQAVPLEK	S376 T378	4

Table 3.2 ***Recombinant MDC1 phospho-peptides identified by mass spectrometry following phosphorylation by recombinant CK2 in vitro.*** *Target consensus motifs are highlighted in red, with '*' denoting putative phosphorylation site. This data was provided by Dr. Nicholas Morrice (University of Dundee).*

3.2.4 MDC1 associates with CK2 in vivo in the presence and absence of damage

To strengthen the evidence for a potential link between MDC1 and CK2, it was deemed important to establish whether or not MDC1 and CK2 associate *in vivo*. Extracts from HeLa cells, either mock-irradiated or irradiated with 10 Gy of IR, were separately incubated with 3 different antibodies raised against regions of the MDC1 protein. Co-immunoprecipitated proteins were then separated by SDS-PAGE and Western blots probed with antibodies against CK2α and CK2α', the catalytic subunits of the kinase.

Interestingly, MDC1 seems to preferentially bind to a CK2 complex containing the α' rather than α catalytic subunit (Figure 3.5). Moreover, this association was unaffected by DNA damage, suggesting that the interaction may be constitutive.

3.2.5 Loss of CK2 results in a persistent DNA damage response following IR

MDC1 plays a number of critical roles during the cellular response to DNA double strand breaks that include recruiting repair proteins to sites of DNA damage, potentiating the phosphorylation of histone H2AX and facilitating the activation of Chk1 (Stewart *et* al, 2003, Lou *et al* 2006, Stucki 2005). To investigate whether the CK2 potentially played a role in activating/regulating MDC1-dependent DDR induced by exposure to IR, the individual catalytic subunits of CK2 were depleted in cells using siRNA and 72 h later the cells were irradiated with 5 Gy of IR. The IR-induced DDR was monitored over a time course post-irradiation, using Western blotting with phospho-specific antibodies to known proteins involved in the ATM-mediated response.

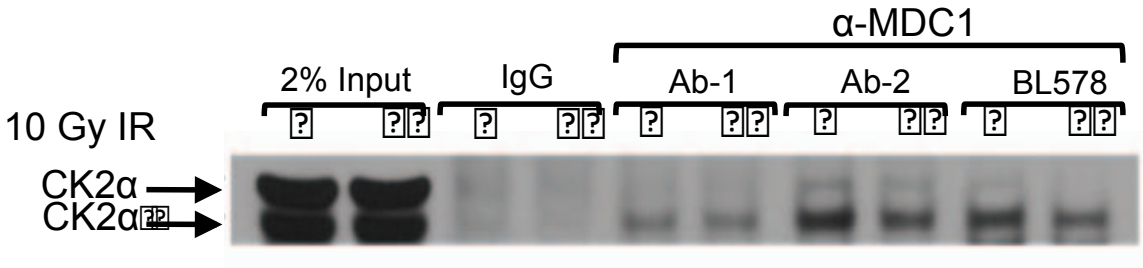


Figure 3.5 ***MDC1 interacts with CK2 in vivo.*** *Extracts from untreated or irradiated (10 Gy and harvested at 8 hr) HeLa cells were separately incubated with 3 different MDC1 antibodies raised against different regions of the protein (Ab-1 aa 326-649, Ab-2 aa 643-1015, BL578 Bethyl) or a non-specific isotype and species-matched control IgG. Immunoprecipitates were then separated by SDS-PAGE, electroblotted and MDC1-associated proteins were detected by incubation of the filters with the indicated antibodies.*

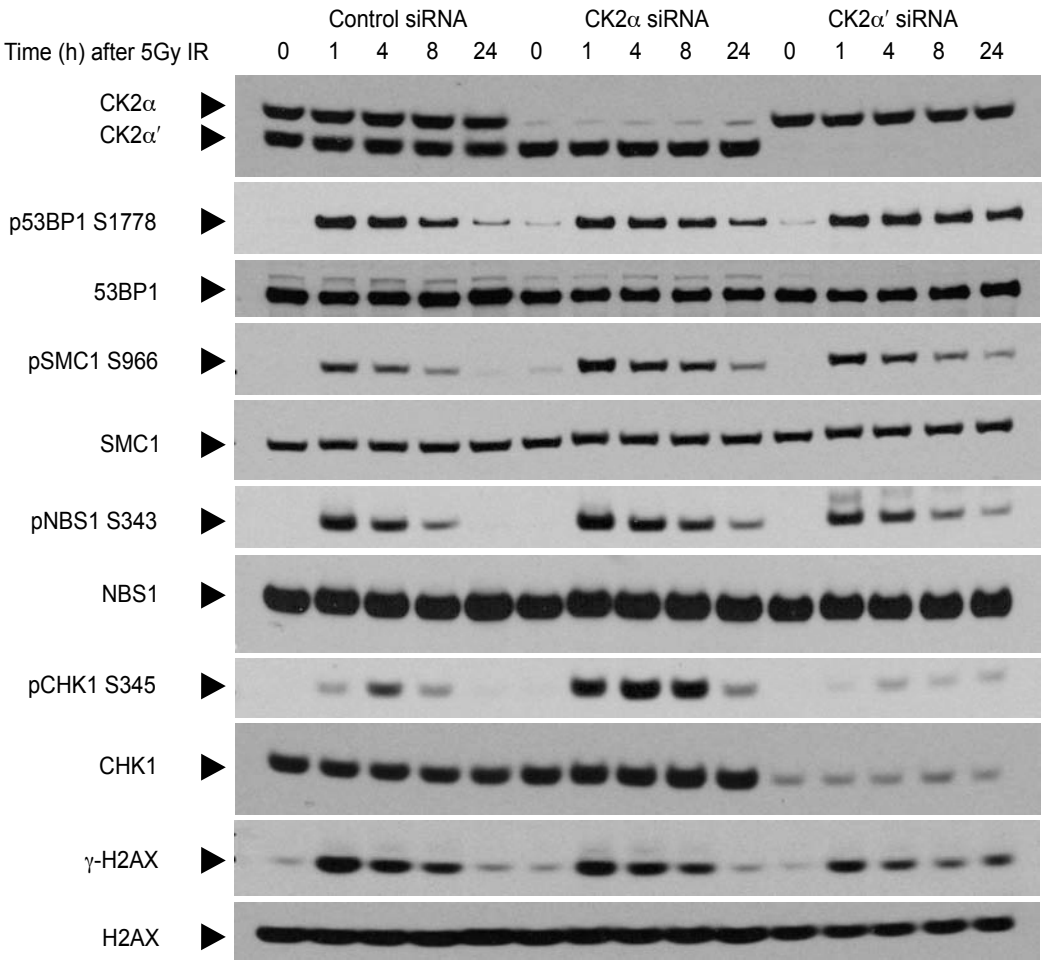


Figure 3.6 ***Loss of CK2 results in a prolonged DNA damage response following IR.*** *HeLa cells were transfected with control, CK2α or CK2α' siRNA. Seventy two hours later the cells were irradiated with 5 Gy of IR and harvested at the indicated time points. Cell extracts were separated by SDS-PAGE followed by Western blotting with the indicated antibodies.*

Loss of the catalytic activity of CK2, in particular the α' subunit, resulted in mild prolonged phosphorylation of many known ATM kinase substrates, including SMC1 and NBS1 (Figure 3.6). This suggests that CK2 activity is may be required for the proper activation/maintenance of the DDR phospho-activity presumably through its ability to phosphorylate a number of molecules required for DNA repair. Strikingly, depletion of the α' subunit of CK2 destabilised endogenous CHK1. Whilst the physiological relevance of this is unknown, it has been demonstrated that CHK1 can bind to the regulatory β subunit of CK2, however the catalytic subunits are not required for this interaction (Guerra *et al.*, 2003). Further investigation would be required to study the loss of native CHK1 following siRNA depletion of CK2α'.

3.2.6 Loss of CK2 via siRNA or mutation of the CK2 target motifs does not affect MDC1 localisation

In response to IR, DDR proteins are recruited to sites of damaged chromatin in a precise and tightly regulated manner. This relocalisation of repair proteins to sites of DNA damage can be visualised by immunofluorescence and are termed Ionising Radiation-Induced Foci (IRIF). MDC1 rapidly forms radiation-induced foci and colocalises with a number of other DDR proteins (Xu and Stern, 2003). To determine whether loss of CK2 influenced the ability of MDC1 to be recruited to sites of DNA DSBs, immunofluorescence was used to study MDC1 foci formation in irradiated U2OS cells transfected with either CK2α and CK2α' siRNA. One hour post-irradiation, cells where fixed and then stained with antibodies to MDC1 and γH2AX, the latter of which was to mark sites of damage. As shown in Figure 3.7 the loss of CK2 catalytic activity did not result in a loss of MDC1 radiation induced foci or indeed its ability to colocalise with γH2AX.

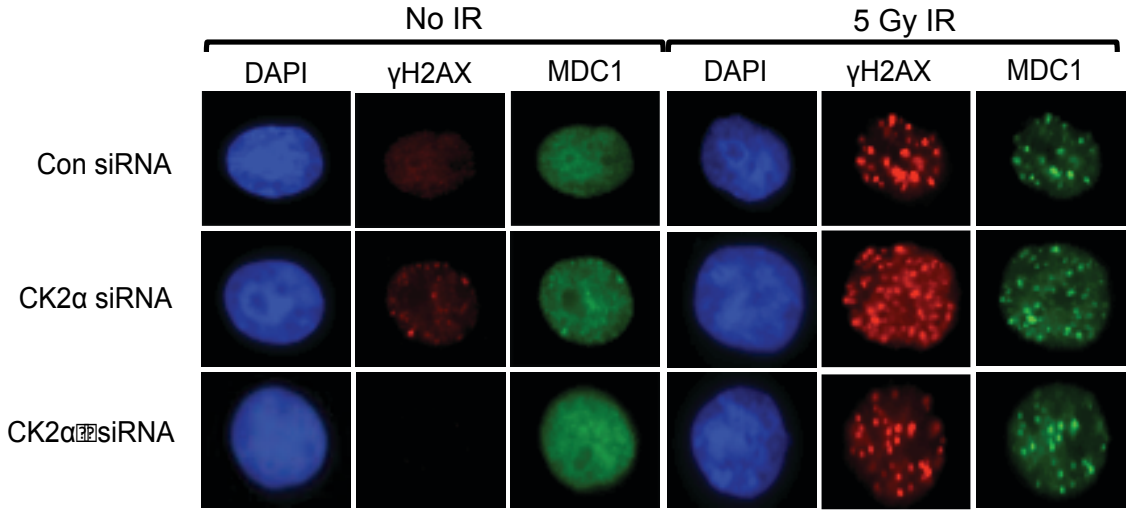


Figure 3.7 ***Loss of CK2 does not affect the relocalisation of MDC1 to radiation-induced foci.*** *HeLa cells were transfected with control, CK2α or CK2α' siRNA and then seeded onto poly-L-lysine coated coverslips. Seventy two hours later cells were irradiated with 5 Gy of IR, pre- extracted and fixed after 1 h, followed by staining with the indicated antibodies. Images are of one cell and are representative of approximately ~1000 cells scored by eye from 3 separate experiments.*

Moreover, to definitively verify that loss of CK2 phosphorylation on MDC1 did not affect MDC1 relocalisation after DNA damage, a number of GFP-MDC1 constructs were made in which either the entire CK2 region was deleted (ΔCK2-MDC1-GFP (aa141-621)) or where the both the serine and threonine residues in each of the SDTD motifs had been mutated to an alanine (1-6 S/T⇒A MDC1-GFP). A further GFP-MDC1 expression construct was made in which Serine-168, which had also been identified as a CK2 phosphorylation site that did not reside in an SDTD motif, had been also been mutated to an alanine (1-6+7 S/T⇒A-MDC1-GFP). These were then transfected into HeLa cells and irradiated with 5 Gy of ionising radiation. Comparable to CK2 knockdown via siRNA, MDC1 lacking the putative CK2 phosphorylation sites was still able to form radiation-induced foci and colocalise with γH2AX (Figure 3.8). This data suggests that phosphorylation of MDC1 by CK2 is not required for either its relocalisation to sites of damage or colocalisation with γH2AX in response to IR.

3.2.7 Loss of CK2 does not impair recruitment of other DNA damage factors following IR

Given that loss of phosphorylation of MDC1 by CK2 did not affect its relocalisation to DNA damage, it is possible that the phosphorylation maybe required for the binding/recruitment of other repair/checkpoint factors known to be downstream of MDC1. In keeping with this, the phosphorylation of XRCC1 by CK2 is not required for its ability to be recruited to SSB but is essential for mediating its binding to either PNKP or APTX (Loizou *et al.*, 2004, Luo *et al.*, 2004). Therefore, to investigate whether the loss of CK2-dependent phosphorylation of MDC1 affected the recruitment of other DNA repair factors to sites of DNA DSBs, HeLa cells seeded onto slides were transfected with either control, CK2α or CK2α' siRNA for 72 h,

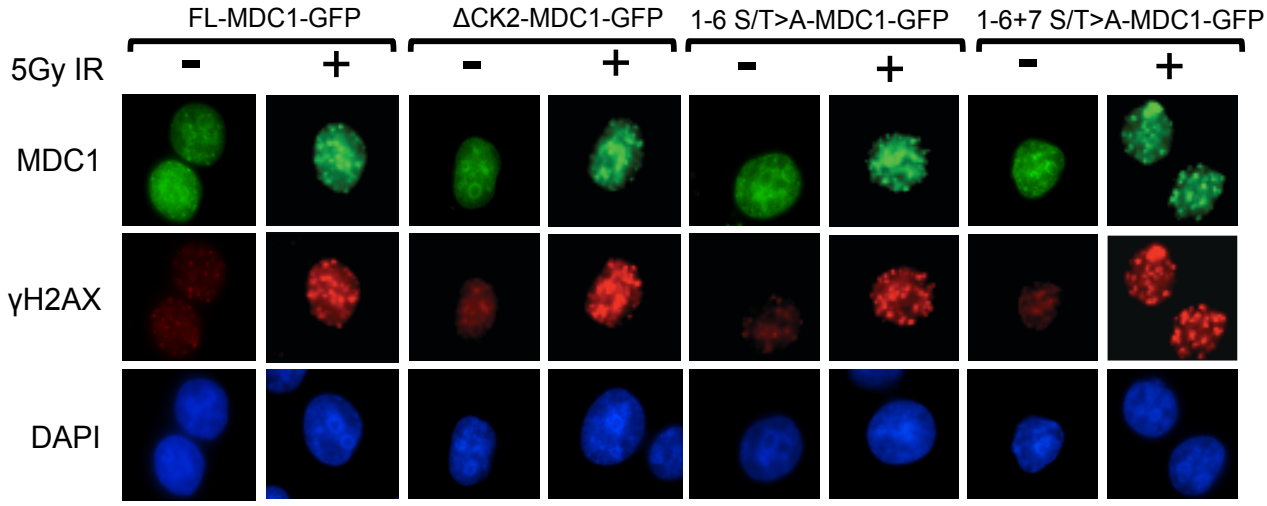


Figure 3.8 ***Loss of the CK2 phosphorylation sites on MDC1 does not affect its relocalisation to radiation-induced foc****i. HeLa cells were transfected with GFP Full-length MDC1 (FL-MDC1 GFP), GFP MDC1 lacking the CK2 domain (ΔCK2-MDC1-GFP), GFP MDC1 where 1-6 SDTD motifs had been mutated (1-6 S/T⇒A MDC1-GFP) and GFP MDC1, where, in addition to 1-6 SDTD motifs mutated, residue 7 had also been mutated (1-6+7 S/T⇒A-MDC1-GFP). Cells were then seeded onto poly-L-lysine coated coverslips cells, irradiated and fixed 1 h later. The cells were then stained with the indicated antibodies. Images are of one cell and are representative of approximately ~1000 cells scored by eye from 3 separate experiments.*

irradiated with 3 Gy of ionising radiation, fixed 8 hour post-irradiation and stained with antibodies to phosphorylated ATM, 53BP1 or BRCA1. The recruitment of each repair protein was determined by immunofluorescence. MDC1 interacts with activated ATM (ATM S1981-P) via its FHA domain, which resides N-terminal to the CK2 domain and thus it is plausible that the phosphorylation of the SDTD motifs aids in the binding of ATM (Lou *et al.*, 2006). However, loss of CK2 did not abolish the ability of ATM to be recruited to sites of DNA damage, as indicated by the presence of phosphorylated ATM (pATM) IRIF in cells depleted of either CK2α or CK2α' (Figure 3.9). The recruitment of 53BP1 and BRCA1 was also investigated following CK2 knockdown. Both of these proteins rely on MDC1 for their localisation to DNA breaks functioning to regulate NHEJ and HRR respectively downstream of MDC1 (Huen *et al*., 2007, Kolas *et al*., 2007, Mailand *et al*., 2007). Similar to pATM, both 53BP1 and BRCA1 formed radiation-induced foci after CK2 knockdown. This implies that phosphorylation of MDC1 by CK2 has no impact on recruitment of a number of major DNA damage proteins.

3.2.8 siRNA-mediated reduction of CK2 results in loss of NBS1 foci following IR

Like ATM, the MRN complex functions early in the DNA damage response, and rapidly forms foci following IR. It has been observed that MDC1 exists in a complex with MRN (Goldberg *et al*., 2003) (Figure 3.11), and that depletion of MDC1 by siRNA leads to a reduction of MRN nuclear foci (Goldberg *et al*., 2003, Lou *et al*., 2006, Lukas *et al*., 2004, Stewart *et al*., 2003). To ascertain whether loss of CK2 activity affected the MDC1-dependent relocalisation of the MRN complex to sites of DNA damage, HeLa cells were seeded onto slides and transfected with either control, CK2α or CK2α' siRNA. The cells were then

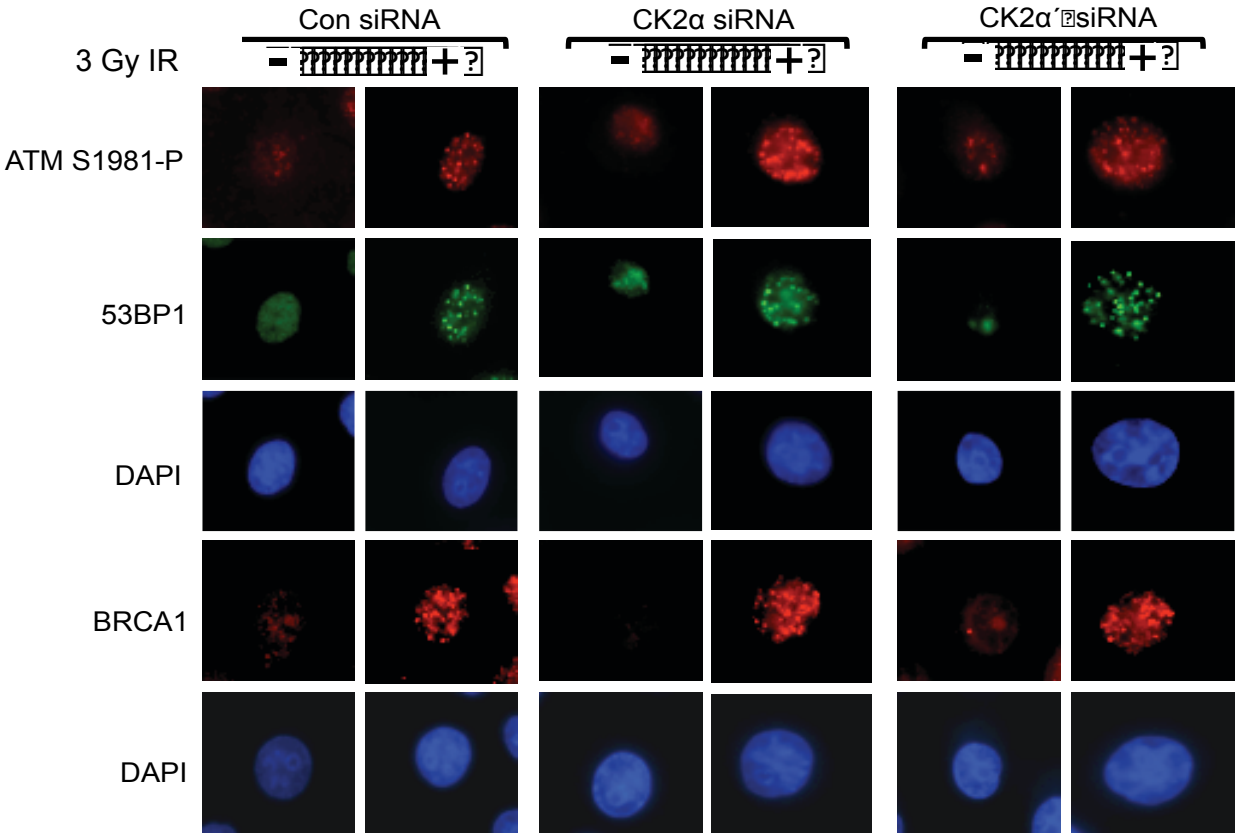


Figure 3.9 ***CK2 depletion by siRNA does not affect the recruitment of other DNA damage factors to radiation-induced foci.*** *HeLa cells were transfected with CK2α, CK2α' or control siRNA. Seventy two hours later the cells were then irradiated with 3 Gy, harvested after 8 h and then fixed. Cells were then stained with the indicated antibodies. Images are of one cell and are representative of approximately ~1000 cells scored by eye from 3 separate experiments.*

irradiated and fixed 8 hours post-irradiation. The cells were subsequently stained with NBS1 and γ-H2AX as a marker of DSBs. Consistent with this hypothesis, when either CK2α or CK2α' was knocked down in HeLa cells using siRNA, NBS1 failed to form foci following 5 Gy of IR compared to cells treated with control siRNA (Figure 3.10). These results suggest that CK2 is required for NBS1, to localise to radiation-induced foci, and that this localisation is possibly mediated through a phospho-dependent interaction on MDC1 involving CK2**.**

It should be noted that all foci images are representative of approximately 1000 cells scored by eye from 3 experimental repeats. However, this method of documenting foci formation sways towards qualitative anaylsis and therefore, has a number of associated caveats. For instance modest impacts may not be detected. Scoring cell number and number of foci per cell would provide quantative analysis of the results, providing a statistical approach to the data.

3.2.9 The SDTD motif of MDC1 mediates its interaction with the MRN complex

Despite having demonstrated that CK2 activity is required for the relocalisation of the NBS1 to sites of DNA damage and that MDC1 and the MRN exist constitutively bound as a complex in the cell, it is unclear whether the CK2-dependent phosphorylation of MDC1 plays a key role in this process. To investigate this, MDC1 was immuno-purified from cells previously treated with either control or CK2 siRNA and Western blots probed with antibodies to NBS1. Strikingly, NBS1 failed to co-precipitate with MDC1 in cells lacking CK2 activity, yet a small amount did in cells treated with control siRNA (Figure 3.11). This finding is consistent with the idea that CK2 phosphorylation is mediating the constitutive interaction of MDC1 with the MRN complex.

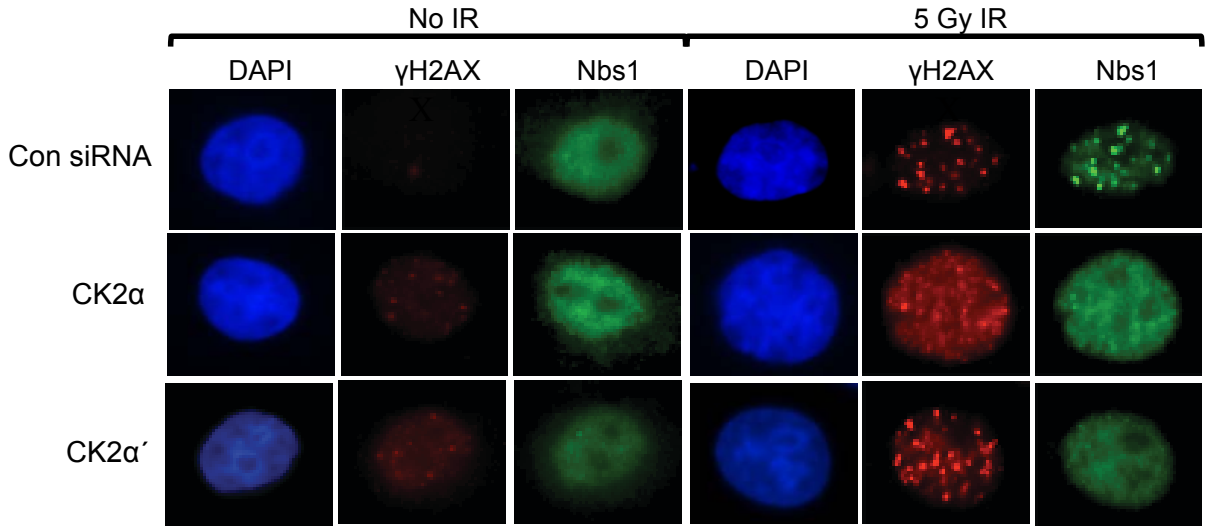


Figure 3.10 ***Loss of CK2 abolishes the recruitment of NBS1 to DNA double-strand breaks.*** *HeLa cells were mock-transfected or transfected with CK2α or CK2α' siRNA for 72 h. Cells were seeded onto coverslips, irradiated with 5 Gy IR of ionising irradiation, fixed after 8 h and stained with the indicated antibodies. Images are of one cell and are representative of approximately ~1000 cells scored by eye from 3 separate experiments.* □

To demonstrate that CK2-dependent phosphorylation of MDC1 was critical in mediating the interaction with the MRN complex, a series of GST-MDC1 fragments were generated, containing mutations in which the threonine residue of each SDTD motif had been sequentially mutated to an alanine. The threonine residue was chosen since FHA domains have a tendancy to preferentially bind phospho-threonine rather than phospho-serine residues (Durocher *et al*., 1999). A GST-pulldown assay was performed, using the recombinant purified MDC1 proteins, to elucidate whether the phosphorylation of MDC1 by CK2 mediates the interaction with the MRN complex. The GST fragments were pre-incubated with CK2 in the presence of ATP and then used to pull down proteins from HeLa nuclear extract. Samples were then separated by SDS-PAGE and Western blots probed with antibodies against members of the MRN complex. In the presence of CK2, the phosphorylated MDC1 WT fragment is capable of pulling down all three members of the MRN complex. However, loss of 3 out of the 6 phospho-SDTD motifs was sufficient to disrupt this interaction (Figure 3.12). Therefore the interaction is phospho-dependent and most probably mediated via NBS1 and the CK2 phosphorylated N-terminus of MDC1.

3.2.10 The CK2 phosphorylated N-terminus of MDC1 interacts with NBS1 via its FHA domain

It has been previously demonstrated that the FHA domain of Nbs1 is required for the MRN to be recruited to DSBs. Interestingly, it has been shown that it is the FHA domains of PNKP and APTX that mediate the binding to CK2 phosphorylated XRCC1 (Loizou *et al*., 2004, Luo *et al*., 2004). It is conceivable that the FHA domain of NBS1 is required for binding to phosphorylated MDC1 and therefore its recruitment to sites of DNA damage. Previous studies

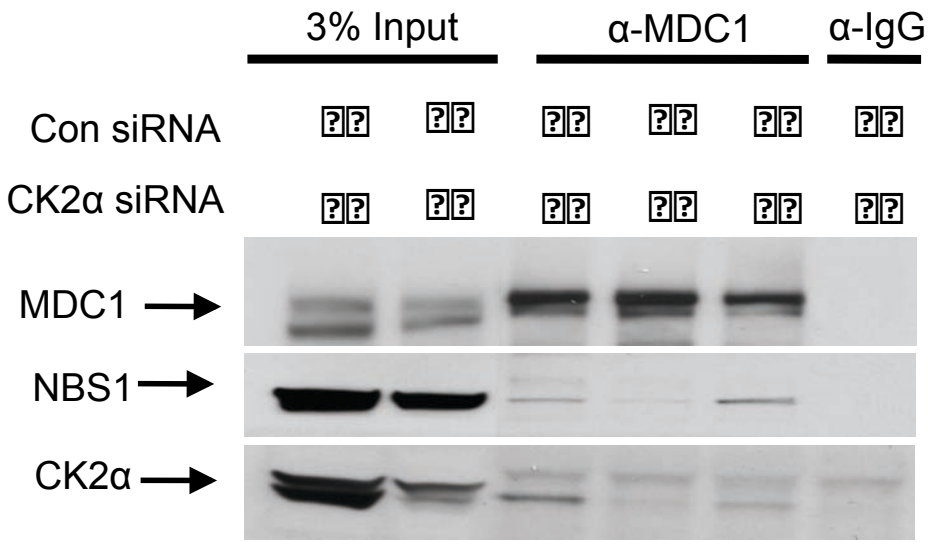


Figure 3.11 ***Knockdown of CK2 using siRNA abrogates the interaction between MDC1 and NBS1 in vivo.*** *HeLa cells were depleted of CK2α or mock-depleted. Seventy two hours later the cells were harvested and extracts were incubated with the indicated antibodies. Immunoprecipitates were then separated by SDS-PAGE followed by Western blotting with the indicated antibodies.*

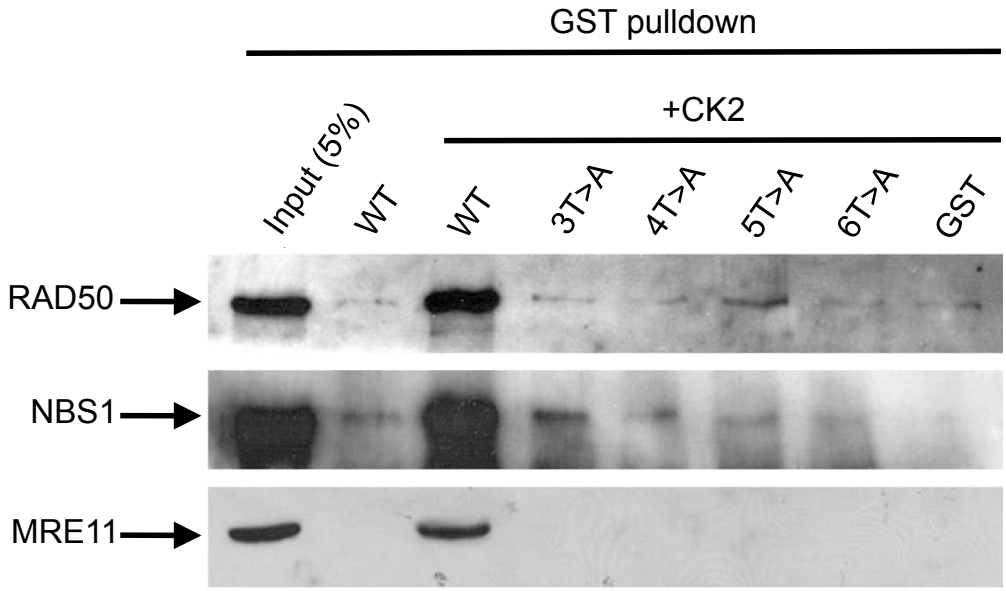


Figure 3.12 ***Mutation of the MDC1 CK2 phosphorylated region prevents binding to the MRN complex in vitro.*** *A MDC1 fragment (aa221-456) derived from mouse where a number of the SDTD motifs had been mutated to SDAD (3T>A: T362A, T387A and T444A: 4T>A: T315A, T362A, T387A and T444A; 5T>A T300A, T315A, T362A, T387A and T444A; 6T>A T222A, T300A, T315A, T362A, T387A and T444A) was generated and expressed as GST fusion proteins in E. coli. The purified fragments were then incubated with recombinant CK2 in the presence of ATP. Fragments were then used to pull down proteins from HeLa nuclear extracts. Proteins were separated by SDS-PAGE and the membranes blotted with antibodies against members of the MRN complex. Figure kindly provided by M.Stucki, Institute of Molecular Cancer Research, University Zürich.*

have shown that a single point mutation (R28A) in the FHA domain of NBS1 abolishes the interaction between MDC1 and the MRN complex and results in loss of MRN accumulation at sites of damage *in vivo* (Horejsi *et al.*, 2004, Lukas *et al.*, 2004a). Therefore, to determine whether MDC1 interacted with NBS1 via its FHA domain, WT MRN and a MRN complex containing the R28A mutant NBS1 were expressed and purified from Sf9 cells. The mutant and WT complexes were then incubated with a purified GST-MDC1 fragment containing the SDTD motifs pre-phosphorylated by CK2. GST-coupled beads were used to isolate MDC1-MRN protein complexes that were subsequently subjected to SDS-PAGE and Western blotting. Interestingly, an interaction between wild-type NBS1 and the MDC1 fragment was observed and this association was significantly increased when MDC1 was phosphorylated by CK2 (Figure 3.13). Strikingly however, this interaction was completely abrogated when the fragments were incubated with the MRN complex harbouring the R28A mutation in NBS1 (Figure 3.13). It can be concluded that phosphorylation of MDC1 by CK2 facilitates the interaction between MDC1 and the MRN complex and that this is mediated via the FHA domain of NBS1.

3.2.11 Abolishing the interaction between MDC1 and NBS1 results in a partial G2/M checkpoint defect

So far, the experimental data has shown that loss of CK2 results in ablation of NBS1 foci following damage, and that CK2 facilitates the interaction between MDC1 and NBS1 through the NBS1 FHA domain. It is also apparent that disruption of this phospho-dependent

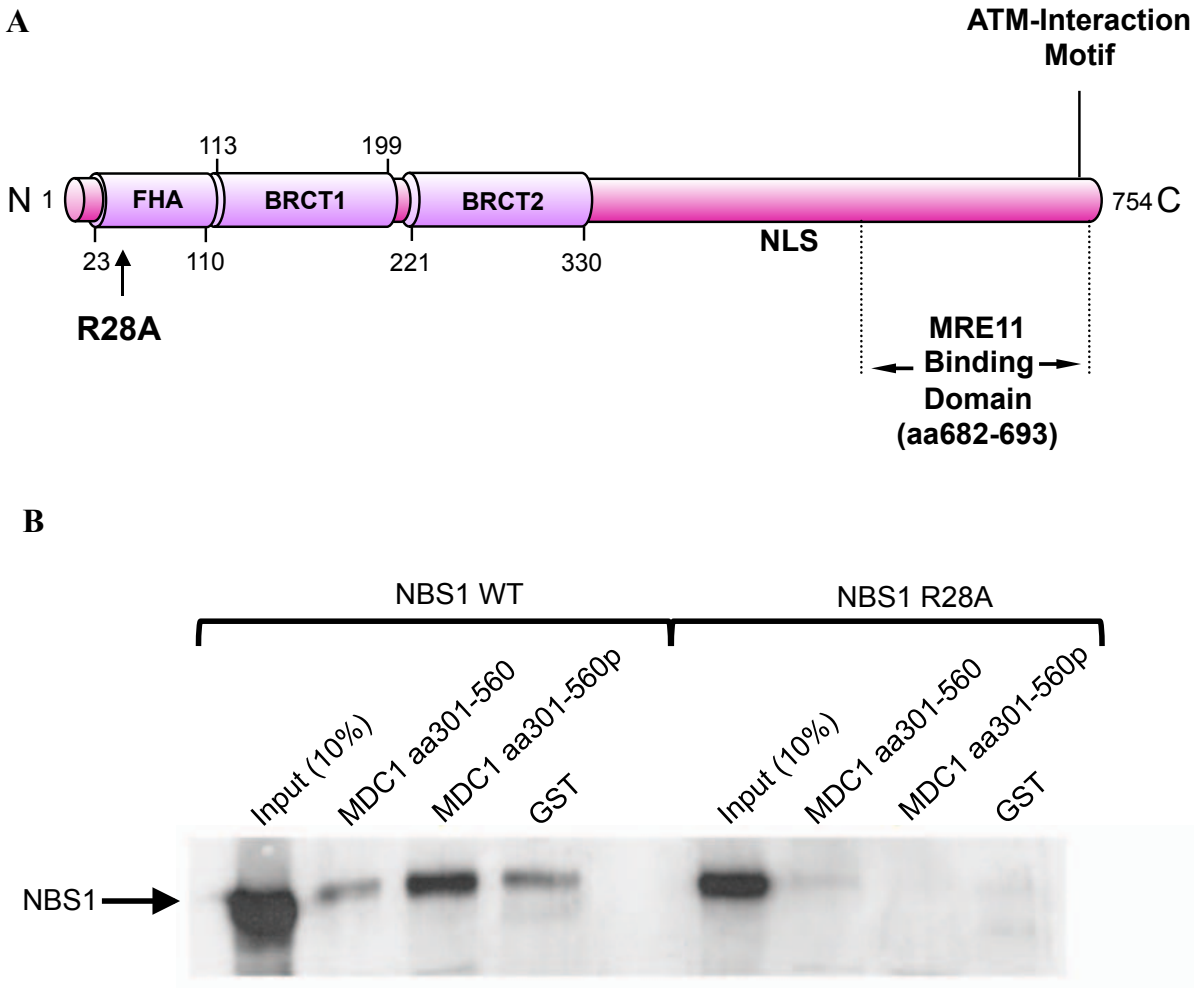


Figure 3.13 ***The CK2 phosphorylated N-terminus of MDC1 interacts with NBS1 via its FHA domain. (A) A Schematic of full length NBS1, showing domains and location of the R28A mutation****. (B) A purified GST-MDC1 fragment (aa301-560) was pre-incubated with CK2 and ATP. The fragment was then incubated with purified MRN complex containing either wild type NBS1 or NBS1 in which the FHA domain had been mutated (R28A). GST-purified protein complexes were separated via SDS-PAGE and the Western blots were probed with an antibody against NBS1. Figure B kindly provided by M.Stucki, Institute of Molecular Cancer Research, University Zürich.*

interaction does not impede the recruitment of early damage response proteins (Figure 3.9). This raises the question as to whether loss of MDC1-dependent NBS1 recruitment to sites of damage translates to a defect in the cellular DDR induced by exposure to IR. To ascertain whether the interaction between MDC1 and Nbs1 is required to mediate this response to DNA double strand breaks, fibroblasts lacking full length NBS1 (NBS-iLB1) were transfected with either WT NBS1 (NBS+WT), or the NBS1 R28A (NBS+R28A) mutant, irradiated with varying doses of IR and then the percentage of mitotic cells determined using phospho-histone H3 serine-10 (p-H3) staining coupled with FACS. As expected the cells transfected with WT NBS1 following damage exhibited a dose-dependent drop in the percentage of mitotic (p-H3 positive) cells, which is indicative of G2 arrest (Figure 3.14.B). As expected cells completely lacking NBS1 exhibited a significantly higher percentage of p-H3 positive cells post-irradiation which suggests a defect in checkpoint activation allowing damaged cells to pass into mitosis (Figure 3.14.B). Interestingly, the cells transfected with NBS1 FHA mutant attenuated activation of the G2/M checkpoint, albeit not as severe as NBS cells lacking NBS1 expression (Figure 3.14.B). Moreover, the observed defect was not due to lower expression of the transduced mutant NBS1 compared to the WT (Figure 3.14.A). This suggests that disruption of the CK2 dependent MRN/MDC1 interaction results in G2/M checkpoint defect, following ionising radiation.

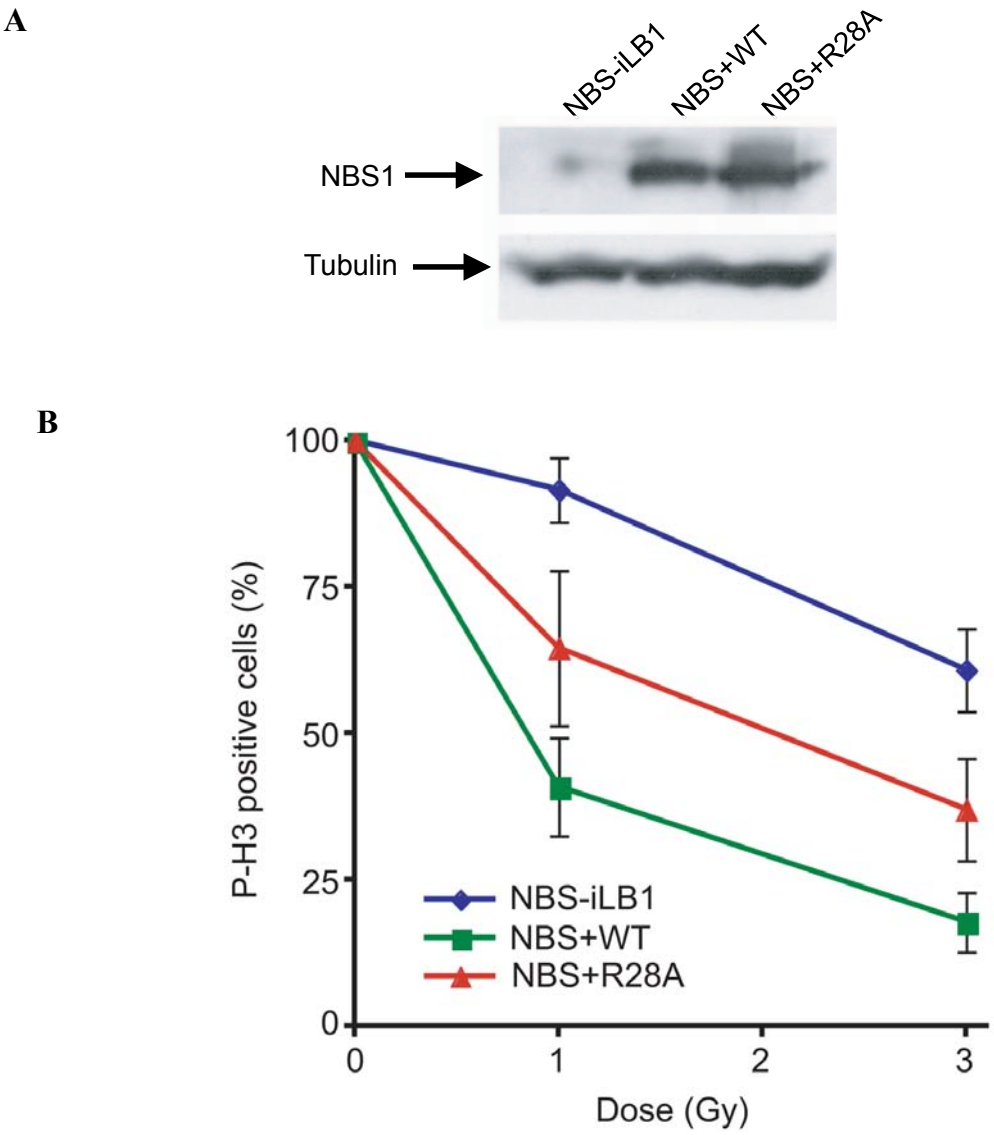


Figure 3.14 ***Abolishing the interaction between MDC1 and NBS1 results in a partial G2/M checkpoint defect.*** ***(A)*** *Cell extracts from NBS-iLB1 fibribroblasts and NBS-iL1 fibroblasts transduced with either WT NBS1 or R28A mutant NBS1. Proteins were separated by SDS-Page followed by Western Blotting with indicated antibodies.* ***(B)*** *NBS fibroblasts (NBS-iLB1) and NBS fibroblasts stably transfected with WT NBS1 (NBS+WT) or the FHA domain mutant of Nbs1 (NBS+R28A) were mock-treated or irradiated with 1 Gy or 3 Gy of IR and harvested 1 hour post-irradiation. Cells were fixed with ethanol and stained with an antibody against phospho-histone H3 serine 10 which is as a marker of mitotic cells. The DNA was stained with propidium iodide and the cells were then analysed using FACS. Error bars represent SD. Figure kindly provided by M.Stucki, Institute of Molecular Cancer Research, University Zürich.*

3.2.12 Identification of a Seckel-like family with a unique mutation in NBS1

Recently, our laboratory has identified a unique family (Family 106) from the United Arab Emirates in which the three affected individuals currently alive, primarily present with moderate microcephaly and growth retardation. Initially this led to a clinical diagnosis of Seckel Syndrome (Figure 3.15 & 3.16). However, following DNA linkage analysis performed by members of Andrew Jackson's laboratory (Institute of Genetics and Molecular Medicine, Edinburgh) a single homozygous mutation was discovered in exon 4 of the *NBS1* gene, which is predicted to result in the inframe deletion of serine 118 (ΔSer118) located within the first BRCT domain (Figure 3.17 & 3.18). Briefly, SNP (Single-nucleotide polymorphism) arrays on the 3 affected individuals and their parents were undertaken using approximately 120,000 of the most informative SNPs. The analysis using Alohomora for data manipulation, graphical relationship representation and Merlin for error checking and removal, and Allegro for linkage analysis, revealed four regions with a positive-linkage signal. Upon receipt of a sample from an unaffected sibling, microsatellites covering these four regions were genotyped, leading to removal of one region due to homozygosity in the unaffected sibling. The remaining three regions were then considered for candidate gene prioritisation based on previous disease associations, cellular function and animal models, resulting in the identification of the mutation in *NBS1*.

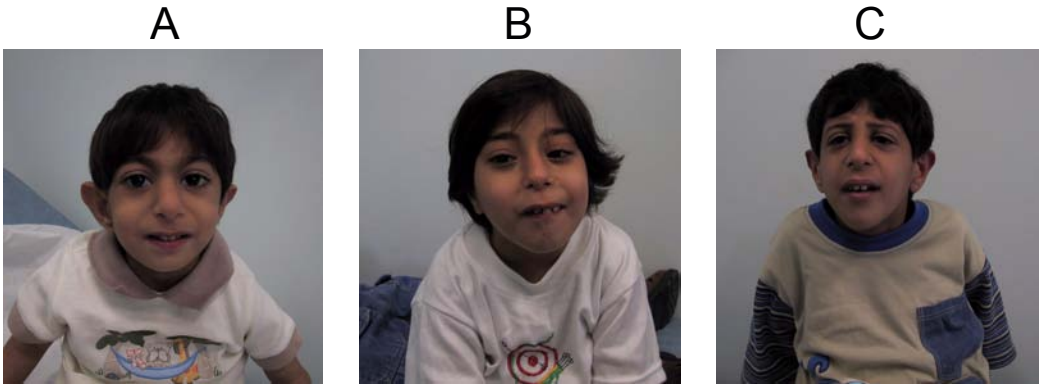
Since it has been reported that in addition to its FHA domain, the BRCT1 domain of NBS1 is also required to mediate its interaction with phospho-MDC1, it could be suggested that the clinical disease exhibited by the affected individuals in this family may specifically result from a failure of MDC1 and NBS1 to interact (Chapman and Jackson, 2008, Hari *et al.*, 2010, Wu *et al.*, 2008). In support of this, structural modeling of this BRCT1 domain mutation,

performed by Scott Williams, highlighted S118 as a critical residue within this domain for mediating its interaction with a phospho-peptide (Figure 3.19). This data was produced via small angle-X-ray scattering based on published work investigating the FHA and BRCT domains of human NBS1 (Williams *et al.*, 2009). Furthermore, this residue had also been previously identified along with another, K160, which is also thought to be important for phospho-peptide interactions within this domain (Lloyd *et al.*, 2009) (Figure 3.19). Interestingly, when compared to classical NBS patients (that typically are homozygous for the 657del5 *NBS1* mutation and do not express full length protein), the NBS BRCT mutant patients (NBS^{BRCT}) lack several of the major clinical/cellular features characteristic of this disorder, such as immunodeficiency, spontaneous chromosome 7;14 translocations and defects in DNA DSB repair (Table 3.3 & 3.4). This observation could indicate that loss of NBS1 binding to MDC1 only contributes to the development of a subset of the clinical features exhibited by classical NBS patients.

3.2.13 ΔSer118 does not result in reduction of NBS1 protein levels or disrupt binding to MRE11 or RAD50

The mutation present in 90% of classical NBS patients (657del5) is hypomorphic, which results in reduced levels of the NBS1 protein and subsequent destabilisation of the other members of the MRN complex. Therefore, it is important to establish whether the clinical features of the NBS^{BRCT} patients is specifically due to the mutation, or also in part caused by a reduction in expression of the NBS1 protein. To assess this, extracts from lymphoblastoid cell lines (LCLs) derived from the 3 affected individuals from family 106 were subjected to Western blotting using antibodies directed against components of the MRN complex and ATM. Extracts from two normal individuals (Normal-1/Normal-2) and a classical 657del5

A



B

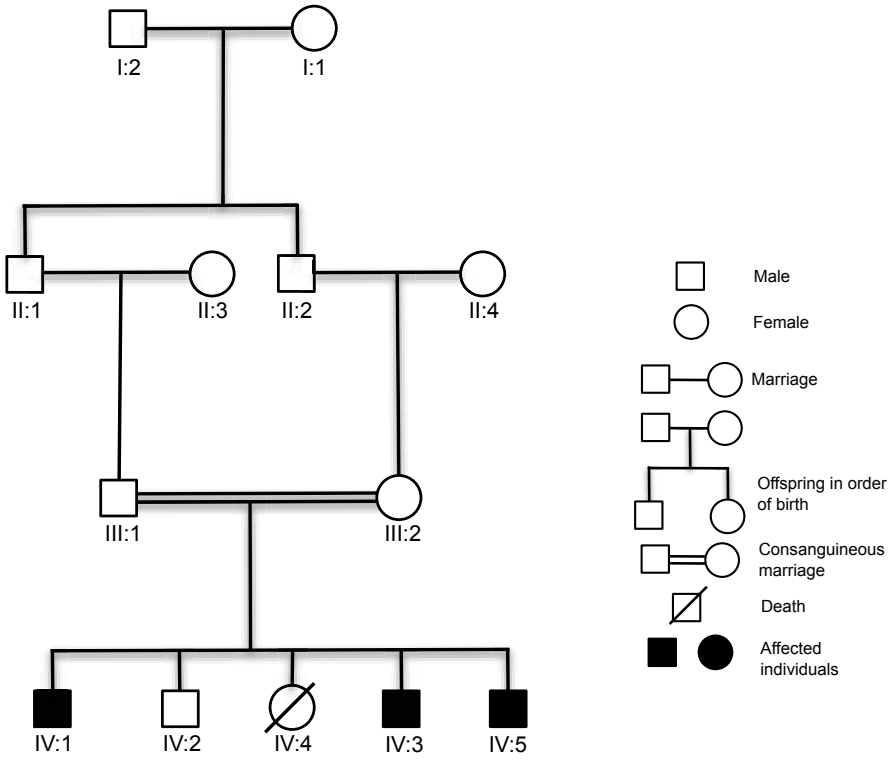
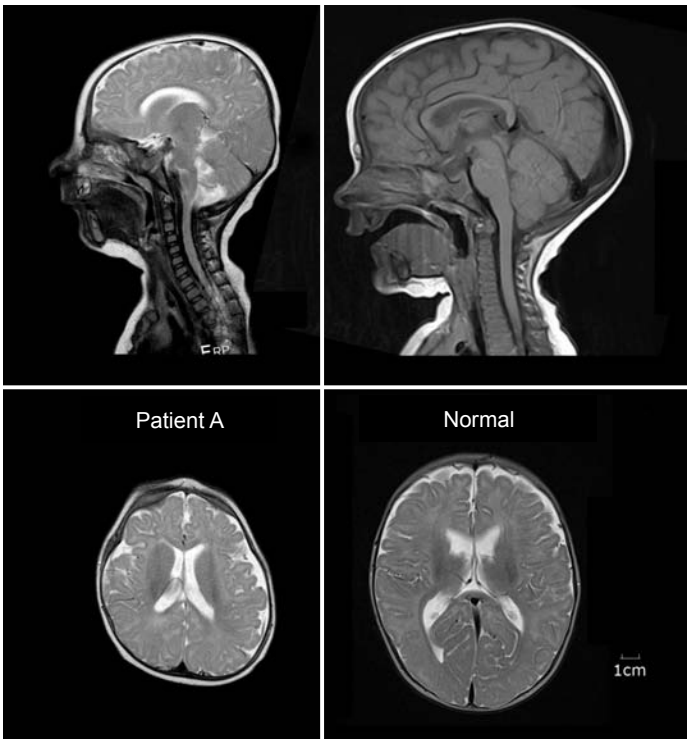


Figure 3.15 ***Family 106. (A)*** *Presentation of microcephaly in three related individuals, patient A, B and C from family 106* ***(B)*** *Genetic pedigree of family 106 (IV:1 patient A, IV:3 patient B, IV:5 patient C).*

A



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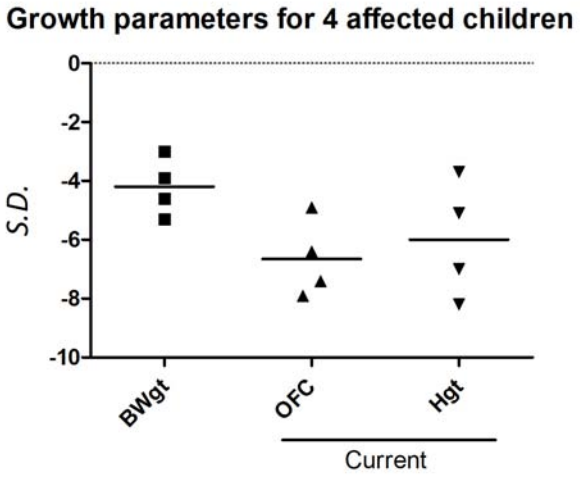


Figure 3.16 ***The NBS^{BRCT} patients from family 106 exhibit microcephaly and growth retardation. (A) MRI scan of NBS^{BRCT} patient A****. One of the affected individuals (patient A) at 7 months (left panel) showing reduced cerebral cortex size compared to that of a normal individual of similar age (right panel).* ***(B) Growth Parameters of the four affected NBS^{BRCT} patients from family 106****. Birth weight (BWgt), occipitofrontal circumference (OFC) and height (Hgt) of the four affected NBS^{BRCT} patients, presented as standard deviations (S.D.) from the norm.*

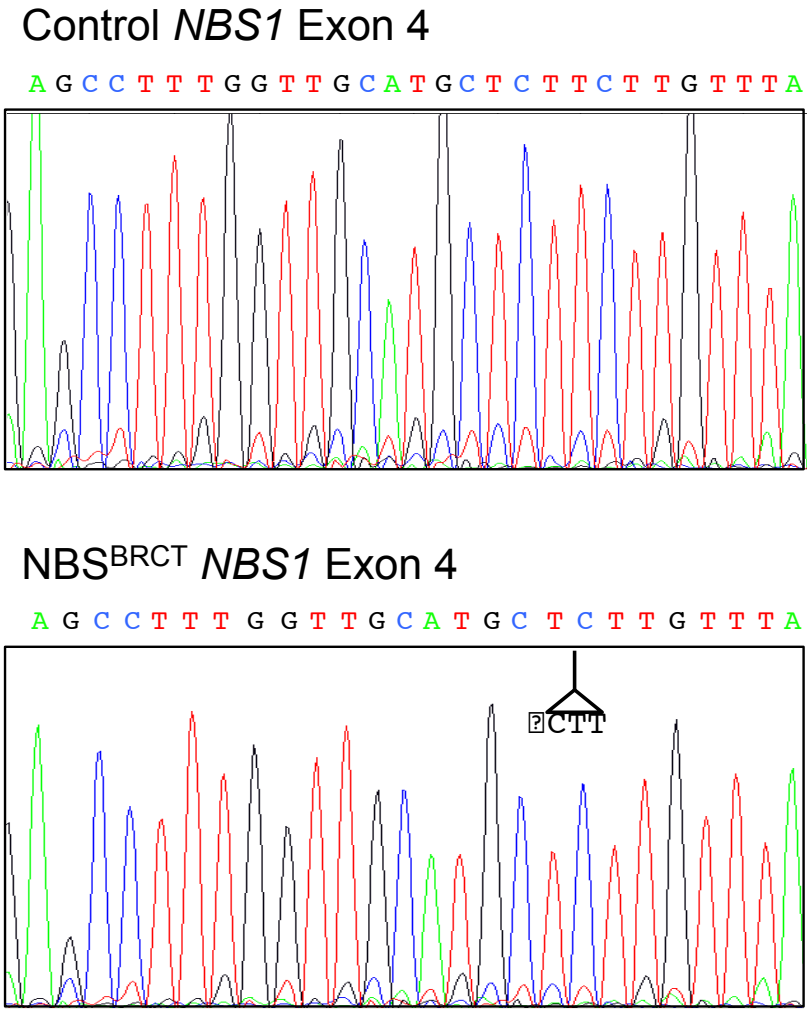
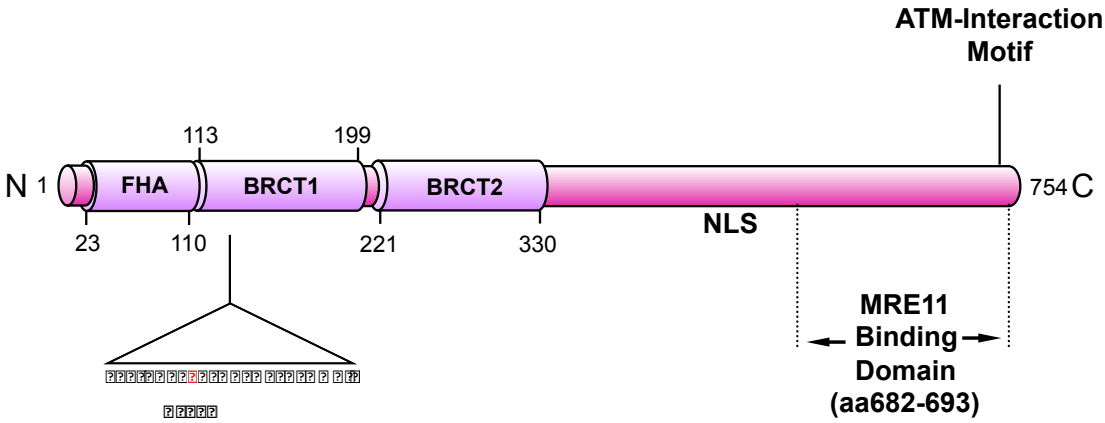


Figure 3.17 ***Identification of a mutation in NBS1, in the affected patients from family 106.*** *A homozygous deletion in exon 4 (ΔCTT) of the patients NBS1 gene, resulting in the loss of a single amino acid was identified through whole genome sequencing (lower panel).*

A



B

Homo sapiens	101	VFGSKFRIEYEPLVACSSCLDVSGKTALNQAILQLGGFTVNNWTEECTHL	150
Pan troglodytes	101	VFASKFRIEYEPLVACSSCLDVSGKTALNQAILQLGGFTVNNWTEECTHL	150
Bos taurus	101	VFESKFRVEYEPLVACSSCLDVSGKTALSHAILQLGGLTVNNWTEECTHL	150
Mus musculus	101	VFESKFRVEYEPLVVCSSCLDVSGKTVLNQAILQLGGLTANNWTEECTHL	150
Rattus norvegicus	101	VFESKFRVEYEPLVVCSSCLDVSGKTVLNQAILQLGGLTANSWTEECTHL	150
Gallus gallus	98	VFESKFRVEYESLVVCSSCLDVAQKTALNEAIQQLGGLVVNEWTKECTHL	147
Danio rerio	88	VFQSKFSLEKECIVVCSSCVDNEGKVTLSQDIRSVGGRLVSSWTSDCTHL	137

Figure 3.18 ***Serine-118 is located in the BRCT1 domain of NBS1 and is conserved across species. (A) Schematic of hNBS1 depicting the location of the ΔS118 on the protein. (B) Amino acid sequence alignment of NBS1 demonstrating S118 is conserved across several species.***

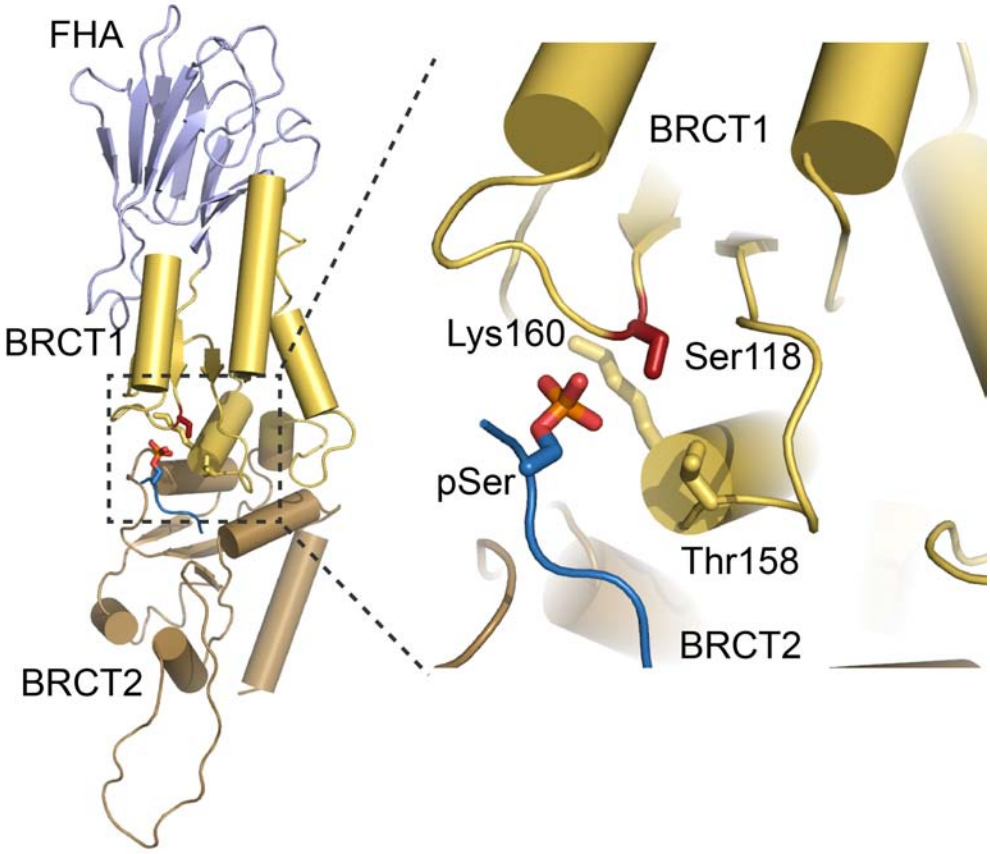


Figure 3.19 ***S118 of NBS1 is essential for binding to phospho-residues.*** *A small angle-X-ray scattering model of the human NBS1 FHA and BRCT1/BRCT2 domains. It is predicted that along with Lys160 and Thr158, S118 interacts with phospho-peptide domains (pSer). This data was provided by Dr. Scott Williams (unpublished).*

Clinical Features	657del5 NBS	NBSBRCT
Microcephaly	**Yes**	**Yes** (4 sbilings)
Ataxia	**No**	**No**
Oculocutaneous telangiectasia	**No**	**Yes** (1 sibling)
Growth retardation	**Yes**	**Yes** (4 sbilings)
Intellectual disability	**Mild-moderate**	**Moderate**
Dvelopmental delay	**Yes**	**Yes**
Dysmorphic facial features	**Yes**	**Yes**
Skin abnormalities	**Yes**	**1 sibling Café-au-lait spots**
Genito-urinary tract abnormalities	**Yes**	**Yes**
Immunoglobulin deficiency	**Yes**	**No**
T cell abnormalities	**Yes**	**No**
B cell abnormalities	**Yes**	**No**
NK cell abnormalities	**Yes**	**No**
Chromosomal translocations	**Yes**	**No**
Radiation chromosome hypersensitivity	**Yes**	**No**
MMC chromosome hypersensitivity	**Yes**	**No**
CPT chromosome hypersensitivity	**Unknown**	**No**

Table 3.3 ***Comparison of clinical and cellular phenotypes exhibited by 657del5 NBS vs NBS^{BRCT} patients. This data was provided by Dr Grant Stewart.*** *Immunophenotyping was carried out by the University of Birmingham's Clinical Immunology Service. Sensitivity to the mentioned DNA damaging agents was assed by via chromosomal breakage.*

	Chromatid gaps	Chromatid breaks	Tri-radial chromosomes	Quadra-radial chromosomes	Other
Normal	2-10	0	0	0	0
A	1	1	0	0	0
B	7	2	1	0	1
C	6	0	0	0	1
NBS 657del	73	27	4	7	6

Table 3.4 ***Cells from the NBS^{BRCT} patients do not exhibit chromosomal radiosensitivity****. The cells of the NBS^{BRCT} patients, A, B and C were treated with 1 Gy of IR and the number of induced chromosome aberrations in G2 were counted in 50 metaphase spreads. These numbers were compared with a normal control and a 657del5 NBS patient. The range of chromosome damage given for the normal individual was derived from the analysis of over 100 individuals. This data was provided by Dr Grant Stewart.*

NBS patient (NBS-Classical) were used for comparison. A complete absence of full length NBS1 protein was observed in the classical NBS patient, compared to the normal (Figure 3.20.A). Strikingly, the levels of NBS1 expression were unaffected in the three affected NBS^{BRCT} patients (NBS-BRCT (A), NBS-BRCT (B), NBS-BRCT (C) (Figure 3.20.A). Moreover, the BRCT1 mutation also did not appear to affect the ability of NBS1 to bind other components of the MRN complex (Figure 3.20.B).

3.2.14 The ΔSer118 mutation disrupts NBS1 binding to MDC1 and ablates NBS1 foci following IR

Given that previous work has highlighted a role for the BRCT1 domain of NBS1 in mediating its association with phosphorylated MDC1, and that predictions from structural modelling highlight the importance of S118 for phospho-peptide binding (Chapman and Jackson 2008, Hari *et al.,* 2010). Taken together these observations suggest that loss of this serine residue would specifically compromise the biochemical link between MDC1 and the MRN complex.

Therefore, to investigate this possibility MDC1 was immunoprecipitated from cell extracts derived from two normal individuals, a classical NBS patient or one of the affected NBS^{BRCT} patients, and the levels of associated NBS1/RAD50 determined by Western blotting. Strikingly, in keeping with the structural prediction, loss of S118 severely compromised the ability of MDC1 and the MRN complex to bind *in vivo* (Figure 3.21). More importantly, cells expressing this mutant protein failed to form NBS1 foci following the induction of DSBs

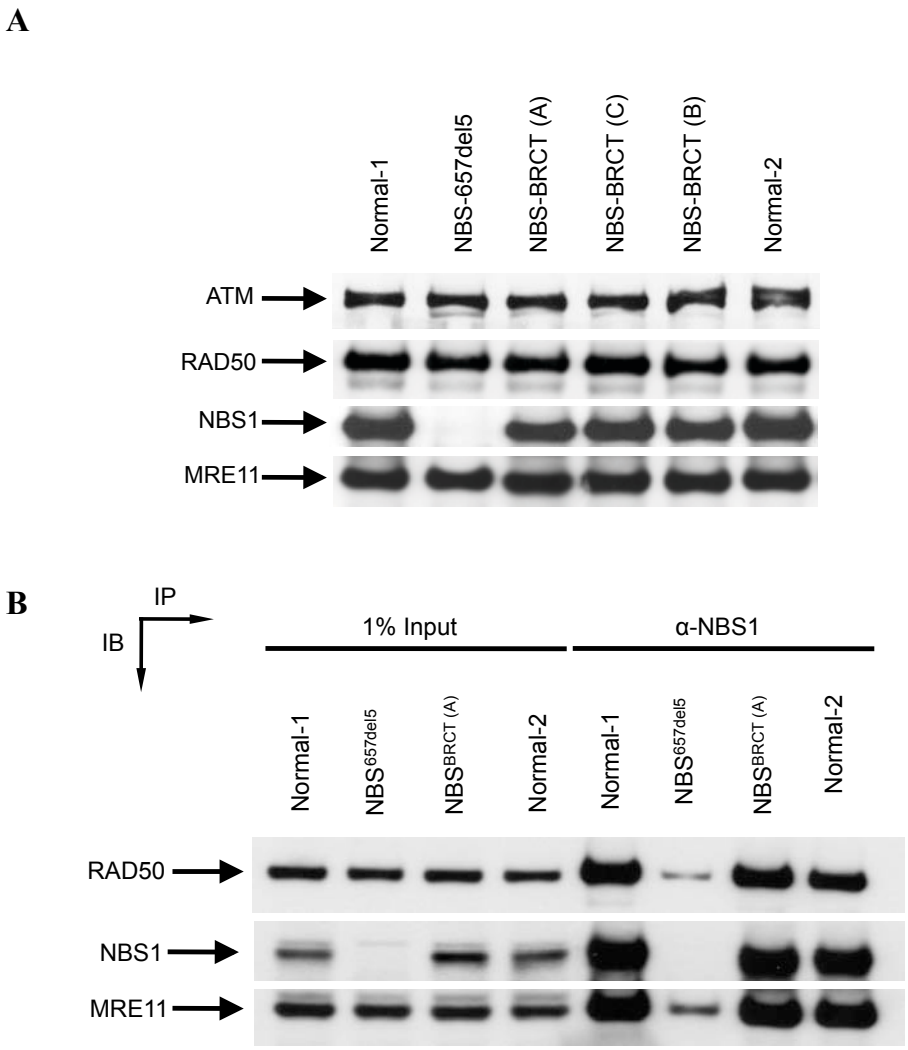


Figure 3.20 ***ΔSer118 does not destabilise the NBS1 protein or disrupt its binding to MRE11 or RAD50. (A) NBS^{BRCT} patients express normal levels of NBS1 protein and members of the MRN complex****. Extracts were prepared from cell lines from two normal individuals (1 & 2), a 657del5l NBS patient and the three affected NBS^{BRCT} patients. Lysates were separated by SDS-PAGE, and Western blotting used to assess the expression of the MRN complex using the antibodies indicated.* ***(B) ΔS118 does not affect MRN complex formation.*** *Extracts from the indicated cell lines were incubated with an antibody against NBS1. Immunoprecipitates were then fractionated by SDS-PAGE and then Western blotting was used to detect co-precipitating proteins.*

despite forming normal γ-H2AX and MDC1 IRIF (Figure 3.22 & 3.23). These data are consistent with deletion of S118 in NBS1 being a pathogenic change that leads to the failure of this mutant protein to bind MDC1, and therefore disrupts its ability to be recruited to chromatin proximal to the sites of DNA damage in an MDC1-dependent manner.

3.2.15 ΔSer118 NBS1 mutation does not result in a G2/M checkpoint activation defect

Since we had previously demonstrated that the R28A FHA domain mutation in NBS1 phenocopies the loss of S118 in the BRCT1 domain in terms of its ability to bind to phospho-MDC1 and be recruited to sites of DSBs, it could be hypothesised that this patient derived *NBS1* mutation would also give rise to a defect in the ability of cells to activate the G2/M cell cycle checkpoint. To investigate this, fibroblasts from a normal individual, a classical 657del5 NBS patient and one of the affected patients (NBS^{BRCT} (C) were treated with different doses of IR and harvested 1 hour post-irradiation. The cells were fixed, stained with an antibody for phospho-histone H3 ser-10 to detect mitotic cells and then subjected to FACS analysis (NB Fibroblasts from the classical NBS patient were only irradiated with a single dose of IR). Strikingly, in contrast to the expected G2/M checkpoint failure typically observed in cells derived from classical NBS patients, the fibroblasts from NBS^{BRCT} patient exhibited a dose-dependent decrease in p-H3 staining indistinguishable from that observed in the normal cell line (Figure 3.24). This suggests that although both the FHA and the BRCT domains of NBS1 are essential for the phospho-dependent interaction with MDC1, it would appear that only the FHA domain is required for G2/M checkpoint activation. In keeping with this apparent disparity between the cells requirement for the FHA domain but not the BRCT domain of NBS1 to activate the IR-induced G2 checkpoint, it has been reported that another NBS1

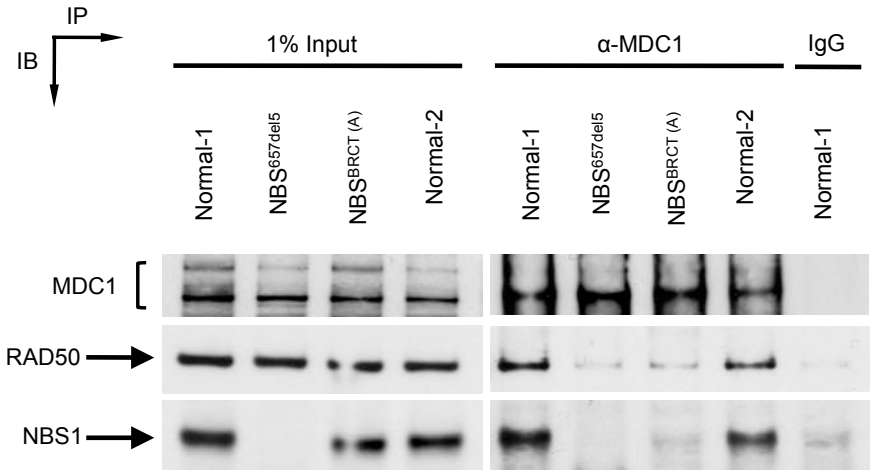


Figure 3.21 ***Loss of NBS1 S118 disrupts its binding to MDC1.*** *Cellular extracts were incubated with an antibody against MDC1 and immunopreciptates were subjected to SDS-Page and Western blotting with the antibodies indicated.*

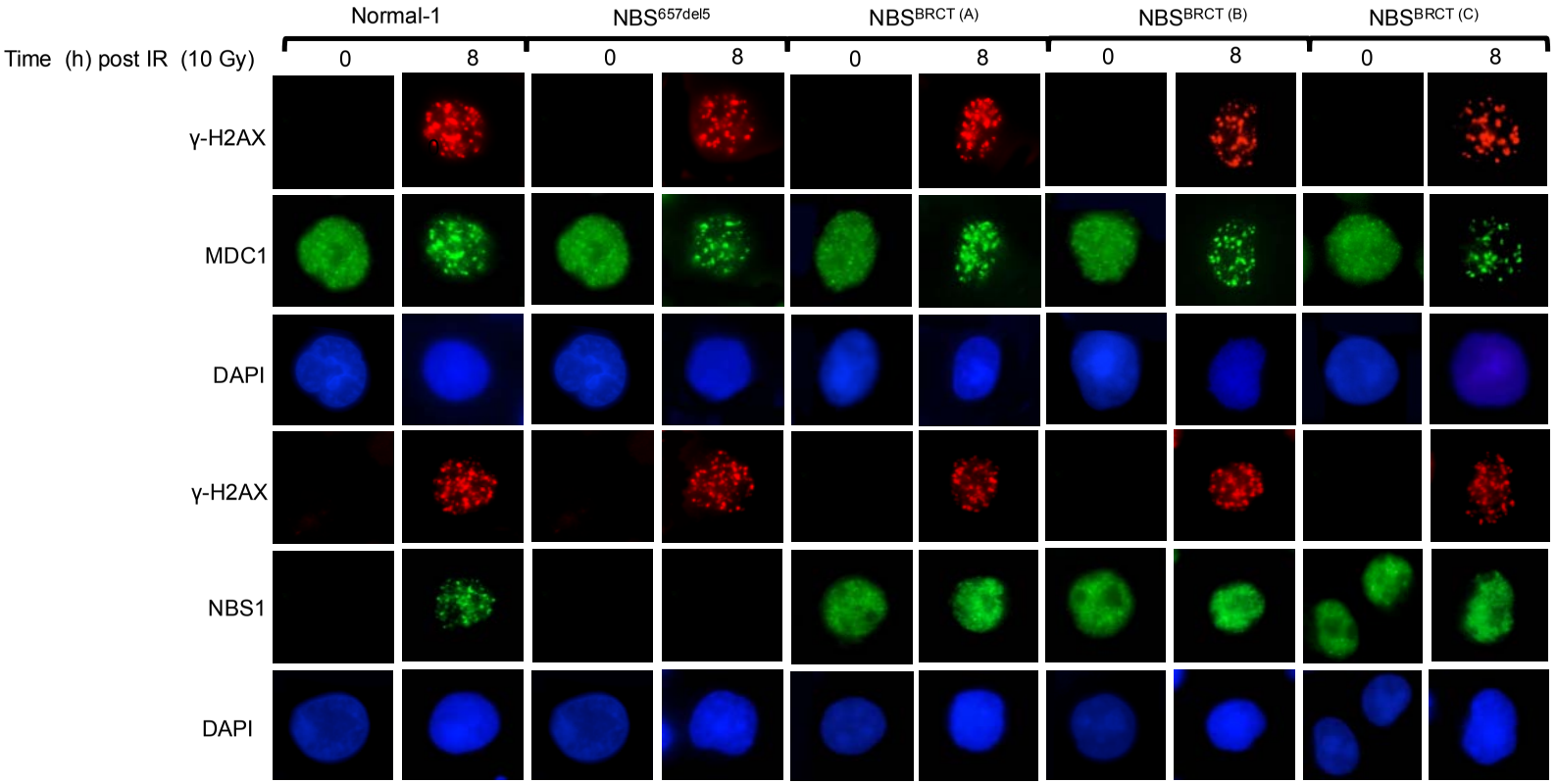


Figure 3.22 ***Loss of S118 of NBS1 compromises its ability to relocalise to sites of DSBs.*** *Lymphoblastoid cells from the three affected NBS^{BRCT} patients, a 'normal' individual (Normal-1) and a classical NBS patient (NBS^{657del5}) were unirradiated (0) or irradiated with 10 Gy of IR and harvested 8 hours later. The cells were then methanol fixed and seeded on to poly-L-lysine coated coverslips. The fixed cells were then immunostained with antibodies against MDC1 or NBS1. An antibody against γH2AX was included as a marker of DSBs. Images are of one cell and are representative of approximately ~1000 cells scored by eye from 3 separate experiments. Images are are representative of approximately ~1000 cells scored by eye from 3 separate experiments.*

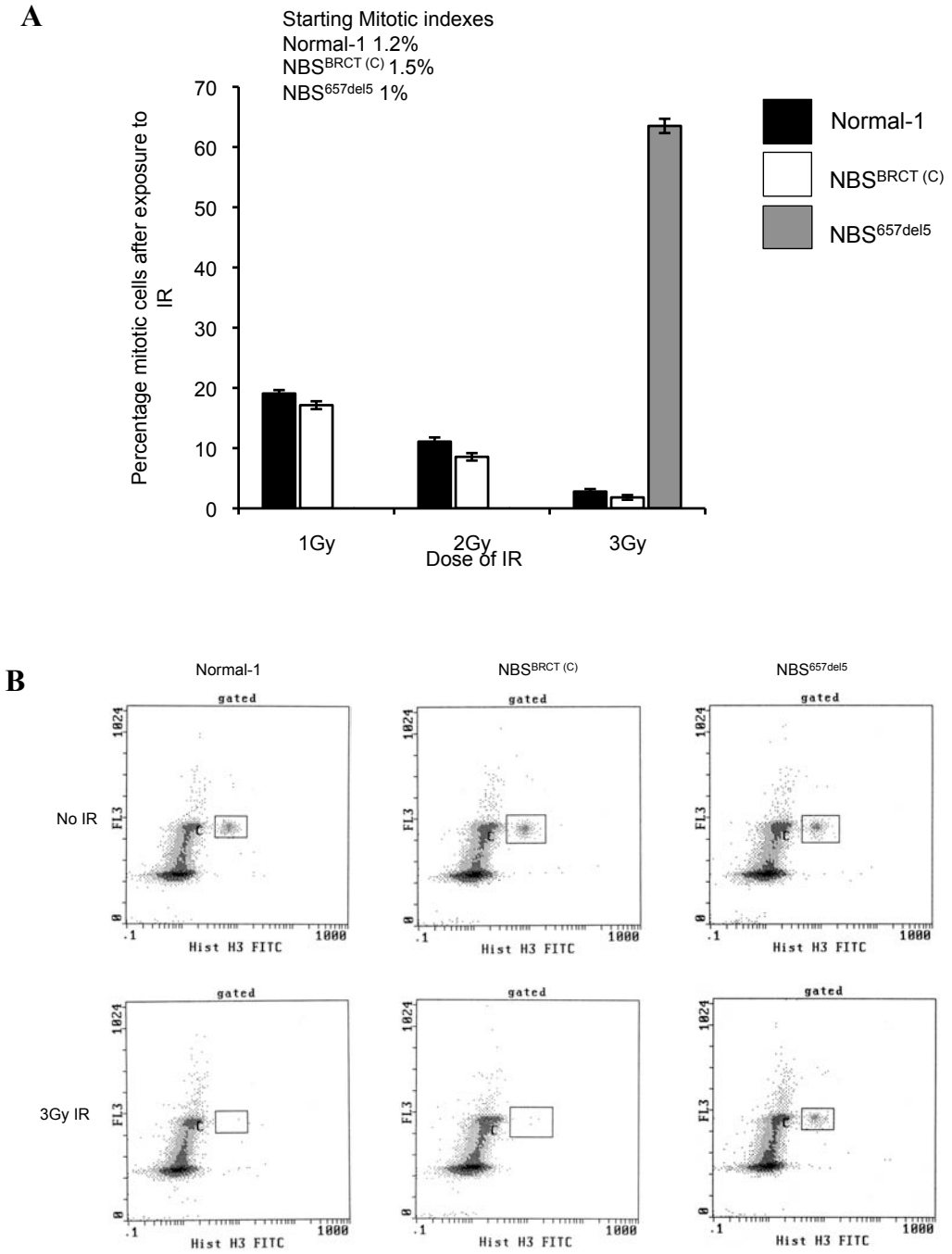


Figure 3.23 ***The IR-induced G2/M checkpoint is intact in cells derived from one of the NBS^{BRCT} patients. (A)*** *Fibroblasts from one of the affected NBS^{BRCT} individuals (NBS^{BRCT}AR), a classical NBS patient (657del5) and a normal (Normal-KB) were irradiated with 1, 2 and 3 Gy of IR and harvested 1 hour later. The cells were then fixed in 70% ethanol and incubated with a phospho-histone H3 serine-10 antibody. Cells were then stained with propidium iodide and analysed using FACs. The data is an average of 3 experiments, error bars represent standard error and data has been normalised to 100%.* ***(B)*** *Representative FACS profiles at 0 Gy and 3 Gy for each cell line tested.*

????

BRCT1 mutation (K160M), that also disrupts MDC1 binding, does not result in an inability to regulate this checkpoint (Hari *et al*., 2010).

3.3 Discussion

MDC1 acts as a molecular scaffold that coordinates both the temporal and spatial regulation of the cellular DDR. This function of MDC1 is mediated through its ability to bind simultaneously to a multitude of different damage response proteins. For instance, the MDC1 FHA domain alone has several documented binding partners, including CHK2, phosphorylated ATM and RAD51 (Lou *et al.*, 2006, Lou *et al.*, 2003b, Stewart *et al.*, 2003, Zhang *et al.*, 2005). Here, we have identified and characterised a novel region of MDC1, termed the SDTD domain, which is constitutively phosphorylated by CK2. This modification facilitates an interaction with the FHA domain of NBS1 , and as a consequence is necessary for the recruitment of the NBS1 to DNA damage foci and activation of the G2/M checkpoint following IR.

The requirement for CK2 phosphorylation to mediate protein-protein interactions within the DDR is not specific to MDC1 and NBS1. As described previous, a similar cluster of CK2 phosphorylation sites on XRCC1 and XRCC4 mediates their ability to bind the FHA domains of either APTX or PNK (Clements *et al.*, 2004, Koch *et al.*, 2004, Luo *et al.*, 2004). This repeated requirement for CK2-dependent phosphorylation to mediate many protein-protein binding highlights this as an essential cellular process. In keeping with this, the budding yeast, *S. cerevisiae*, homolog of NBS1, Xrs2, binds in a phospho-dependant manner to the XRCC4 homolog Lif1, via its FHA domain of Xrs2 (Matsuzaki *et al* 2008, Palmbos *et al* 2008). Taken together, this conservation of phosphorylated SDTD motifs mediating binding to FHS/BRCT

domain containing proteins suggest that this may represent a fundamental mechanism for controlling protein function/localization (Lloyd *et al* 2009).

The observations from this study show MDC1 and NBS1 exist in a complex, which is dependent on CK2. However, based on data presented here and from work by others there is evidence to suggest that not all NBS1 is bound to MDC1. Upon CK2 knockdown, therefore disrupting its association with MDC1, NBS1 foci are abolished (Figure 3.10). Yet, the NBS1-dependent accumulation of phosphorylated-ATM is not affected, suggesting that NBS1 is still present at the site of a DNA double-strand break (Figure 3.9). Furthermore, cells lacking MDC1 or CK2 can still efficiently activate the ATM-dependent DDR (Figure 3.6) (Lou *et al*., 2006, Stewart *et al*., 2003). This is further supported by the observation that NBS1 is still able to accumulate within striped laser damage, in the presence of MDC1 lacking the SDTD region (Chapman & Jackson 2008). Moreover, early research showed that cells transfected with an NBS1 R28 FHA mutant still displayed ATM activation following IR, yet failed to form NBS1 IRIF (Horejsi *et al* 2004). Therefore, it would seem a fraction of NBS1 not bound to MDC1 is able to localise to the sites of DNA damage, and that this pool of NBS1, along with the other members of the MRN complex, is responsible for the activation of ATM and the ATM-dependent DDR. It could be speculated that once the ATM-dependent signalling cascade has been activated, the second pool of NBS1/MRN, that which is bound to MDC1, is recruited to chromatin via the interaction between MDC1 and γH2AX. The localisation of this second pool of NBS1/MRN could serve to amplify the intra-cellular DNA damage signal by further activation of ATM and γH2AX accumulation. This amplification step may be necessary to reach a signalling threshold for cell cycle checkpoints to become activated, which is supported by the partial G2/M checkpoint defect observed in cells expressing an NBS1 FHA domain mutant, and an intra-S pahse checkoint defect in MDC1 deficient cells

complemented with a mutant lacking the SDTD repeat domain (Figure 3.14) (Wu *et al.*, 2008). It could be inferred that although there is partial activation of the G2/M checkpoint at low doses of IR in NBS cells expressing an FHA point mutation, the CK2-dependent MDC1/MRN interaction is required to fully activate the checkpoint. Overall, these observations imply that following ATM activation at a DNA DSB, the focal recruitment of NBS1 to the chromatin is required to amplify the intra-cellular signal induced by low but not high levels DNA damage. In keeping with this, previous work has shown that a different NBS1 FHA domain mutant, H45A, also displays a partial G2/M checkpoint at low but not high does of IR (Difilippantonio *et al* 2007).

If only a proportion of NBS1 is constitutively bound to MDC1, what are the mechanisms that regulate the interaction? Mirroring XRCC1, it could be that multiple different proteins interact in a phospho-dependent manner with the same site on MDC1 and that the regulatory control occurs at the level of the binding partner rather than whether the scaffold protein is phosphorylated or not. In this respect Aprataxin and TopBP1 have been shown to interact with SDTD domain of MDC1 via their FHA or BRCT domains respectively (Becherel *et al* 2009, Wang *et al* 2011). Therefore, it is possible that this cluster of phosphorylation sites on MDC1 may also provide a binding platform for other as of yet unidentified phospho-peptide binding domain-containing proteins that prevents its interaction with NBS1. Despite the vast majority of CK2-dependent phosphorylation being constitutive, some degree of cellular regulation could be achieved by a stimulus-mediated increase or decrease in the association of CK2 with its substrate. In this respect it has been shown that the localisation of CK2 can change after the exposure to cells to specific types of genotoxic agent and that this may be involved in promoting DNA damage-inducible phosphorylation of a subset of CK2 substrates e.g. HP1-β, histone H4 or p53 (Ayoub *et al.*, 2008, Cheung *et al.*, 2005, Keller and Lu, 2002,

Keller *et al.*, 2001, Olsen *et al.*, 2012, Yamane and Kinsella, 2005b). Furthermore, it is tempting to speculate that the level of regulating CK2-mediated phospho-interactions occurs at the level of dephosphorylation. Following completion of repair, the cell needs to disassemble the DNA repair machinery and inactivate the checkpoint machinery in order to re-enter into the cell cycle. It has been reported that this can, in part be achieved through the dephosphorylation of H2AX mediated by PP2A, PP4 and PPM1D (Cha *et al.*, 2010, Chowdhury *et al.*, 2005, Chowdhury *et al.*, 2008, Moon *et al.*, 2010, Nakada *et al.*, 2008). Based on this it could be postulated that the SDTD motifs of MDC1 may also be a target for these phosphatases and that this could be one mechanism with which the MDC1/MRN-dependent DDR is terminated of how different pools of MDC1 and NBS1 are maintained. However, this would require further investigation.

The importance of the interaction between MDC1 and NBS1 (MRN complex), described here, is highlighted by the identification of a novel NBS family from the United Arab Emirates, in which the affected individuals carry a homozygous *Nbs1* gene mutation that disrupts this association. This mutation is a single amino acid deletion, ΔS118, in the BRCT1 domain of NBS1, that not only not only abolishes the interaction between MDC1 and NBS1, but also completely blocks the ability of NBS1 to form IRIF (Figure 3.21, 3.22 & 3.23). These observations are supported by work from other groups, demonstrating that the BRCT domains of NBS1 are also required to mediate binding to the SDTD domain of MDC1 (Chapman and Jackson, 2008, Melander *et al*., 2008, Spycher *et al*., 2008, Wu *et al*., 2008, Hari *et al.*, 2010). However, unlike mutating the FHA domain of NBS1, the BRCT1 S118 mutation does not result in a G2/M checkpoint defect (Figure 3.24). This preliminary analysis recapitulates much of what is known about this domain obtained from studies using the K160M mutation (Hari *et al.*, 2010, Lloyd *et al.*, 2009). Moreover, evidence from one of these

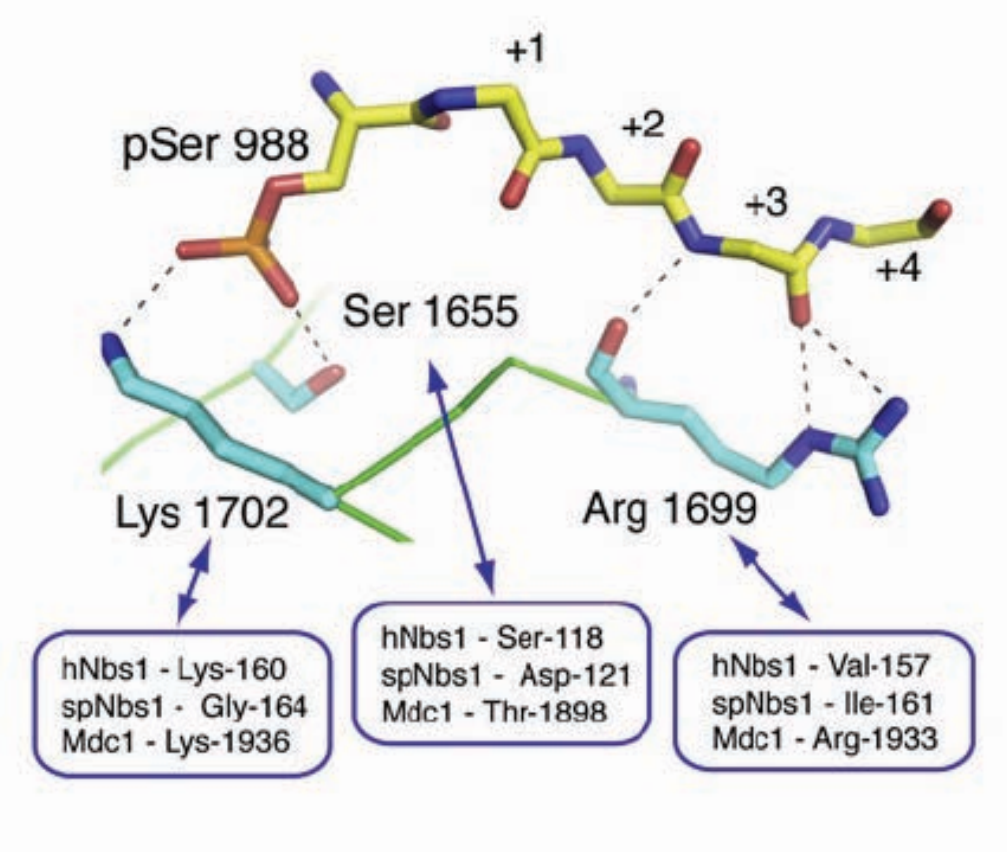


Figure 3.24 ***Residues necessary for BRCA1 BRCT/BACH1 phospho-dependent interactions are partially conserved in human NBS1 (hNbs1).*** *Stick model taken from Lloyd et al., 2009, showing conservation of NBS1 K160/K1702 and S118/ S1655 in BRCA1, respectively.*

studies showed, via structural analysis, that residues analogous to K160 and S118 of NBS1 are positionally conserved in the BRCT domains of other proteins, supporting the evidence presented here that both these residues are critical for phospho-dependent interactions (Figure 3.19 & 3.25) (Lloyd *et al.*, 2009). Despite both these domains being required for focal recruitment of NBS1 and its interaction with MDC1, why is a G2/M checkpoint defect only observed following mutation of the FHA domain? It could be speculated that the FHA domain of NBS1 may have an as yet unidentified binding partners, and it is this association with these proteins that is required for checkpoint activation in the presence of DNA DSBs. In this respect, the *S.pombe* end-processing factor Ctp1 contains a domain similar to the SDTD region of MDC1. Several studies have demonstrated that this region is potentially phosphorylated by CK2 and interacts also with the FHA domain of NBS1 (Dodson *et al.*, 2010, Hari *et al.*, 2010, Lloyd *et al.*, 2009, Williams *et al.*, 2009). Therefore, it is possible that the human orthologue, CtIP, may be another potential binding partner for the phospho-dependent binding activities of NBS1 that is regulated by CK2. Indeed a direct interaction has been seen with CtIP and NBS1 in human cells (Chen *et al.*, 2008). Moreover, CtIP has previously been implicated in activation of the G2/M checkpoint (Yu and Chen, 2004). Theses observations suggest that the MDC1/NBS1 interaction and foci accumulation, although perhaps still necessary for signal amplification, may not be required for G2/M checkpoint activation.

Intriguingly, the affected patients from this family only exhibit some of the clinical abnormalities commonly associated with NBS patients carrying the hypomorphic 657del5 mutation. Moreover cells from these patients lack many of the major cellular phenotypic defects associated with mutations in this gene, the major one being genome instability. Initial characterisation of cells derived from the affected patients has shown that the ΔS118 mutant

NBS1 protein is expressed at normal levels and binds efficiently to MRE11 and RAD50 (Figure 3.20, 3.21). Strikingly cells from these patients do not appear to have a defect in the repair of DNA DSBs, as judged by chromosomal breakage studies and the lack of immunodefciency (Table 3.4). This suggests that although the ΔS118 mutation disrupts the ability to bind to MDC1 and foci formation, it does not appear to impact on the DDR to DSBs. Therefore, it could be speculated that the ΔS118 mutation in the patients described here may disrupt other functions of NBS1.

The microcephaly and developmental defects presented in the affected individuals from this unique NBS family have phenotypic similarities that overlap with other human disorders associated with developmental defects, such as Seckel Syndrome (SCKL). Based on neurodevelopmental characterisation of the $ATR^{sckl/sckl}$ mouse model, it has been proposed that the underlying cause of these phenotypes is caused by a failure to overcome replication stress that occurs during embryonic development (Murga *et al.*, 2009). It is therefore tempting to speculate that the clinical symptoms presented in the NBS^{BRCT} patients may be as a result of problems in dealing with replication stress, rather than due to an overt DSB repair defect. There are several lines of evidence that support this hypothesis: Firstly, NBS1 has been shown to be required for efficient activation of ATR in response to replication damage and secondly, cells from NBS patients exhibit similar problems restarting stalled replication forks as those found in cells with a compromised ATR pathway, signalling is defective in NBS cells, which also display problems in recovery following replication stalling, suggesting NBS1 and ATR function in the same pathway (Stiff *et al.*, 2005, Stiff *et al*., 2008). Given that the 657del5 NBS patients and the NBS^{BRCT} patients both present comparable severity of microcephaly and growth defects, it could be postulated that the BRCT of NBS1 is critical for protecting neuroprogenitor cells from replication stress. Since replication stress is prevalent in

these cells due to their highly proliferative nature, it seems likely that if not dealt with appropriately the accumulation of abnormal replication intermediates represents the most likely cause of neuronal cell death. If theses lesions are not deal with, the accumulation of replication-born DSBs could result in cell death. Indeed, published data has shown that the deletion of NBS1 in mouse neuronal tissue triggers excessive apoptosis in proliferating neuroprogentitor cells (Zhou *et al.*, 2012). Furthermore, it was suggested that this was caused by the accumulation of endogenous replication intermediates, resulting from improper fork resolution, being converted to DSBs, and activating a p53-dependent cell death pathway (Bruhn *et al.*, 2014). Thus, NBS1 clearly has a function during replication. However, whether it is loss of this function of the protein that gives rise to the neurodevelopmental phenotype exhibited by the NBS^{BRCT} patient's remains to be elucidated. Despite this, it is clear that the study of these extremely rare patients could provide an opportunity to study the role of the NBS1 protein during the cellular response to replication stress, independently of its role in DSB repair. From this, the overall goal will be to attribute some aspects of the clinical phenotype exhibited by patients with mutations in this gene to a specific function of the protein.

CHAPTER 4

Chapter 4 Identification of putative CK2 motifs on Mre11

4.1 Introduction

Like many DDR proteins, MRE11 is post-translationally modified before and after the induction of DNA damage, but how these regulate its specific functions remains unclear. It has been demonstrated that MRE11 is constitutively methylated on arginine residues within its GAR domain by PRMT1 and this modification is essential for its exonuclease activity, checkpoint activation and end-resection-dependent HR (Boisvert *et al.*, 2005a, Yu *et al.*, 2011). However, in contrast, little is known about what role, if any, phosphorylation plays in controlling the MRE11-dependent DDR. Several large phospho-proteomic screens have identified both constitutive and DNA damage-inducible phosphorylation sites on MRE11 but their functional relevance has not been thoroughly addressed (Beausoleil *et al.*, 2004, Dephoure *et al.*, 2008). A single study using an *in vitro Xenopus* egg extract system has shown that the DNA damage-inducible phosphorylation of MRE11 mediated by ATM and other PIKKs is required to reduce its ability to tether DNA ends and maintain ATM-dependent signaling (Di Virgilio *et al.*, 2009). The authors suggest that the phosphorylation of MRE11 in response to DNA damage is modulated by phosphatases, which ensure that the MRN complex does not disassociate from the chromatin prematurely, but at the same time prevents MRN from spreading uncontrollably along the chromatin surrounding the break. They propose that lack of regulation in this scenario could be harmful to the cell by potentially prolonging DDR signalling, cell-cycle arrest and/or inducing apoptosis (Di Virgilio *et al*., 2009). Thus, the phosphorylation-dependent removal of the MRN complex from the chromatin could constitute a means through which the cell can 'switch off' the DDR and trigger re-entry into the cell cycle.

In addition to PIKK-dependent phosphorylation of MRE11, a report published by Kim in 2005, identified another phosphorylation site within MRE11 (serine-649) and suggested that this residue is constitutively modified *in vivo* (Kim, 2005). Interestingly, the author suggested that this site might be targeted by CK2 indicating that this kinase may not only regulate the ability of the MRN complex to bind to MDC1 as previously described in the previous chapter, but may also modulate directly one or more activities of the complex itself. In support of this potential biochemical link between MRE11 and CK2, additional *in vivo* phosphorylated sites on MRE11 have been identified by mass spectrometry that lie within amino acid sequences that conform to the consensus CK2 phosphorylation motif (Beausoleil *et al*., 2004, Dephoure, *et al*., 2008).

Given the growing body of experimental evidence documenting a major role for CK2 in regulating a variety of different cellular DDR signalling pathways, and our own observations demonstrating that CK2-dependent phosphorylation is essential for the interaction between the MRN complex and MDC1, the aims of this study were:

i) *Identify the sites of CK2-dependent phosphorylation of MRE11.*

ii) *Investigate the contribution of CK2-dependent MRE11 phosphorylation of MRE11 to regulation of cellular DDR.*

4.2 Results

4.2.1 A C-terminal region of MRE11 is phosphorylated by CK2 in vitro

MRE11 has previously been shown to be phosphorylated by CK2 on serine 649 (S649) *in vivo* (Kim 2005). The results from this study, and the large number of proteins that contain putative CK2 phosphorylation motifs prompted the search for other potential CK2 target sites within MRE11. Referring to published, large-scale phospho-protein screens and several commercial websites (http://scansite.mit.edu/, http://phospho.elm.eu.org/, http://www.phosphosite.org/, (http://www.dabi.temple.edu/disphos) designed to identify putative phosphoryaltion sites, two additional MRE11 CK2 sites were identified up-stream of S649 at serine 688 (S688) and 689 (S689) (Beausoleil *et al.*, 2004, Dephoure *et al.*, 2008) (Table 4.1.). Using an online protein alignment tool, Clustal omega ((http://www.ebi.ac.uk/Tools/msa/clustalo/), all three sites were shown to be conserved across Human (*H.sapiens*), Chimpanzee (*P. troglodytes*), Mouse (*M.musculus*), Rat (*R.norvegicus*) and Frog (*X.laevis*), which suggests that these residues may have a functional significance (Figure 4.1).

To investigate whether the identified residues were CK2 targets, a GST-tagged expression construct encompassing a C-terminal fragment of MRE11 containing the 3 putative CK2 phosphorylation sites (GST-MRE11 (aa360-708)) recived from Dr Xiahua Wu, TRSI, California, was expressed in bacteria and the induced protein purified using glutathione beads. The purified Mre11 protein was then incubated with recombinant CK2 and radio-labelled $\gamma(^{32}P)$-ATP, for 10 minutes at 30°C. The kinase reactions were run on an SDS-PAGE gel, which was then stained with Coomassie Brilliant Blue and phosphorylated proteins were

Phosphorylation Site	Peptide	Reference
S649	EVIEVDES*DVEEDIF	Kim, 2005. Dephoure, *et al*, 2008 http://scansite.mit.edu/ http://phospho.elm.eu.org/ http://www.phosphosite.org/ http://www.dabi.temple.edu/disphos/
S688	SKGVDFES*SEDDDDD	Beausoleil, *et al*, 2004. Dephoure, *et al*, 2008. http://scansite.mit.edu/ http://phospho.elm.eu.org/ http://www.phosphosite.org/ http://www.dabi.temple.edu/disphos/
S689	KGVDFESS*EDDDDDP	Beausoleil, *et al*, 2004. Dephoure, *et al*, 2008. http://scansite.mit.edu/ http://phospho.elm.eu.org/ http://www.phosphosite.org/ http://www.dabi.temple.edu/disphos/

Table 4.1 ***Summary of potential CK2 phosphorylation sites (*) in MRE11 identified in vivo.***

Figure 4.1 ***MRE11 putative CK2 phosphorylation residues are conserved between species.*** *The C-terminal region of human MRE11 (H.sapiens) containing the putative CK2 phosphorylation motifs was aligned with several other vertebrate species, Pan troglodytes (P.trolodytes), Mus musculus (M.musculus), Rattus norvegicus (R.norvegicus) and Xenopus laevis (X.laevis) to identify any evolutionary conservation, using an internet based multiple sequence alignment programme, Clustal omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). The potential CK2 sites are shaded in grey.*

visualised by audioradiography. Consistent with the data published by Kim (2005), the GST-MRE11 fragment containing the CK2 phosphorylation motifs was robustly phosphorylated by CK2 *in vitro* compared to the GST control (Figure 4.2). This data indicated that CK2 can target MRE11 directly for phosphorylation, albeit *in vitro*.

To assess which of the 3 phosphorylated serine residues were targeted within the MRE11 C-terminus identified as putative CK2 targets, three new recombinant GST-MRE11 (aa360-708) fragments were produced. In each of these fragments the serine of each potential CK2 motif had been mutated to alanine. The fragments were named 1A (S649A), 2A (S688A/S689A) and 3A (S649A/S688A/S689A). Following sequential rounds of mutagenesis, the constructs were sequenced to ensure that no additional mutations had occurred during the amplification steps. The mutant recombinant proteins were again expressed in bacteria, purified as previously described and then used as substrates in an *in vitro* CK2 kinase assay. A specific CK2 inhibitor, 4,5,6,7-Tetrabromobenzotriazole (TBB), was added to half the kinase reactions to verify that any observed phosphorylation of the mutant MRE11 proteins was not due to a contaminating kinase (Battistutta *et al.*, 2001, Sarno *et al.*, 2001). Interestingly, in disagreement with the observations by Kim (2005) mutation of serine 649 to alanine did not reduce the *in vitro* phosphorylation of MRE11 by CK2 (Figure 4.3). However in contrast, mutation of serines 688/689 appeared to compromise the CK2-dependent phosphorylation of MRE11. Moreover, a similar reduction *in vitro* phosphorylation was also seen following sequential mutation of all 3 putative CK2 sites, suggesting that at least *in vitro* serine 649 is not a major target for CK2-dependent phosphorylation, and that serines 688/689 may be the preferred sites for modification, although this would require further investigation (Figure 4.3). Despite this, a significant amount of MRE11 phosphorylation was still observed even when all three serine residues were mutated in combination. This observation suggested the

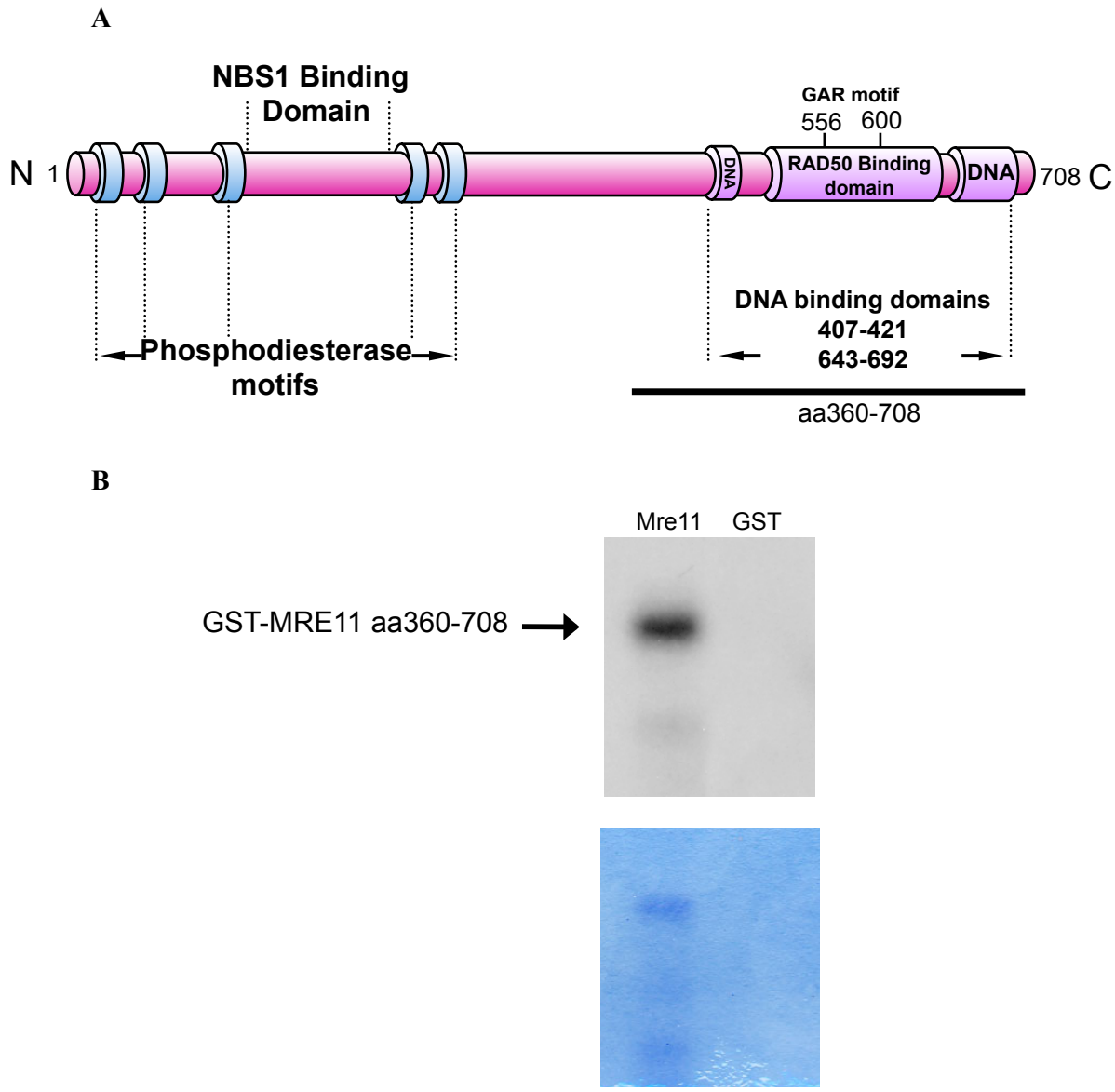
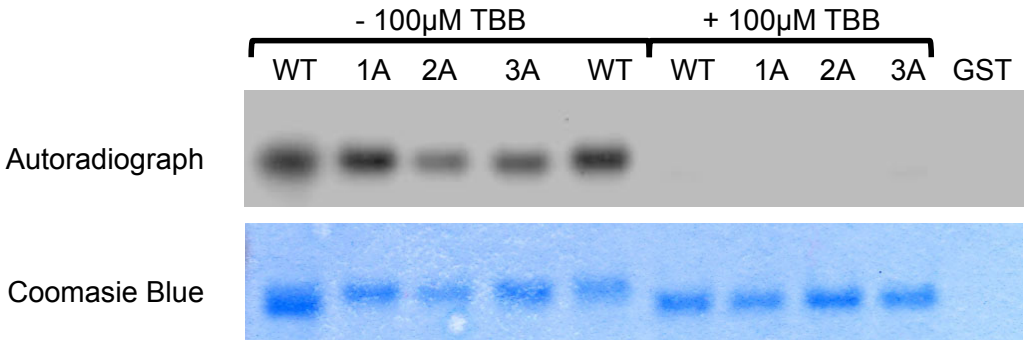


Figure 4.2 ***The C-terminal region of MRE11 is phosphorylated by CK2 in vitro. (A) A schematic of MRE11 showing known domains and the location of the amino acid 360-708 MRE11 GST fragment****. (B) A C-terminal region of MRE11 is phosphorylated by CK2 in vitro****. A GST-tagged MRE11 C-terminal fragment spanning amino acids 360-708 was expressed in E.coli and purified. 1 µg of the GST MRE11 or GST alone was then incubated with recombinant CK2 and radio-labelled ATP (γ-^{32}P) at 30°C for 10 minutes. Proteins were separated by SDS-PAGE, stained with Coomassie blue and dried. Radio-labelled proteins were detected via autoradiography.*



646vde**s**dveedifpttsktdqrwssts**670**

671sskimsqsqvskgvdfe**ss**eddddd**695**

Figure 4.3 ***In vitro phosphorylation of mutant MRE11 lacking putative CK2 phosphorylation sites.*** *WT or mutant recombinant MRE11 was used as a substrate in a CK2 in vitro kinase assay as described before in Figure 4.2. WT denotes a wild type MRE11 protein fragment encompassing amino acids 360-708. Mutant MRE11 protein fragments 1A, 2A and 3A refer to the S649A, S688A/S689A and S649A/S688A/S689A mutations respectively. The CK2 inhibitor 4,5,6,7-Tetrabromobenzotriazole (TBB) was added to some kinase reactions to rule out any non-specific phosphorylation catalysed by a contaminating kinase(s). Kinase reactions were fractionated by SDS-PAGE, stained with Coomassie blue and dried. Radio-labelled proteins were detected by autoradiography.*

existence of an additional site(s) of CK2-dependent phosphorylation within the C-terminal half of the MRE11 protein.

4.2.2 Generation of ATLD2 stable cell lines

Whilst it was evident from the *in vitro* kinase assays that additional potential CK2 phosphorylation exist in the C-terminal half of MRE11, only serines 649, 688 and 689 had been identified as being phosphorylated *in vivo*. Therefore, it was decided that this project would be split into two halves:

1) Determine the physiological relevance of the *in vivo* phosphorylation of Mre11 on S649, S688 and S689

2) Identify additional sites on Mre11 phosphorylated by CK2 using *in vitro* kinase assays (This aspect of the project will be discussed in section 4.2.6)

In order to investigate the physiological relevance of these putative CK2 phosphorylation sites on MRE11, it was necessary to model a mutant MRE11 lacking these residues *in vivo*. Cells derived from the ATLD1/2 patients have a homozygous nonsense mutation in *MRE11* (1897C→T, R633 stop) that gives rise to the expression of a highly unstable truncated protein (Stewart *et al*., 1999). As a consequence of this *MRE11* mutation, the overall stability of the MRN complex is significantly reduced, and as such the expression of both NBS1 and RAD50 are also affected. These cells therefore provide an ideal system with which either exogenous WT or mutant MRE11 can be re-expressed without interference from the endogenous protein. Using site-directed mutagenesis, a retroviral expression vector (received from Dr Matthew Weitzman), containing full-length, FLAG-tagged *MRE11* was used to produce a panel of

MRE11 expression constructs ablating the putative CK2 phosphorylation sites: 1A (S649A), 2A (S688/689A) and 3A (S649/688/689A). These constructs were transfected into the 293FT packaging cell line and the media containing the infectious viral particles was used to transduce hTERT immortalized ATLD2 fibroblasts. Individual clones were cultured and selected on whether they exhibited physiological levels of MRE11 protein determined by Western blotting (Figure 4.4). Those clones that expressed levels of exogenous Mre11, comparable to the level of endogenous MRE11 expressed in a normal fibroblast, were used for future experiments. The presence of the mutated *MRE11* was also confirmed by sequencing across the mutated region using cDNA from the complemented cell lines (data not shown).

4.2.3 Mutation of MRE11 putative CK2 sites does not lead to instability of the MRN complex or loss of MRE11 arginine methylation.

It has previously been shown that loss of members of the MRN complex, either through mutation or siRNA-mediated depletion leads to a reduction in expression of the other members and instability of the complex (Stewart *et al.*, 1999, Zhong *et al.*, 2005). To determine whether mutation of the identified CK2 motifs in *MRE11* affected expression of the members of the MRN complex, stably transfected ATLD2 cell lines were lysed and the cell extracts were separated by SDS-PAGE. The proteins were transferred to nitrocellulose membranes and the blots were probed with antibodies against components of the MRN complex. The ATLD2 cell line containing an empty vector (V) and, therefore, lacking full-length WT MRE11, showed a significant reduction in the levels of RAD50 and NBS1 (Figure 4.5.A). In contrast, the WT and, more importantly, the three mutant stable ATLD2 cell lines, 1A, 2A and 3A all exhibited normal expression of RAD50, NBS1 and MRE11 itself

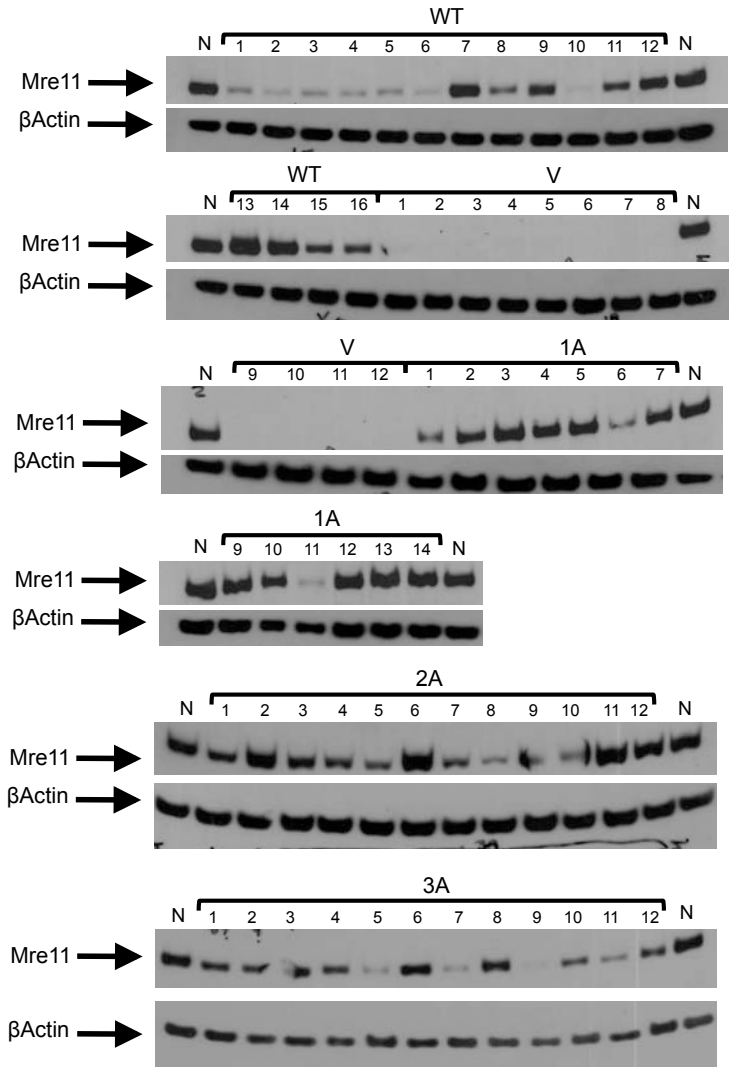


Figure 4.4 ***Colony screening of ATLD2 stable cell lines expressing Flag-tagged MRE11.*** *ATLD2 fibroblasts were infected with retroviruses expressing either WT, 1A, 2A, 3A MRE11 or an empty vector. Infected cells were selected using neomycin. Individual clones were isolated and then screened by Western blotting for expression of the exogenous MRE11 protein. 1A, 2A and 3A refer to the S649A, S688A/S689A and S649A/S688A/S689A MRE11 mutations respectively, V refers to the ATLD2 fibroblasts infected with an empty vector and N refers to a fibroblast cell line expressing endogenous levels of MRE11.*

indicating that mutation of the CK2 sites on MRE11 does not affect the stability of the other subunits of the MRN complex (Figure 4.5.A).

Since the three potential CK2 phosphorylation sites reside in close proximity to the RAD50 binding site, it is possible that mutation of these sites may compromise the ability of Mre11 to bind RAD50. Therefore, to investigate this, NBS1 was immunoprecipitated from cell extracts made from each of the complemented ATLD2 cell lines, separated the purified proteins by SDS-PAGE and then probed Western blots with antibodies to all three components of the MRN complex. In keeping with the ability of the MRE11 phospho-mutants to rescue the stability of RAD50 and NBS1 in the ATLD2 cells, similar amounts of RAD50 and MRE11 co-precipitated with NBS1 from both WT and mutant MRE11 ATLD2 cell extracts. This observation indicated that mutations in the putative CK2 phosphorylation sites in MRE11 do not affect the ability of the MRN complex to assemble *in vivo*. As expected there was a significant reduction in the levels of all members of the MRN complex in ATLD2-Vector only cell extracts that lack full-length MRE11 (Figure 4.5.B).

As the putative CK2 phosphorylation sites are located near to the GAR domain of MRE11, a second immunoprecipitation experiment coupled with Western blotting was performed to ascertain whether mutation of these phospho-sites affected the ability of PRMT1 to target this domain for modification. MRE11 immunoprecipitates from the ATLD2 cell lines were separated by SDS-PAGE, and Western blots probed with an arginine-methylated MRE11 specific antibody. The level of arginine methylation of the mutant Mre11 proteins was found to be comparable to those observed for the WT protein, demonstrating that compromising the phosphorylation on serine 649, 688 and 689 of MRE11 does not have any influence on methylation of the MRE11 GAR domain (Figure 4.6)

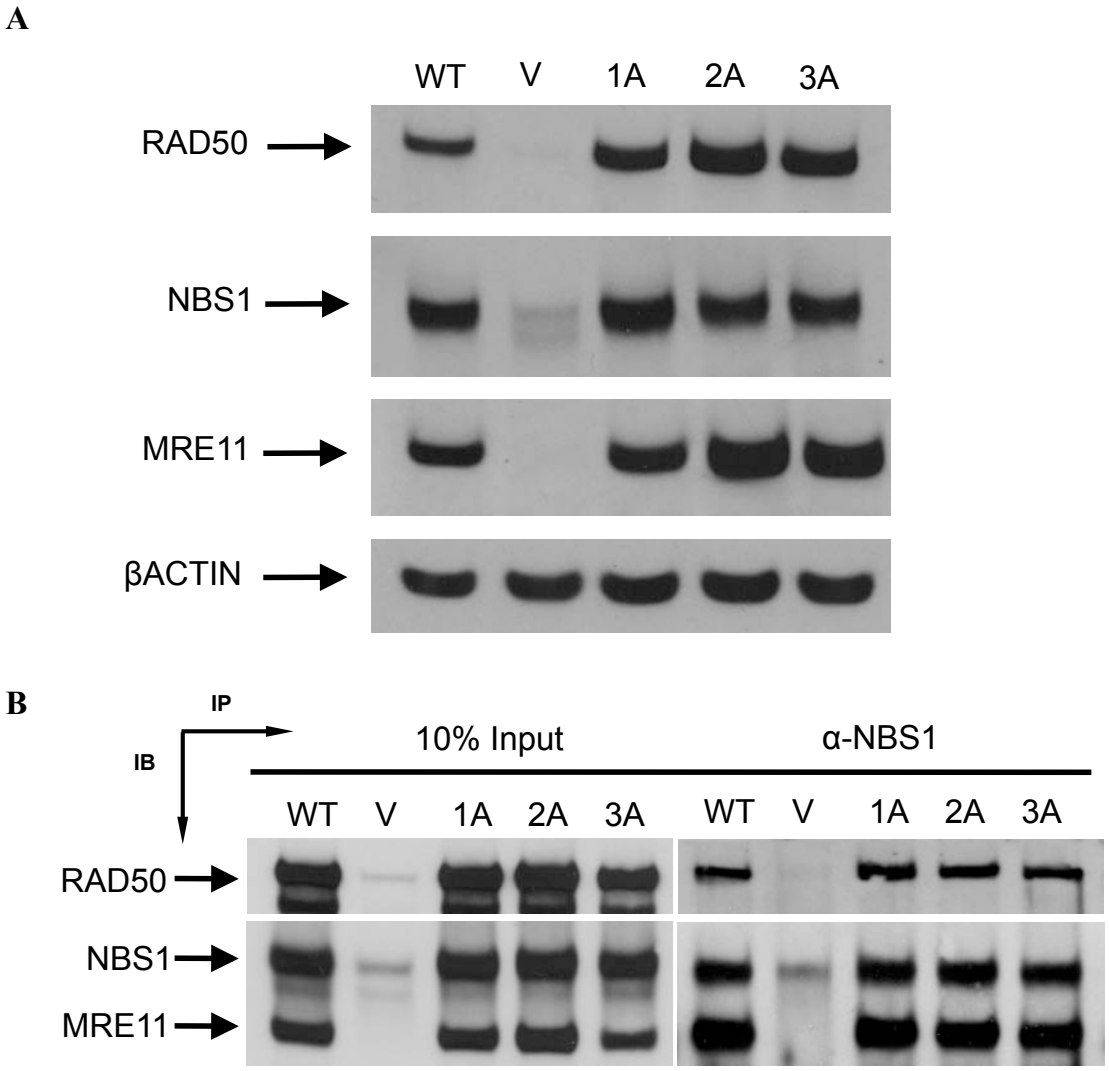


Figure 4.5 ***Mutation of the putative CK2 phosphorylation sites on MRE11 does not compromise its binding to NBS1 and RAD50.*** *(A) Western blot showing the expression of MRE11, RAD50 and NBS1 in selected stable ATLD2 fibroblast clones expressing either WT or mutant MRE11, 1A, 2A and 3A refer to the S649A, S688A/S689A and S649A/S688A/S689A MRE11 mutations respectively or an empty vector (V). β-Actin was used as a loading control. (**B**) NBS1 immunoprecipitates from ATLD2 cell extracts were subjected to SDS-PAGE and Western blotting using the antibodies indicated.*

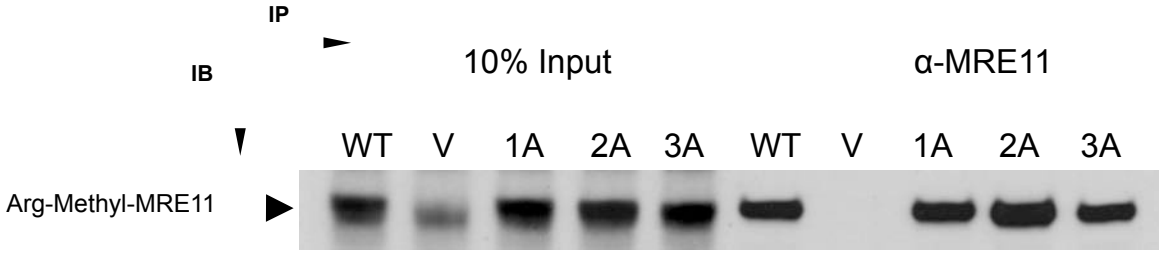


Figure 4.6 ***Mutation of the putative CK2 phosphorylation sites on MRE11 does result in loss of arginine methylation****. MRE11 immunoprecipitates from ATLD2 cells transduced with retroviral constructs 1A, 2A or 3A were subjected to SDS-PAGE and Western blotting using an antibody specific to arginine-methylated MRE11. 1A, 2A and 3A refer to the S649A, S688A/S689A and S649A/S688A/S689A MRE11 mutations respectively.*

4.2.4 Mutation of MRE11 CK2 phosphorylation sites does not prevent the MRE11 protein from forming ionising radiation-induced foci (IRIF)

Given that the putative CK2 phosphorylation sites are located near to the C-terminal DNA binding domain of MRE11, and that it has been previously shown that MRE11 phosphorylation regulates its retention on damaged chromatin (Di Virgilio *et al.*, 2009). It is possible that the constitutive phosphorylation of MRE11 on these sites may influence its ability to be recruited to sites of DNA damage in a manner comparable to the binding of NBS1 to CK2 phosphorylated MDC1. To examine this, ATLD2 fibroblasts complemented with either WT MRE11, empty vector or the individual serine-to-alanine mutants (1A, 2A and 3A) were mock-irradiated or irradiated with 10 Gy of IR and harvested at 0 h and 8 h post-irradiation. The cells were then fixed and stained with antibodies against MRE11 and γH2AX, the latter of which was used as a marker of DSBs.

As shown in Figure 4.7, WT MRE11 and all 3 phospho-site mutants formed radiation-induced foci following exposure to IR that colocalised with γH2AX. In contrast, the ATLD2 cells containing an empty vector failed to form any MRE11 IRIF (Figure 4.7). Therefore, it can be concluded that the putative CK2 phosphorylation sites on MRE11 are not required for its re-localisation to sites of DNA damage induced by IR.

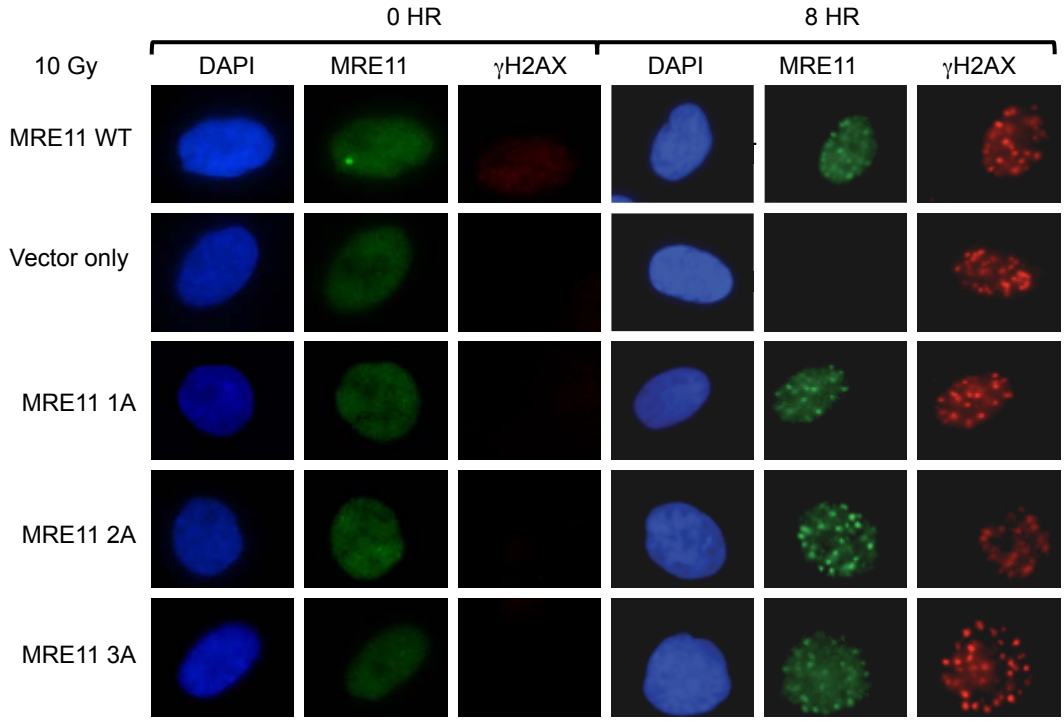


Figure 4.7 ***Mutant MRE11 lacking the putative CK2 phosphorylation sites accumulates normally at sites of DNA double-strand breaks.*** *ATLD2 cell lines stably expressing either WT MRE11, mutant MRE11 (1A (S649A), 2A (S688A/ S689A) and 3A (S649A/S688A/S689A)) or an empty vector, were mock treated or irradiated with 8 Gy of IR and harvested at 0 and 8 h post-irradiation. The cells were then fixed and stained with antibodies against MRE11 and γH2AX. Images are of one cell and are representative of approximately ~1000 cells scored by eye from 3 separate experiments.*

4.2.5 Mutant CK2-putative phosphosite (MRE11) transduced ATLD2 cell lines are proficient in repair of DNA DSBs

Whilst loss of the putative CK2 phosphorylation sites on MRE11 did not prevent its recruitment to damaged chromatin, it is still possible that these residues may be important for other aspects of MRE11 function, such as DNA repair. To test this, the stable ATLD2 cell lines were exposed to 2 Gy of IR and fixed at 0, 1, and 24 h post irradiation. Fixed cells were stained with antibodies against 53BP1 and γH2AX, and the IR-induced foci in a minimum number of 300 cells per experiment were quantified using fluorescent microscopy. 53BP1 foci were used as markers of DNA double strand breaks in conjunction with γH2AX in the event that mutations in *MRE11* compromised the ability of ATM to phosphorylate H2AX.

In keeping with the documented defect in DSB repair in ATLD cells, the ATLD2 cell line complemented with the empty vector (V) showed a significant number of residual 53BP1/γH2AX foci at 24 h post-irradiation compared to the WT cell line (Figure 4.8). In contrast, the mutant MRE11-expressing cell lines exhibited resolution of 53BP1 and γH2AX foci comparable to those observed in the WT MRE11 complemented ATLD2 cells, indicating that mutation of the putative CK2 phosphorylation motifs in MRE11 does not compromise repair of DNA DSBs induced by IR (Figure 4.8). It should be noted, as cell cycle progression was not controlled for with these cell lines, foci determination can be problematic as the lines used may cycle at different rates. If this experiment were to be repeated, evidence of how these four cell lines cycle could benefit the result, in addition to providing a positive control, for instance using a cell treated with an ATM inhibitor. The major role of the MRN complex is activating the ATM-dependent DDR following the induction of DSBs, and therefore it is conceivable that the phosphorylation of MRE11 by CK2 could affect this process. In support of this, it has been previously observed that the phosphorylation of SMC3

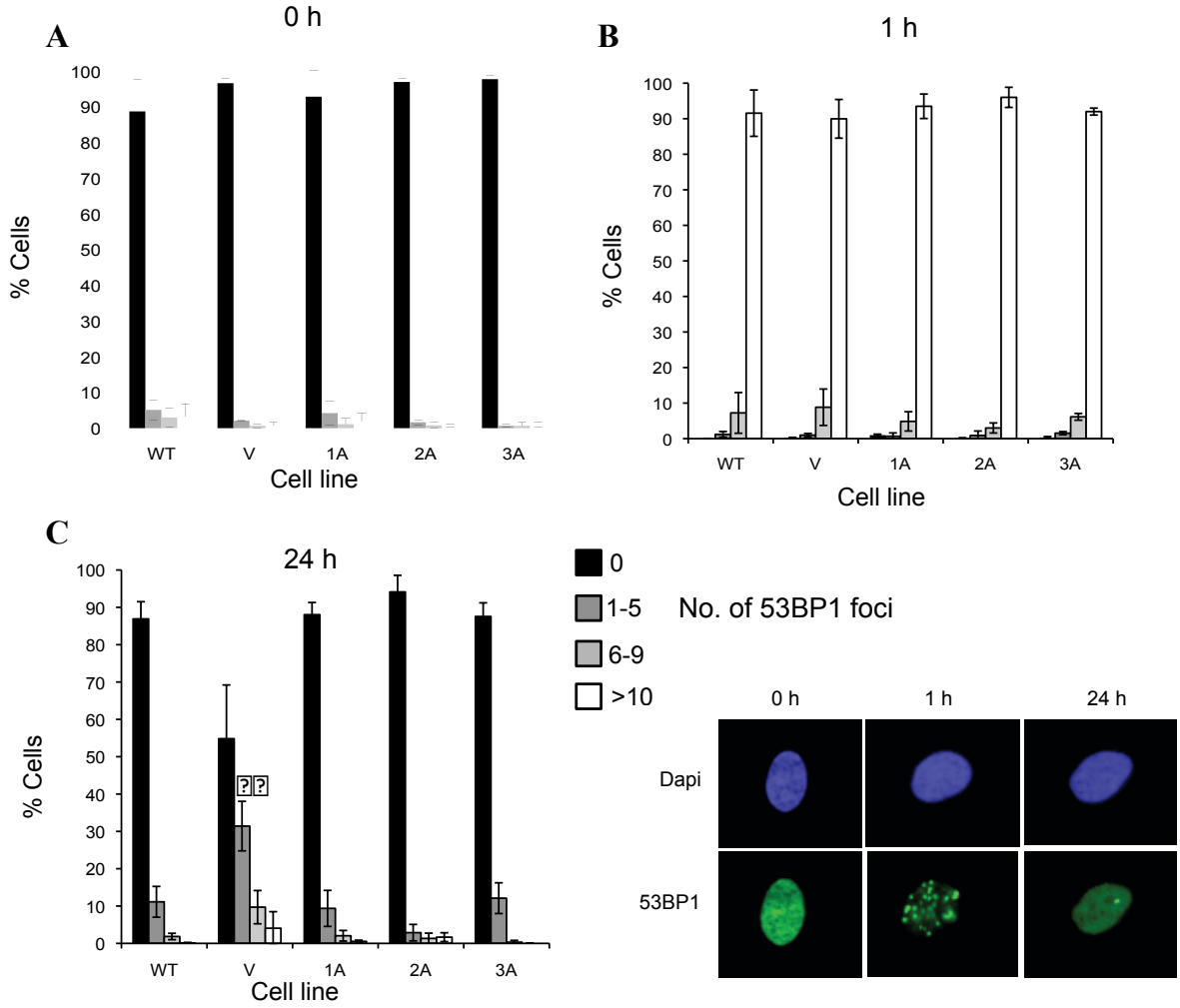


Figure 4.8 ***ATLD2 cell lines stably expressing phospho-site mutants of MRE11 repair DSBs normally.*** *Each of the stable ATLD2 cell lines were mock treated or irradiated with 2 Gy of IR. The cells were then harvested at 0hrs, 1 h and 24 h post-irradiation, fixed and stained with antibodies against 53BP1 and γH2AX. 53BP1 foci were then counted in each of the five cell lines for each time point, 0hrs (**A**), 1 h (**B**) and 24 h (**C**) and the percentage of cells with foci was calculated (n=300). Values represent the mean of three experiments ± standard error. * = <p=0.05 (t-test) compared to wildtype.*

by ATM requires its prior phosphorylation by CK2 on a neighbouring residue (Luo *et al*., 2008). To assess this, the panel of stable ATLD2 cell lines were irradiated with 2 Gy, harvested at 0, 1, and 24 h post-irradiation and then extracts from these cells subjected to SDS-Page and Western blotting using phospho-specific antibodies that recognize a range of known ATM substrates. After performing this assay numerous times it became apparent that a substantial variation in the expression of the non-phosphorylated form of many of these ATM targets, such as SMC1, H2AX and RPA, occurred between the different isogenic cell lines as well as between different clones of the same cell line (data not shown). As a result, this variation made drawing any conclusions as to whether mutation of the putative CK2 phosphorylation motifs in MRE11 affected activation of the ATM-dependent DDR extremely challenging. In addition to this, despite being hTert immortalised, the growth of the cloned ATLD2 complemented cell lines started to slow down significantly over time, and eventually reached a point where they stopped growing at all. Similar growth defects were observed in multiple different isolated clones of each cell line, as well as following revival of earlier passages of the same cell line from stocks stored in liquid nitrogen. The expression of hTERT was verified again by PCR in each of the cloned cell lines to rule out the possibility that these growth defects arose from loss of its expression, and as a consequence the induction of senescence. However, this did not seem to be the case.

Since the quality of the foetal calf serum (FCS) added to culture media can have a considerable affect on the ability of patient-derived cell lines to grow *in vitro*, especially fibroblasts, alternative sources of the foetal calf serum were used in different concentrations to determine whether this maybe the underlying cause for the growth failure of these cells. Unfortunately this failed to rectify the problem.

Taken together, it was decided that a different cell system was required to determine whether loss of putative CK2-dependent phosphorylation of MRE11 affected the cellular response to DNA damage.

4.2.6 Identification of an additional putative MRE11 CK2 phosphorylation motif

Previous *in vitro* kinase assays demonstrated that mutating all three *in vivo* putative CK2 phosphorylation sites within the C-terminus of MRE11 did not completely abolish its phosphorylation. This observation indicated the existence of additional residues targeted by CK2 at least *in vitro* (Figure 4.3). In light of this, reducing the stringency with which two web-based phospho-protein search tools identify putative kinase phosphorylation motifs within an amino acid sequence resulted in serine 541 (S541) of MRE11 being suggested as another putative CK2 phosphorylation target (Table 4.2). Furthermore, aligning the amino acid sequence of human MRE11 with those of other vertebrate species revealed S541 to be conserved (Figure 4.9).

A new GST-MRE11 (aa360-708) fragment was generated in which S541 was mutated to alanine on a 3A background, termed 4A (S541A/S649A/S688A/S689A). This new MRE11 mutant protein was expressed in bacteria, purified and then used in an *in vitro* CK2 kinase assay, alongside the WT MRE11 C-terminal fragment, the 1A, 2A and the 3A mutant fragments. Strikingly, a complete loss of CK2-dependent phosphorylation was seen in the 4A fragment, implying that at least *in vitro*, S541 is major site for the phosphorylation of MRE11 by CK2 (Figure 4.10). This supports the residual phosphorylation of the 3A construct seen in this and previous experiments (Figure 4.3 & 4.10).

Phosphorylation Site	Peptide	Reference
S541	EESASAFS*ADDLSI	http://scansite.mit.edu/ http://www.dabi.temple.edu/disphos/
S649	EVIEVDES*DVEEDIF	Kim, 2005. Dephoure, *et al*, 2008 http://scansite.mit.edu/ http://phospho.elm.eu.org/ http://www.phosphosite.org/ http://www.dabi.temple.edu/disphos/
S688	SKGVDFES*SEDDDDD	Beausoleil, *et al*, 2004. Dephoure, *et al*, 2008. http://scansite.mit.edu/ http://phospho.elm.eu.org/ http://www.phosphosite.org/ http://www.dabi.temple.edu/disphos/
S689	KGVDFESS*EDDDDDP	Beausoleil, *et al*, 2004. Dephoure, *et al*, 2008. http://scansite.mit.edu/ http://phospho.elm.eu.org/ http://www.phosphosite.org/ http://www.dabi.temple.edu/disphos/

Table 4.2 ***Identification of an additional putative CK2 phosphorylation site on MRE11.***

Figure 4.9 ***Conservation of S541 of MRE11 across species.*** *The region of human MRE11 (H.sapiens) containing the newly identified putative CK2 phosphorylation motif was aligned with several other vertebrate species, Pan troglodytes, Mus musculus (M.musculus), Rattus norvegicus (R.norvegicus) and Xenopus laevis (X.laevis) to identify any evolutionary conservation, using an internet based multiple sequence alignment programme, Clustal omega (http:// www.ebi.ac.uk/Tools/msa/clustalo/). The putative CK2 site is highlighted in grey.*

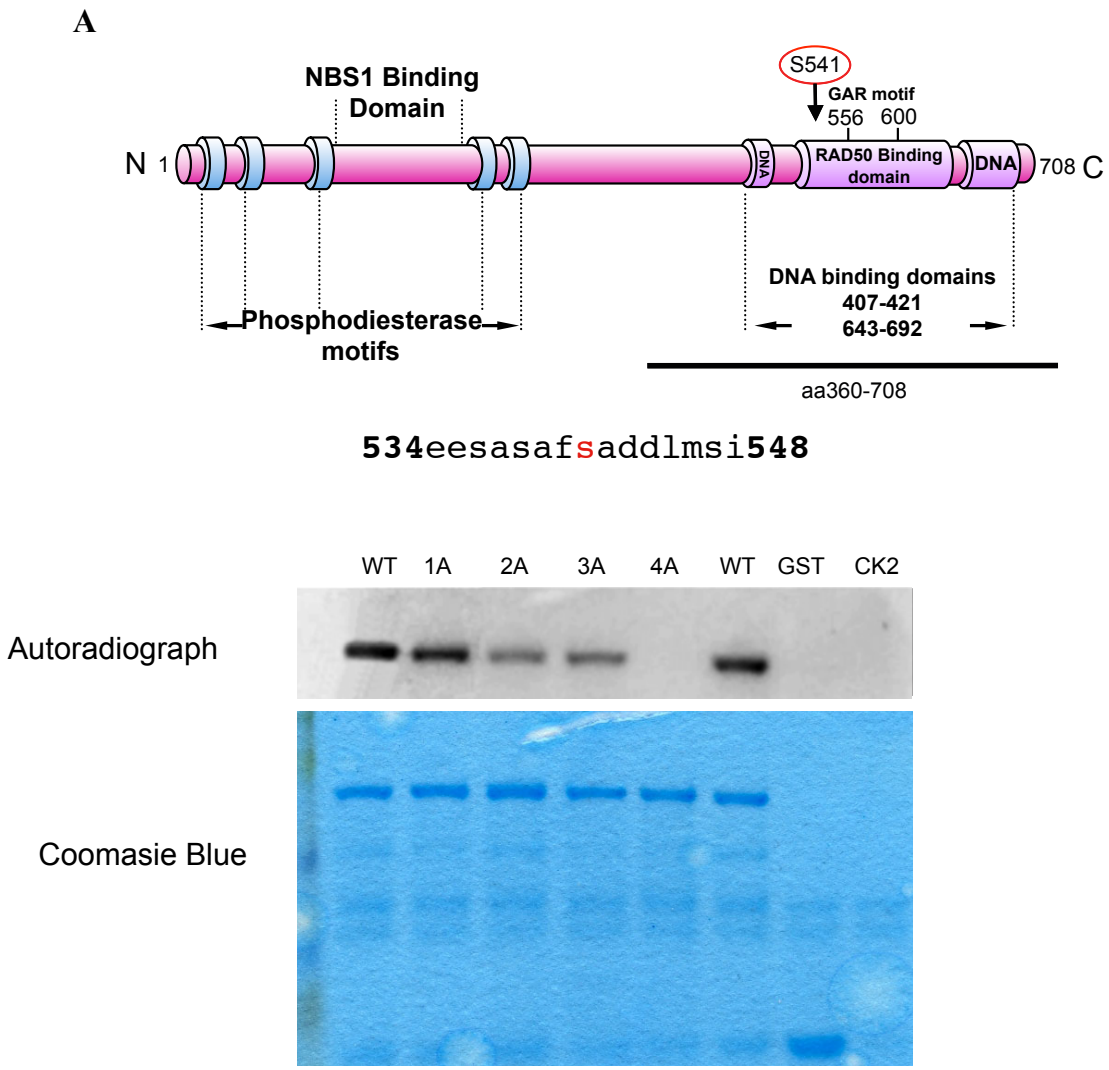


Figure 4.10 ***Mutation of S541 of MRE11 abrogates its CK2-dependent phosphorylation in vitro****. **(A)** A schematic of MRE11, showing the location of the additional CK2 site (circled in red). **(B)** A GST fragment (aa360-708) was generated where all four putative CK2 target sites had been mutated, 4A (S541A/ S649A/S688A/S689A). The fragment was expressed in E. coli and purified. The new fragment, along with the 3 other GST-MRE11 mutants, were incubated with recombinant CK2 and radio-labelled ATP (γ-^{32}P) for 10 minutes at 30°C. Proteins were separated by SDS-PAGE, stained with coomassie blue and dried. Radio-labelled proteins were detected by autoradiography.*

4.2.7 HCT116 Flp-In/T-Rex in vivo system

Due to the problems with the growth of stable ATLD2 cells expressing WT or mutant MRE11, an alternative *in vivo* system was utilised. The HCT116 colorectal tumour cell line has a compound heterozygous mutation in a poly-T track residing in intron 4 of the *MRE11* gene that results in aberrant splicing and loss of exons 5-7, caused by a Mis-match repair (MMR) defect, that results in Micro-satellite instability (MSI) (Furuta *et al.*, 2003, Giannini *et al.*, 2004, Giannini *et al.*, 2002, Takemura *et al.*, 2006, Wen *et al.*, 2008). As a consequence, these cells express low levels of endogenous full-length MRE11 protein and an unstable truncated mutant protein. Despite this, HCT116 cells have a relatively high level of homologous recombination, due to the repair of divergent DNA sequences incurred due to defective MMR. As such this phenotype has been repeatedly exploited to generate somatic gene knockout or knockin cell models to study protein function, including a multitude of DNA repair/cell cycle regulatory proteins, for example ATRIP, CHK1 and KU80 (Cortez *et al.*, 2001, Jardim *et al.*, 2009, Li *et al.*, 2002a). Taken together, this indicated that the HCT116 cell line would be a suitable system for modeling *MRE11* mutations in an isogenic genetic background that would overcome the problematic growth encountered when using patient-derived cell lines. An additional benefit is that a derivative of this cell line (HCT116 Flp-In/T-Rex) had been created to stably express the Tet-repressor, and also contain a single copy of an integrated FRT (Flp recombination target) site. This allows for the rapid generation of a cell line stably expressing an integrated copy of a gene of interest under the control of a tetracycline/doxycycline-inducible promoter, without the use of retro- or lenti-viruses. An overview of the details of this cell system can be found on the Invitrogen website (http://www.lifetechnologies.com).

To check that the HCT116 Flp-In/T-Rex derivative exhibited a similar reduction in MRE11 protein to those previously reported for this cell line, the expression of MRE11, RAD50 and NBS1 were assessed by Western blotting, and compared to a parental HCT116 cell line, obtained from the American Type Culture Collection (ATCC). In addition, another colorectal tumour cell line (HT29) was also included as it displays WT levels of MRE11 expression. As expected, both the parental HCT116 cell line and the Flp-In/T-Rex derivative exhibited reduced levels of MRE11, RAD50 and NBS1 expression when compared to the HT29 cell line (Figure 4.11).

4.2.8 Doxycycline-induced expression of MRE11 in HCT116 Flp-In/T-Rex cells

To create a stable doxycycline-inducible cell line expressing full-length MRE11, WT or mutant *MRE11* was sub-cloned into a modified pcDNA5 FRT vector containing an N-terminal Flag tag. The pcDNA5-Flag FRT *MRE11* constructs were then transfected into the HCT116 Flp-In/T-Rex cell line along with a pOG44 plasmid, which expresses the Flp recombinase. Cells containing the gene of interest integrated into the FRT site, rather than randomly elsewhere within the genome, lose their resistance to zeocin, (encoded for within the FRT integration site) and become resistant to hygromycin. Therefore, following selection with hygromycin, resistant cells were pooled and checked for their sensitivity to zeocin. Those cell populations that met these criteria were taken forward for assessment of the level of inducible MRE11 expression. Since the expression of MRE11 could be induced following treatment of the cells with either doxycycline or tetracycline, 1 μg/ml of each antibiotic was added to the cells for 24 h to determine which of these would give the best inducible expression of MRE11. Extracts from these cells were separated by SDS-PAGE and the membranes probed with antibodies against MRE11 as well as the other members of the MRN

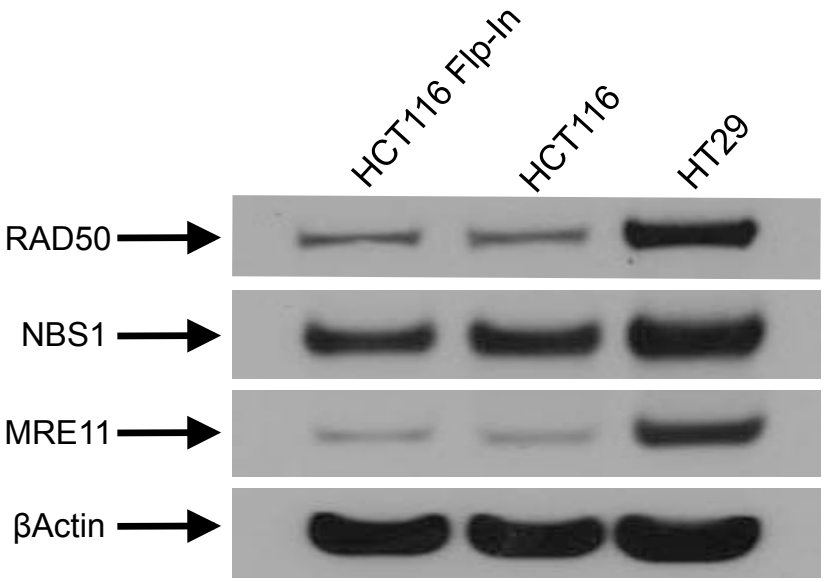


Figure 4.11 ***MRN protein expression levels in the HCT116 Flp-In/T-Rex cell line.*** *Western blotting was used to compare the expression of components of the MRN complex in the HCT116 Flp-In/T-Rex cell line to those expressed in an unmodified HCT116 cell line (obtained from the ATCC) and another colorectal tumor cell line HT29 (obtained from the ATCC), previously reported to have WT MRE11.*

complex (Figure. 4.12.A). A robust induction of Flag-tagged exogenous MRE11 was observed following the addition of both doxycycline and tetracycline (Figure. 4.12.A). Notably, induction of Flag-tagged MRE11 expression in the HCT116 Flp-In/T-Rex cell line resulted in an increase in the levels of both endogenous RAD50 and NBS1. For the remainder of the study doxycycline was chosen as the antibiotic used to induce MRE11, as it known to be more stable than tetracycline in solution.

The requirements for the study involved treating the cell lines with DNA damaging agents and harvesting at different time points. For this reason it was necessary to examine whether the expression of MRE11 following induction could be maintained for longer than 24 h. For this, the cells were treated with 1 μg/ml of doxycycline and then harvested at 24, 48 and 72 h. The expression of induced MRE11 was sustained after 24 h, and up to 72 h (Figure 4.12.B). However, at 72 h the levels of exogenous MRE11 began to decrease, suggesting that 72 h is the limit at which expression of MRE11 in this system could be maintained at an experimental level. Lastly, to monitor the levels of expression of the different mutant MRE11 proteins and to compare them to the levels of the WT protein, 1 μg/ml of doxycycline was added to HCT116 Flp-In/T-Rex cells expressing either WT MRE11, mutant MRE11 containing the phospho-site mutations (1A, 2A, 3A and 4A) or an empty vector for 24 h, after which the cells were harvested. The lysates were then separated on SDS-PAGE gels and the expression determined by Western blotting using the indicated antibodies. As shown in Figure 4.13, each of the phospho-mutant MRE11 proteins were expressed at levels comparable to that of the WT protein.

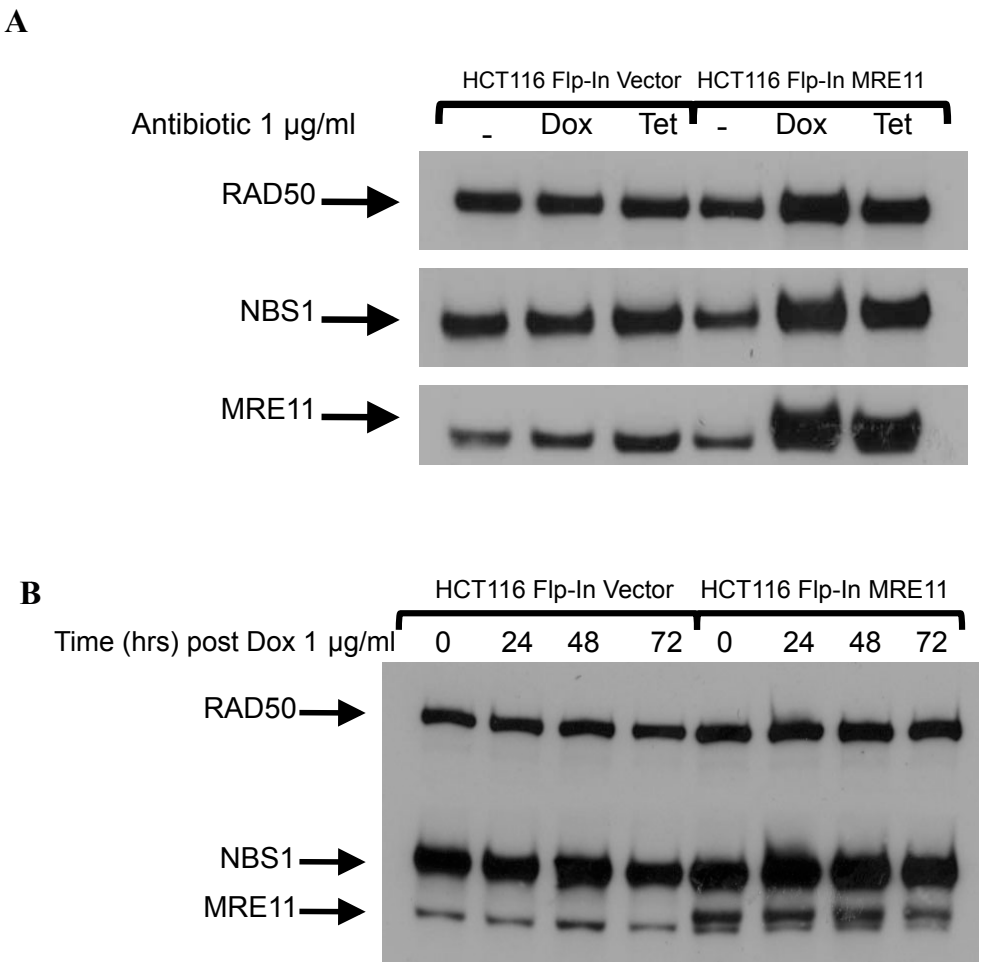


Figure 4.12 ***A doxycycline inducible system for the expression of exogenous MRE11 in HCT116 Flp-In/T-Rex cells.*** *(**A**) Either 1 µg/ml of doxycycline or tetracycline was added HCT116 Flp-In/T-Rex cells containing a Flag-tagged WT MRE11 expression construct for 24 h, the cells harvested and extracts were separated by SDS-PAGE. Expression of MRE11, RAD50 and NBS1 was assessed by Western Blotting. (**B**) 1 µg/ml of doxycycline was added to HCT116 Flp-In/T-Rex cells containing a Flag-tagged WT MRE11 expression construct for times indicated. The cells were then harvested, extracts separated by SDS-PAGE and MRE11, RAD50 and NBS1 expression was determined by Western blotting.*

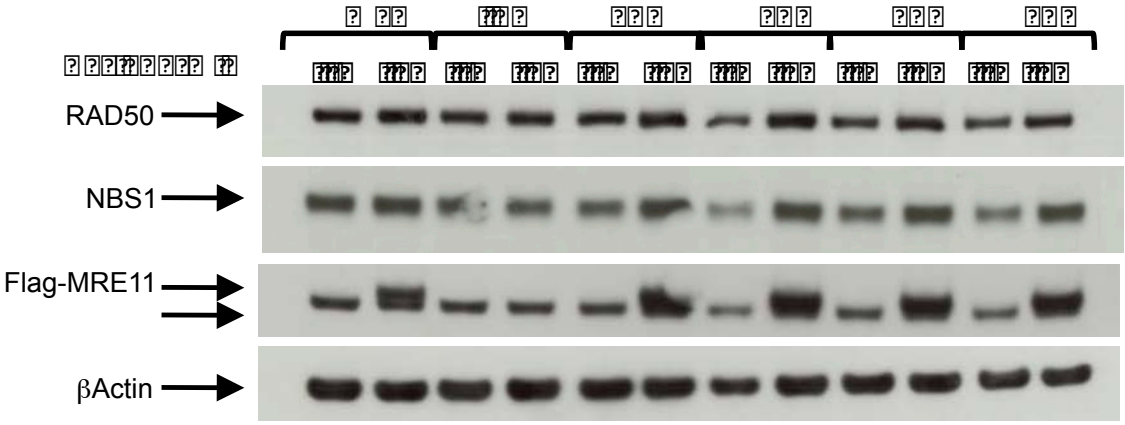


Figure 4.13 ***Expression of a doxycycline-induced mutant MRE11 in HCT116 Flp-In/T-Rex cells.*** *1 μg/ml of doxycycline was added to HCT116 Flp-In/T-Rex cells expressing either inducible WT MRE11, an empty vector (V), or phospho-mutant MRE11 (1A, 2A, 3A, 4A). The cells were harvested 24 hrs after induction of expression with doxycycline and the extracts were separated by SDS-PAGE. Expression of exogenous Flag-tagged MRE11 was determined by Western blotting using the indicated antibodies.*

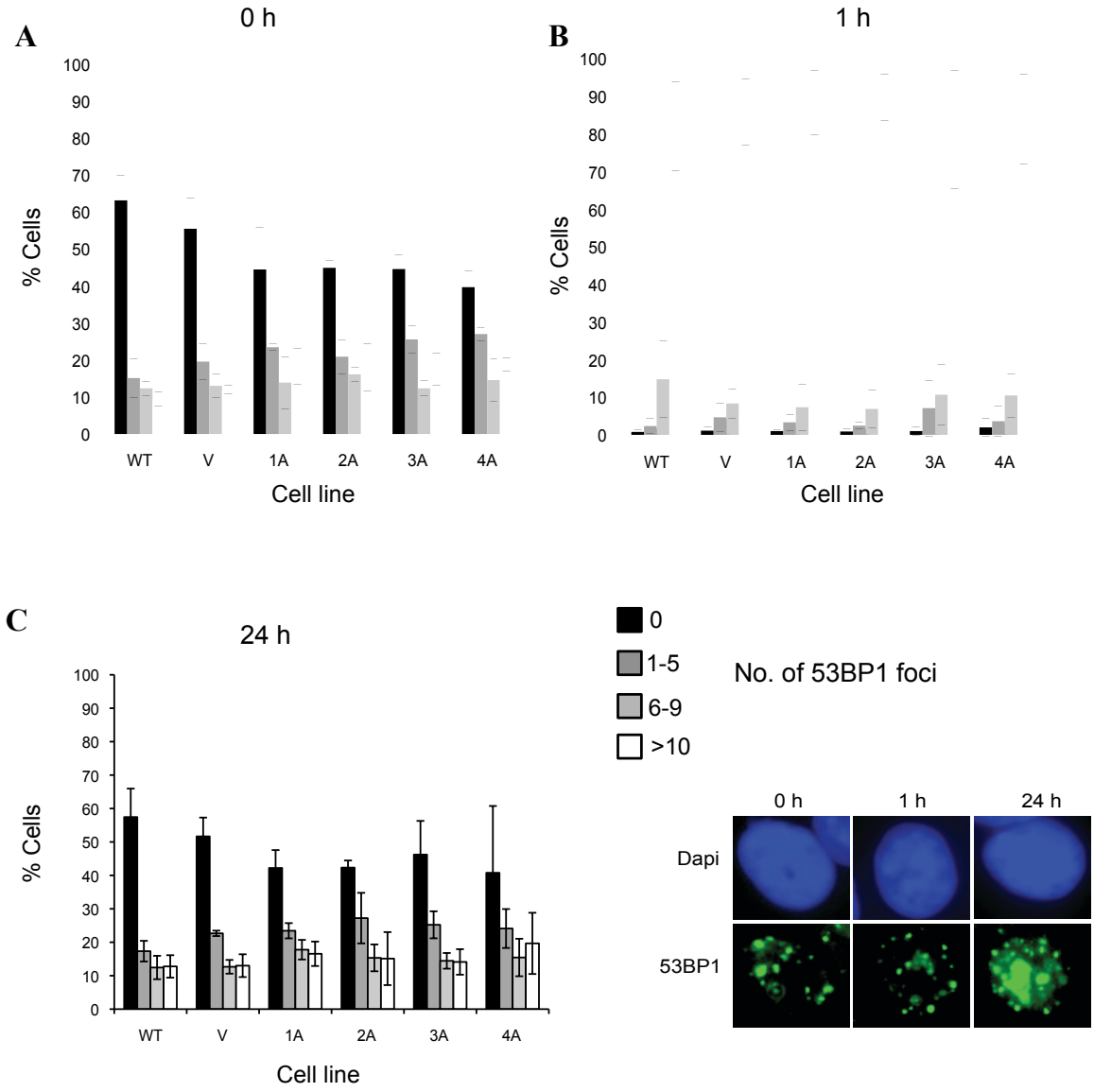


Figure 4.14 ***HCT116 Flp-In/T-Rex cells display constitutive 53BP1 foci.*** *MRE11 expressing HCT116 Flp-In/T-Rex cell lines were incubated in 1 μg/ml of doxycycline for 24 h, either mock-irradiated or exposed to 2 Gy of IR and then fixed at 0 h, 1h and 24 h post-irradiation. Cells were then stained with antibodies agaisnt 53BP1 and γH2AX. 53BP1 foci were then counted in each of the five cell lines at each time point, 0 h (**A**), 1 h (**B**) and 24 h (**C**) and the percentage of cells with foci calculated (n=300). Values represent the mean of three experiments ± standard error.*

4.2.9 HCT116 Flp-In/T-Rex cells show aberrant DNA double-strand breaks

To determine whether expression of either the WT or phospho-defective MRE11 was capable of correcting the DSB repair defect resulting from the endogenous *MRE11* mutation in the inducible HCT116 Flp-In/T-Rex cell system, each cell line was treated with doxycycline 24 h prior to irradiation with 2 Gy of IR. The cells were then fixed and stained with antibodies to 53BP1 and γ-H2AX 1 hr or 24 h post-irradiation. Again, the percentage of 53BP1 and γ-H2AX foci remaining at late times post-irradiation were determined by immunofluorescence microscopy, and used as a measure of DSB repair as in Figure 4.8. Unfortunately, it was clear that HCT116 cells exhibit relatively high levels of 53BP1 foci even before treatment, and that expression of WT MRE11 was not sufficient to correct this (Figure 4.14). Indicating that this cell line is not a suitable model system to study defects in the MRE11-dependent DDR. Moreover, preliminary analysis of the IR-dependent activation of ATM signaling cascade by Western blotting in HCT116 cell lines revealed that they express very low levels of ATM (Figure 4.15). Using the COSMIC database (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/) to look for mutations identified in this cell line, a heterozygous mutation (p.A1127V) in ATM has previously been reported. However, whether this would result in the low levels of ATM protein observed by western blot analysis would require further investigation. Taken together, it appears that the inducible HCT116 cell system is not conducive for investigating the IR-induced DDR.

4.2.10 MRE11 expression in a U2OS Flp-In/T-Rex system

Due to the unexpected problems encountered using the HCT116 Flp-In/T-Rex system, a different inducible Flp-In/T-Rex cell was used. U2OS cells were chosen based on their

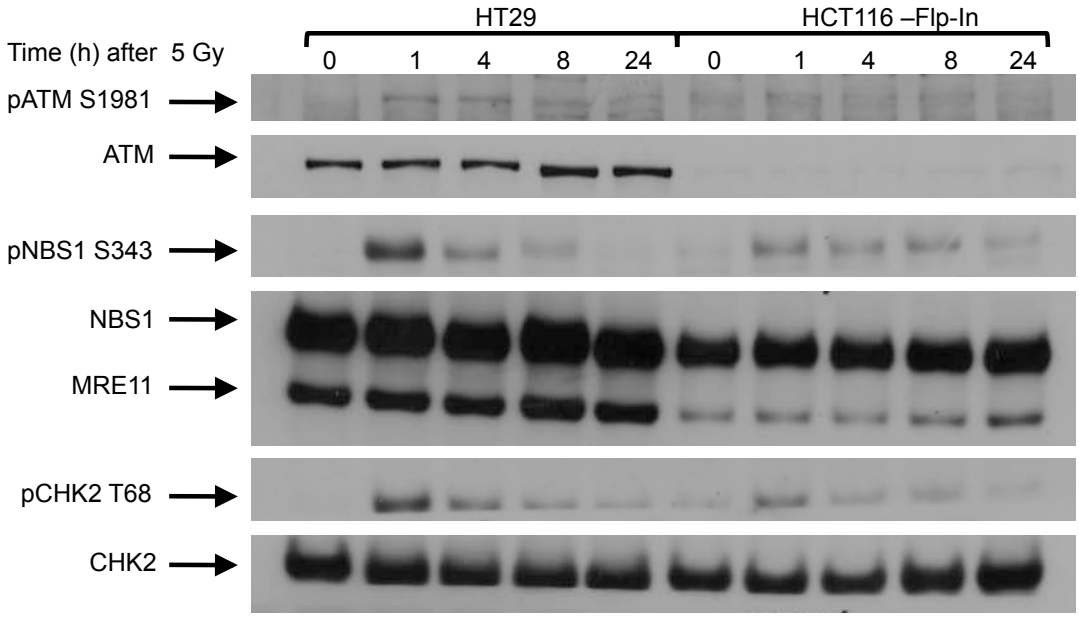


Figure 4.15 ***ATM signalling in HCT116 Flp-In/T-Rex cells following IR.*** *Cell lines were irradiated with 5 Gy of IR and harvested at the indicated time points. Lysates were fractionated by SDS-PAGE, and ATM-dependent DNA damage signaling assayed by Western blotting using the indicated antibodies.*

extensive use to study the cellular DNA damage response including those assessing regulation of ATM/MRN-mediated intracellular signaling induced by different DNA damaging agents (Gately *et al.*, 1998, Yuan and Chen, 2010). However, in contrast to the HCT116 cell line, a disadvantage of using of U2OS cells is that they express WT levels of MRE11, which consequently means that before any analysis of mutant MRE11 is carried out endogenous MRE11 would have to be depleted with siRNA.

As previously described, U2OS Flp-In/T-Rex cells were transfected with the Flp-In *MRE11* plasmids containing either WT *MRE11*, and empty vector, the 3A *MRE11* mutant lacking all the putative CK2 phosphorylation sites identified *in vivo*, the 4A *MRE11* mutant that also included loss of the major *in vitro* CK2 phosphorylation site (S541A) or a single mutant *MRE11*, in which only serine-541 had been mutated to alanine. Again the cells were selected with hygromycin and a polyclonal population of cells generated to counteract any artifacts caused by cloning. To assess the expression of the exogenous MRE11, each of the polyclonal U2OS MRE11 Flp-In/T-Rex cell lines were treated with 1 μg/ml doxycycline for 24 h and Western blotting on whole cell extracts was carried out using antibodies to MRE11, RAD50 and NBS1. As shown in Figures 4.16A and B, WT and the different mutant MRE11 proteins were expressed at comparable levels, which was observed to be approximately 2 fold higher than the endogenous MRE11.

4.2.11 Depletion of endogenous MRE11 in U2OS Flp-In/T-Rex cells using a 5´ UTR-directed siRNA

Unlike the HCT116 Flp-In/T-Rex cell lines, the U2OS Flp-In/T-Rex cells do not have a pathogenic mutation in *MRE11* that results in reduced expression of the protein. Since the *MRE11* Flp-In expression constructs generated were not siRNA resistant, a previously

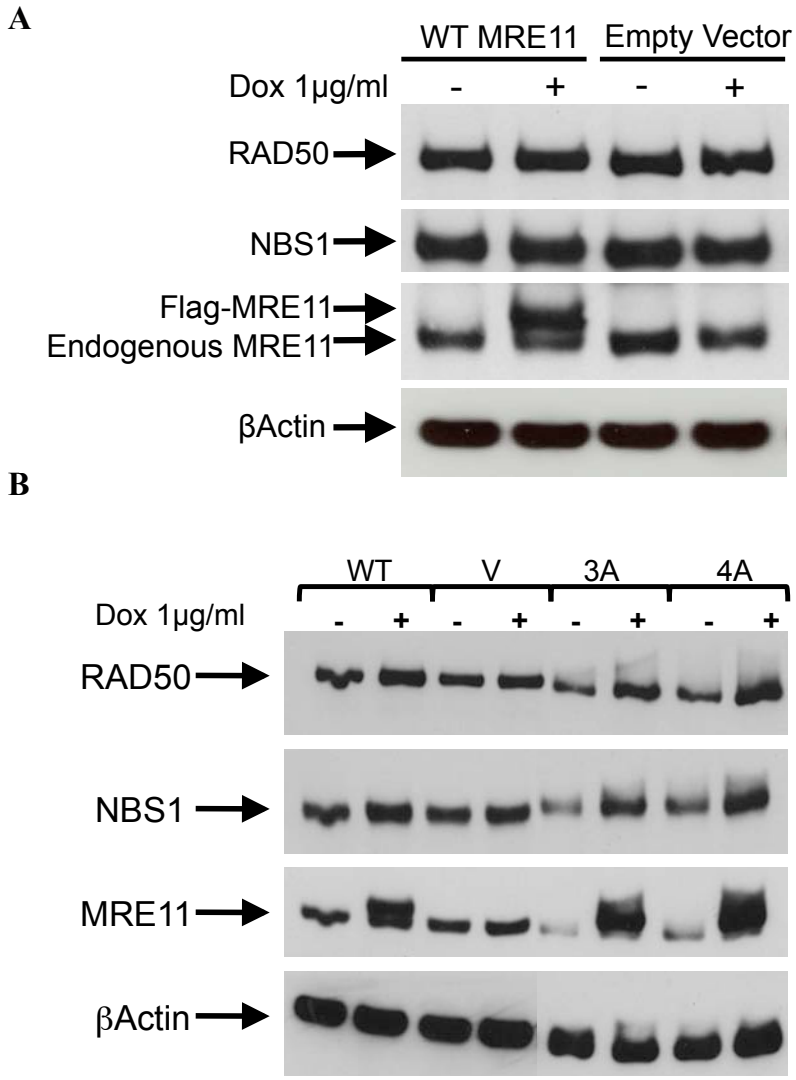


Figure 4.16 ***A doxycycline inducible system for expression of MRE11 in U2OS Flp-In/T-Rex cells. (A)*** *1 µg/ml of doxycycline was added to U2OS Flp-In/T-Rex cells stably expressing either WT MRE11 or an empty vector. After 24 h, the cells were harvested, extracts separated by SDS-PAGE and the expression of MRE11, RAD50 and NBS1 was assessed by Western blotting. An antibody against β-Actin was used as a loading control.* ***(B)*** *1 µg/ml of doxycycline was added to U2OS Flp-In/T-Rex cells stably expressing either WT MRE11, an empty vector, the 3A (S649A/S688A/S689A) or 4A (S541A/S649A/S688A/S689A) MRE11 mutants for 24 h. Lysates were then separated via SDS-PAGE and MRE11 expression determined by Western blotting. NB The autoradiograph has been altered to omit unnecessary data.*

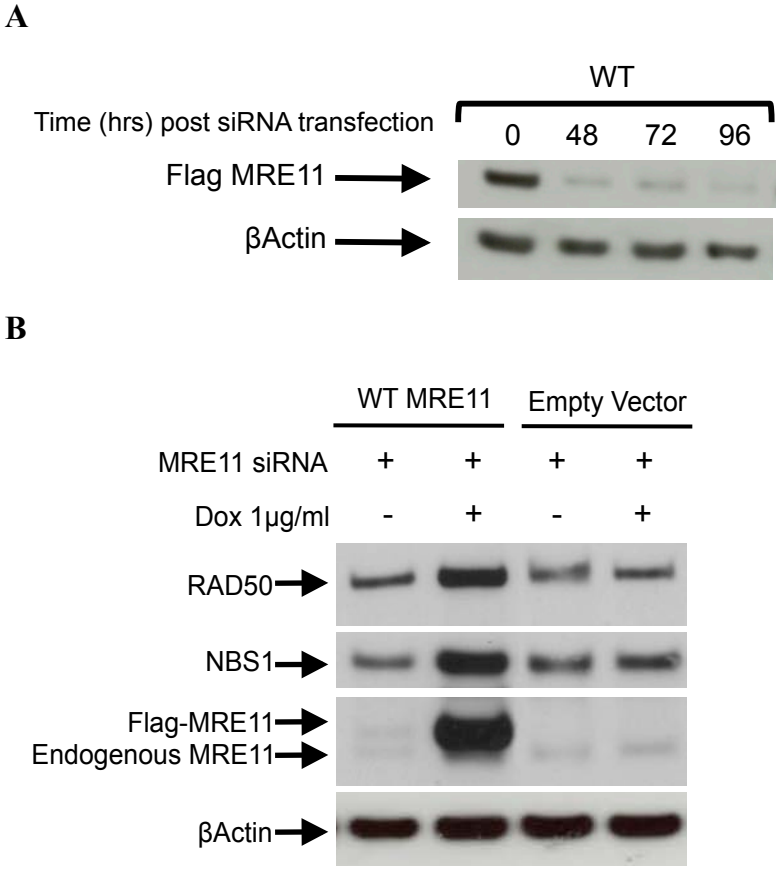


Figure 4.17 ***Depletion of endogenous MRE11 using a 5' UTR-directed siRNA and inducible expression of exogenous MRE11 protein*** *(A) U2OS Flp-In/T-Rex cells expressing WT tagged exogenous MRE11 were transfected with MRE11 5'UTR siRNA in the absence of doxycycline and harvested 48, 72, or 96 h post-transfection. Lysates were separated by SDS-PAGE and the efficiency of MRE11 depletion was assessed by Western blotting. (**B**) Cells were treated as in part (A) except doxycycline was added 48 h post-tranfection and cells harvested 24 h later.*

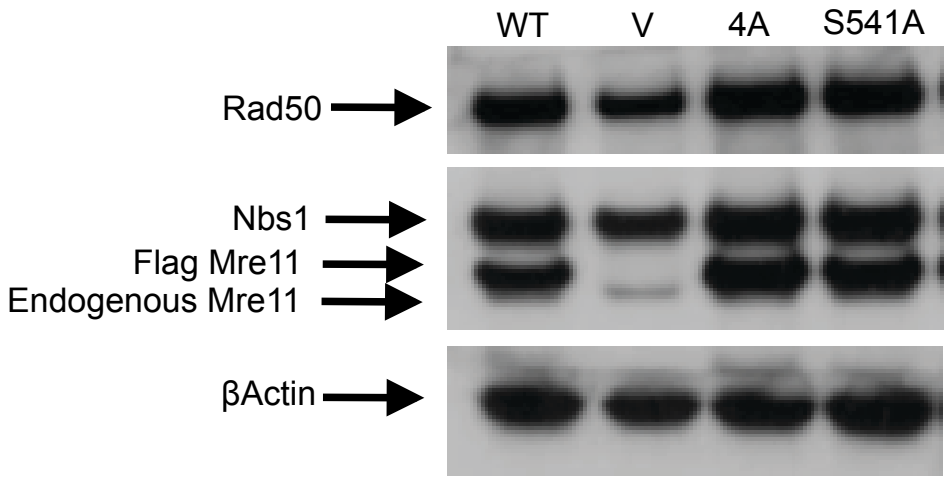
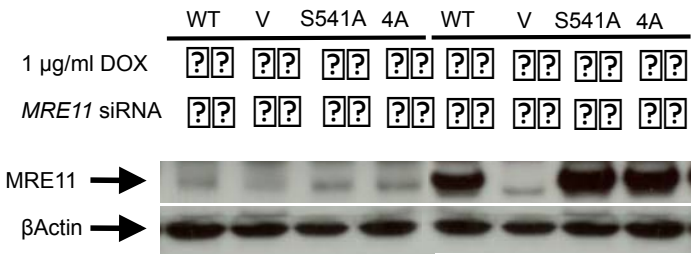


Figure 4.18 ***Mutation of a fourth MRE11 putative CK2 motif does not affect the stability or expression of the MRN complex.*** *U2OS Flp-In/T-Rex cells expressing either WT, an empty vector, a 4A (S541A/S649A/S688A/S689A) mutant or a single S541A mutant MRE11 were transfected with MRE11 siRNA for 72 h. Twenty four hours prior to harvesting, the cells were treated with 1 μg/ml of doxycycline. The cells were then harvested and lysates separated by SDS-PAGE. Western blotting was then used to assess MRN complex stability and expression using indicated antibodies.*

published siRNA to *MRE11*, directed towards a sequence in its 5' untranslated region (UTR) was used to deplete the endogenous, but not exogenous MRE11 (Zhuang *et al.*, 2009). Initially, it was necessary to determine whether this siRNA could efficiently deplete endogenous MRE11 protein in this cell line, and also how long this depletion lasted. Cells were transfected with 100 nM of the 5'UTR *MRE11* siRNA, and were then harvested at 0, 48, 72 and 96 h after transfection. Depletion of MRE11 was assessed by Western blotting. A significant reduction in endogenous MRE11 levels was observed 48 h post-siRNA transfection that lasted up to 96 h (Figure 4.17.A). To confirm the specificity of the 5' UTR siRNA for the endogenous but not exogenous *MRE11* (which lacks any UTR sequence), cells were transfected with siRNA and then treated with doxycycline for 24 h, 48 h post-transfection. Expression of MRE11 and other components of the MRN complex were then determined by Western blotting on extracts made from these cells. As shown in Figure 4.17B, exogenous WT MRE11 protein was efficiently expressed in cells transfected with the 5'UTR MRE11 siRNA and this was capable of restabilising the cellular pools of both RAD50 and NBS1. In addition, U2OS Flp-In/T-Rex expressing MRE11 protein containing the single S541 mutated to an alanine did not disrupt expression or stability of the MRN complex (Figure 4.18). Following depletion of endogenous MRE11 and re-expression of inducible mutant protein.

Before any characterisation of the cellular DNA damage response was carried out using these cells, FACS analysis was used to assess whether the siRNA transfection or the doxycycline-inducible expression of the WT/mutant protein had any detrimental affects on the cell cycle that could potential bias any results using these cells. As shown in Figure 4.19, siRNA-mediated depletion of endogenous MRE11 and re-expression of exogenous WT or mutant

A



B

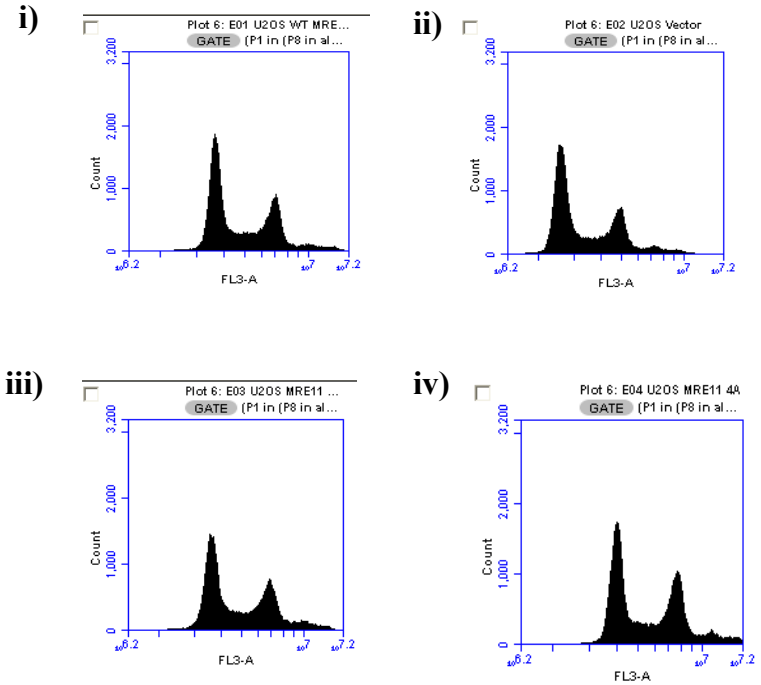


Figure 4.19 ***Stable U2OS Flp-In/T-Rex cell lines have similar cell cycle profiles following siRNA-mediated depletion of endogenous MRE11 and re-expression of exogenous MRE11. (A)*** *Western blot confirming depletion of MRE11 by siRNA and induction of exogenous MRE11 in U2OS Flp-In/T-Rex cell lines.* ***(B)*** *Cell lines were harvested and fixed in 70% ethanol. Following permeablisation, the cells were stained with propidium iodide and analysed by FACS analysis. The four figures represent cell cycle profiles for:* ***i)*** *WT MRE11,* ***ii)*** *V, empty vector* ***iii)*** *S541A MRE11 and* ***iv)*** *4A (S541/649/688/689A) MRE11. The panels represent one among three experiments.*

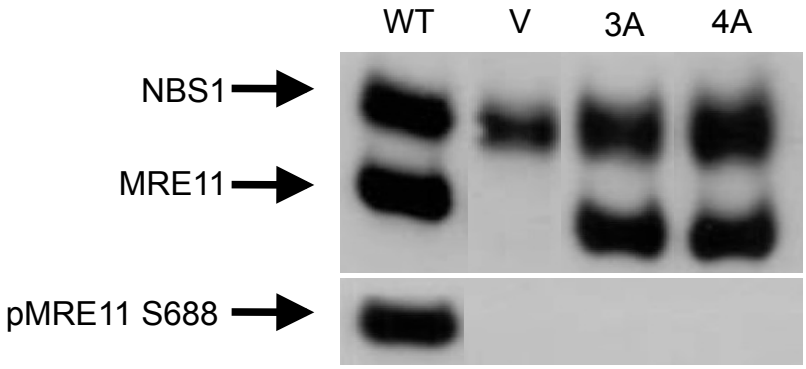


Figure 4.20 ***S688 is phosphorylated in vivo****. Cell lysates were prepared from WT, V, 3A and 4A U2OS Flp-in/T-REX MRE11 cell lines that had been transfected with MRE11 5'UTR siRNA for 72 h, followed by treatment with doxycycline for 24 h to induce exogenous MRE11. The lysates were separated by SDS PAGE, and phosphorylation of MRE11 was assessed using an antibody against phospho-serine 688. NB. The autoradiograph is part of a larger figure, but has been adjusted to display relevant result.*

MRE11 had very little impact on cell cycle distribution. Although that it should be noted that induction of the 4A MRE11 phospho-mutant did result in an increase of cells in G2 phase.

4.2.12 Phosphorylation of S688 is constitutive

Phosphorylation of proteins by CK2 often occurs in the absence of any stimuli, which is a reflection of the constitutive activity of CK2. The availability of an antibody against phosphorylated S688 of MRE11 (Abcam) allowed the investigation as to whether this was the case for this residue *in vivo*. Following transfection with 5'UTR *MRE11* siRNA and doxycycline induction of exogenous protein. Whole cell extracts were prepared from the MRE11 WT, the vector only and the 3A and 4A mutant U2OS Flp-In/T-Rex cell lines, which were then separated by SDS-PAGE. The Western blots were probed with an antibody against phosphorylated S688 of MRE11. Cells expressing WT MRE11 were phosphorylated on this residue, whereas the vector-only, and more importantly the 3A and 4A cell lines were not (Figure 4.20). This observation implies that S688 is phosphorylated *in vivo* and that the phosphorylation is constitutive.

4.2.13 Mutation of the four putative CK2 phosphorylation sites in MRE11 does not affect its ability to relocalise to sites of DNA damage or to promote repair of DSBs.

To confirm that loss of the putative CK2 phosphorylation sites on MRE11, including the newly identified *in vitro* phosphorylation site on serine-541, did not affect the ability of the MRE11 protein to relocalise to sites of DNA damage in U2OS cells, endogenous MRE11 was depleted using siRNA in U2OS Flp-In/T-Rex cell. The cell lines expressed either WT MRE11, an empty vector or a mutant MRE11, in which serine-541 had been mutated to

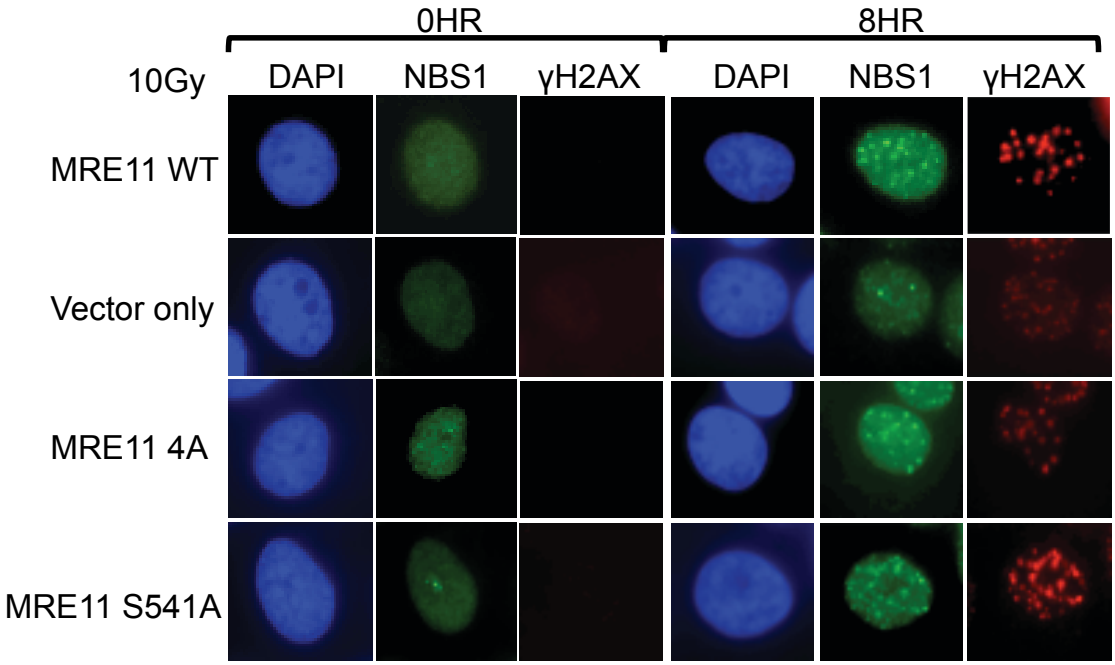


Figure 4.21 ***Loss of putative CK2 phosphorylation of MRE11 does not affect its ability to relocalise to sites of DNA damage in U2OS cells.*** *U2OS Flp-In/T-Rex cells expressing either WT, an empty vector, a 4A (S541A/S649A/S688A/S689A) mutant or a single S541A mutant MRE11 were transfected with MRE11 siRNA for 72 h. Twenty four hours prior to harvesting, the cells were treated with 1 μg/ml of doxycycline. The cells were then irradiated with 8 Gy of IR and fixed at 8 h, followed by staining with the indicated antibodies. Images are of one cell and are representative of approximately ~1000 cells scored by eye from 3 separate experiments.*

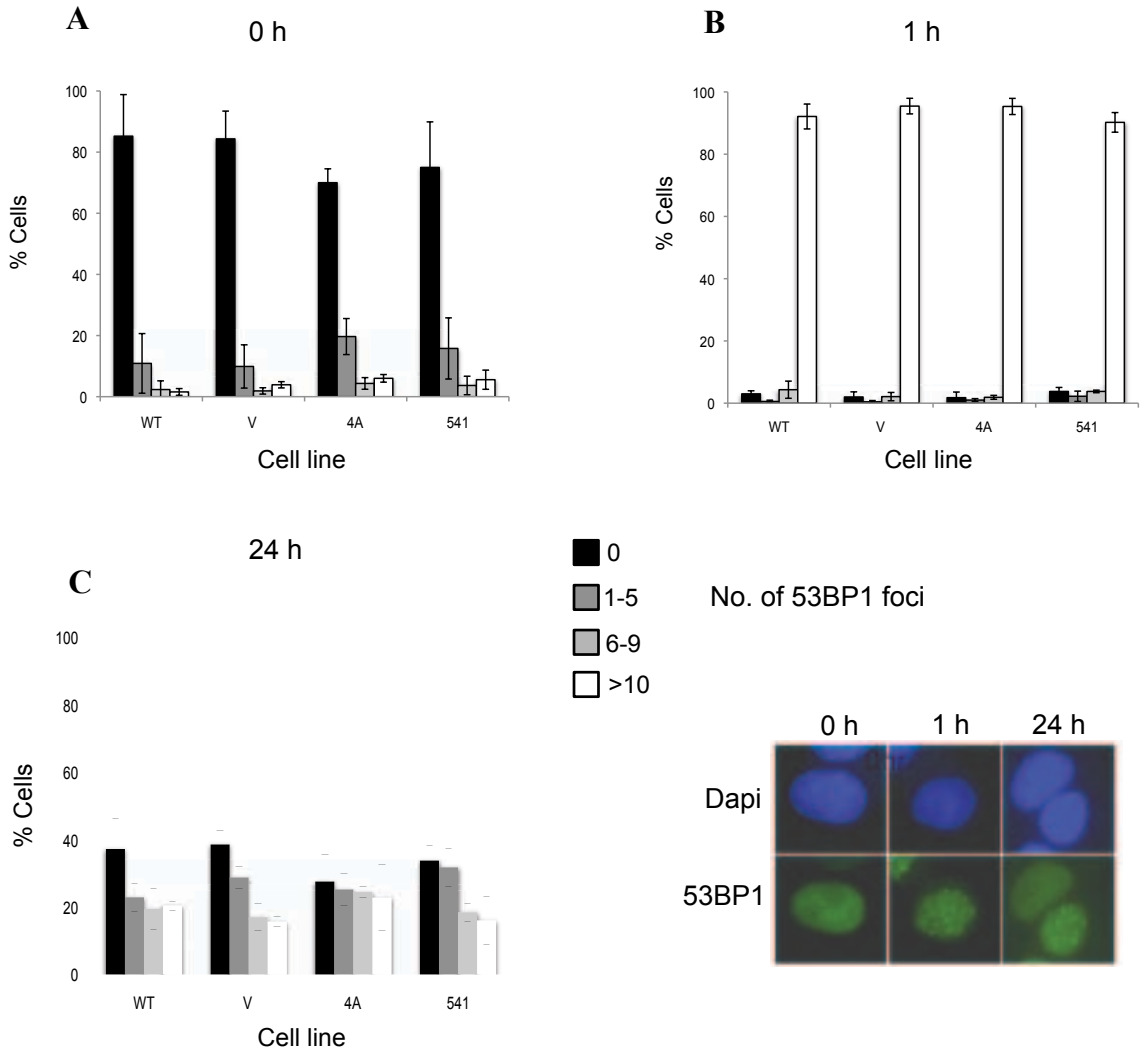


Figure 4.22 ***Assessment of DSB repair kinetics in U2OS Flp-In/T-Rex cells expressing WT MRE11, empty vector or mutant MRE11****. The cells were then irradiated with 2 Gy and fixed at 0* ***(A)****, 1* ***(B)****, and 24 h* ***(C)*** *post-irradiation. The Cells were stained with the above antibodies and the percentage of cells with 53BP1/γ-H2AX ???? foci counted (n=300) using immunofluorescence microscopy. Values represent the mean of three experiments ± standard error.*

alanine alone, or in conjunction with serines 649, 688 and 689. Exogenous MRE11 was then overexpressed following incubation with doxycycline for 24 h. The cells were then either mock treated or irradiated with 8 Gy of IR, fixed 8 h post-irradiation and stained with antibodies to MRE11 and γ-H2AX. In keeping with previous observations, mutation of all the putative CK2 target phosphorylation sites, including S541, did not affect the ability of MRE11 to form IRIF (Figure 4.21).

Next to investigate that a defect in DSB repair caused by depletion of MRE11 could be detected in the U2OS Flp-In/T-Rex system, and to assess whether loss of the putative CK2 phosphorylation sites on MRE11, including serine-541, had any impact on repair, the resolution of 53BP1 and γ-H2AX foci following exposure to low dose IR was monitored by immunofluorescence, as previously described in section (4.2.5). Unfortunately, similar to the HCT116 cells, the U2OS cells also exhibited high levels of residual 53BP1 and γ-H2AX foci remaining at 24 h post-irradiation that could not be complemented with the re-expression of WT MRE11 (Figure 4.22). This observation in conjunction with those obtained using HCT116 cells indicated that tumour-derived cell lines are not good *in vitro* cellular models with which to assess the contribution of MRE11 in promoting the repair of DSBs, due to the high levels of endogenous damage.

4.2.14 Mutation of putative CK2 phosphorylation sites on MRE11 affects its phosphorylation of S676 after exposure to IR.

Despite an absence of a detectable DSB repair deficiency caused by depletion of MRE11 in the U2OS Flp-In/T-Rex system, it is possible that these cells would still display defects in ATM-dependent intra-cellular signalling. To address this, endogenous MRE11 was depleted in all cell lines using siRNA and the exogenous MRE11 was induced 24 h prior to IR exposure. Each cell line was irradiated with 5 Gy and cells harvested at 1 h and 6 h post-

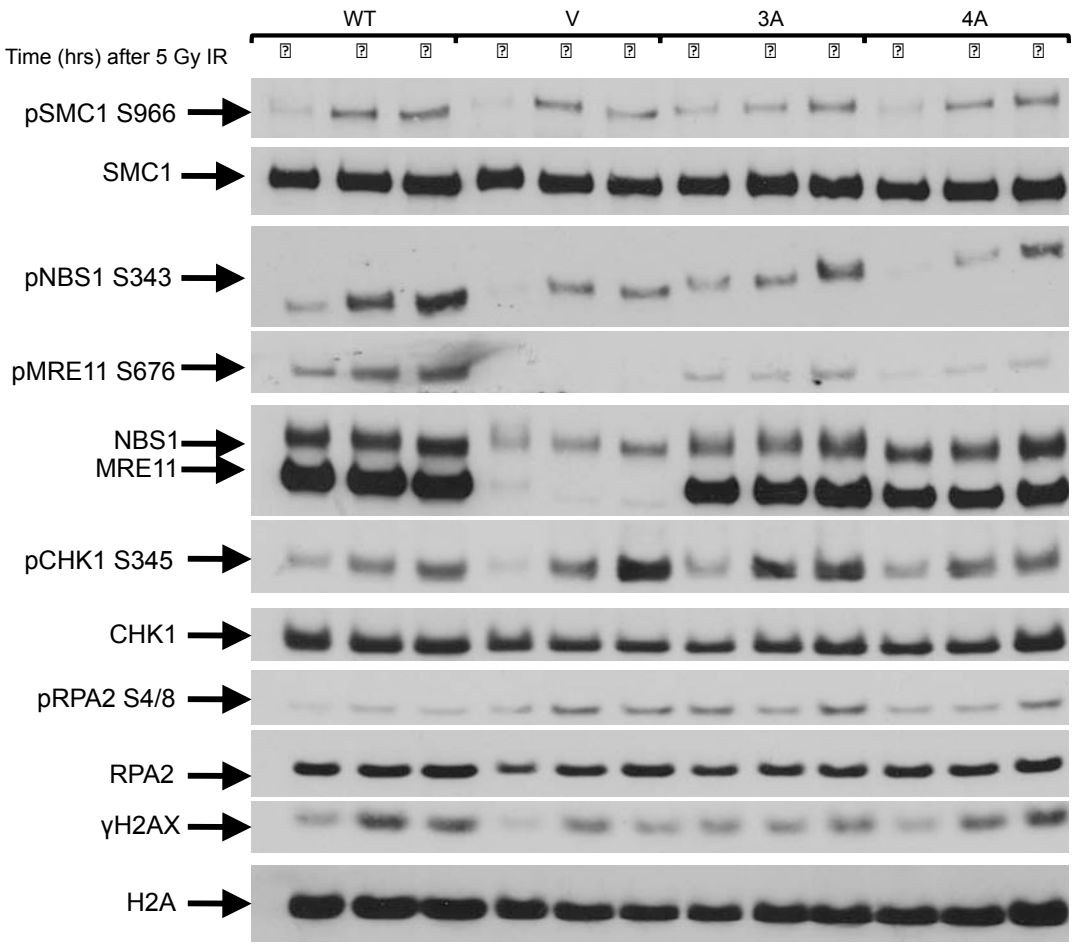


Figure 4.23 ***DNA damage signaling in U2OS Flp-In/T-Rex cell lines expressing mutant MRE11 induced by IR.*** *Following transfection with MRE11 siRNA for 72 h and treatment of cells for 24 h with doxycycline to induce either WT or mutant MRE11, cells were irradiated with 5 Gy of IR and harvested at the above time points. Proteins were then fractionated by SDS-PAGE and Western blots incubated with the indicated antibodies.*

treatment. Cell extracts were then subjected to SDS-PAGE and Western blotting using phospho-specific antibodies to known ATM substrates. Despite getting robust knockdown of endogenous MRE11, only an extremely mild defect in the ATM-dependent phosphorylation of H2AX was observed in the U2OS Flp-In/T-Rex cells complemented with an empty vector (Figure 4.23). Unexpectedly, the IR-induced phosphorylation of SMC1 on serine-966 that has been reported to be MRN-dependent, did not show any significant decrease in the vector complemented cells (Kim et al., 2002, Yazdi et al., 2002). Furthermore, it has also been demonstrated that the phosphorylation of CHK1 following the induction of DSBs is also MRN-dependent (Myers and Cortez, 2006). However, in contrast to this, loss of endogenous MRE11 appeared to result in an increase in CHK1 phosphorylation (Figure 4.23). No consistent defects in the phosphorylation of any ATM target proteins were observed in cells in which either the 3A or 4A MRE11 mutant had been re-expressed, with the exception of MRE11 itself, which seemed to be reduced in cells expressing either mutant.

4.2.15 Camptothecin-induced activation of the cellular DDR in the U2OS Flp-In/T-Rex cell lines expressing mutant MRE11 is unaffected.

The MRN complex plays a major role in repairing DNA damage and activating ATM/ATR-dependent intracellular checkpoint signalling particularly during S-phase (Olson *et al.*, 2007a, Olson *et al.*, 2007b). Camptothecin (CPT) is a topoisomerase I (Top1) poison that gives rise to DSBs in S-phase when a replication fork collides with an abortive Top1 cleavage complex. This complex consists of Top1 covalently bound to the DNA, proximal to an unligated DNA single strand break. It is known that CPT is a potent inducer of MRN-dependent signalling, DNA end-resection and cell cycle checkpoint activation (Kousholt *et al.*, 2012, Robison *et al.*, 2005, Takemura *et al.*, 2006). In this respect loss of components of the MRN complex

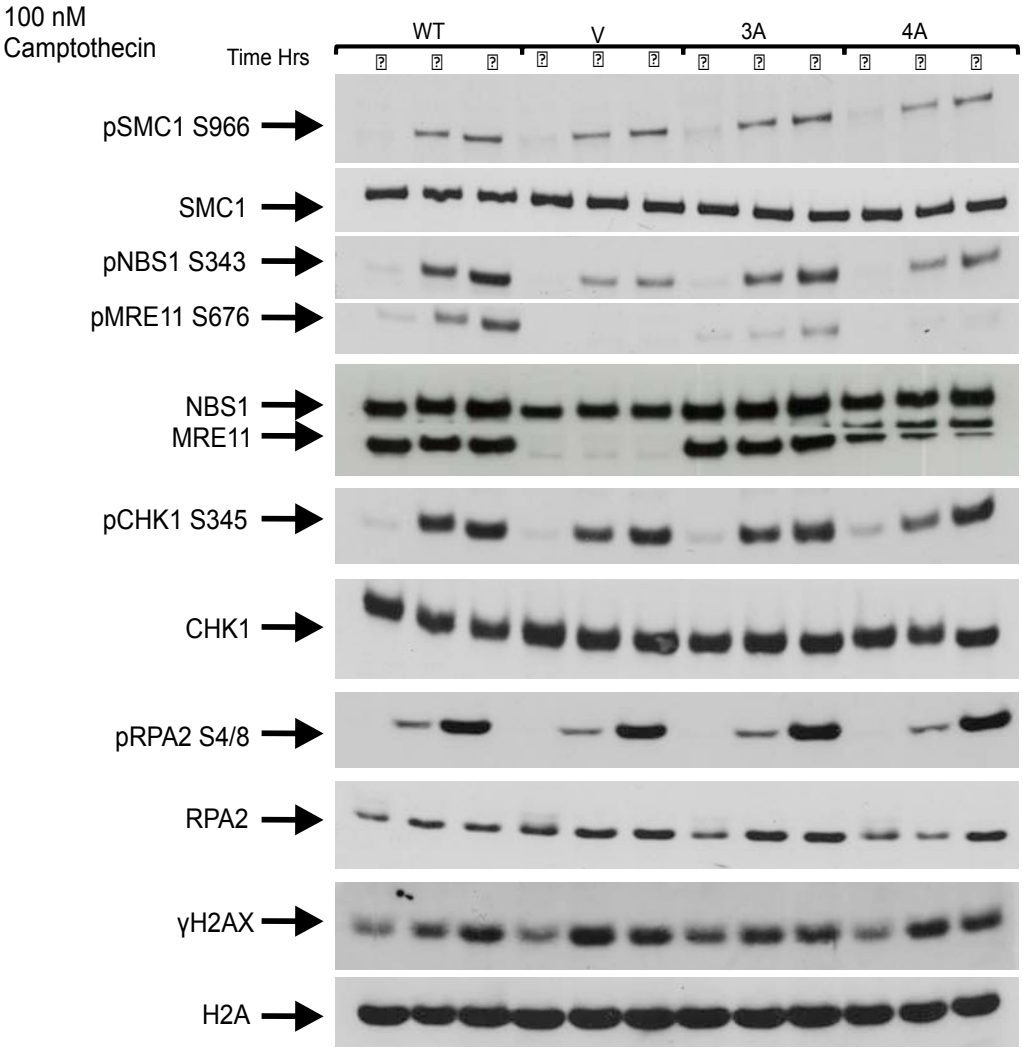


Figure 4.24 ***DNA damage signaling in U2OS Flp-In/T-Rex cell lines expressing mutant MRE11 induced by CPT.*** *Following transfection with MRE11 siRNA for 72 h and treatment of cells for 24 h with doxycycline to induce either WT or mutant MRE11, cells were incubated with 100 nM CPT and harvested at the above time points. Proteins were then fractionated by SDS-PAGE and Western blots incubated with the indicated antibodies.*

strongly compromises DNA end-resection and consequently HR-mediated repair of the damage that can be visualised by a reduction in RPA2 and CHK1 phosphorylation. To determine whether loss of the putative CK2 phosphorylation sites on MRE11 affected DNA damage signaling induced by CPT-mediated replication damage, cells expressing either WT MRE11, an empty vector or mutant MRE11 were transfected with *MRE11* siRNA, treated with doxycycline and then exposed to low dose CPT for either 1 or 6 hours. Western blots of cell extracts were then probed with phospho-antibodies to known ATM/ATR target proteins, including RPA2 S4/8-P and CHK1 S345-P to monitor integrity of the MRN-dependent signaling cascade (Figure 4.24). Again, loss of endogenous MRE11 alone did not give rise to an observable defect in the CPT-induced phosphorylation of any of the ATM/ATR substrates tested indicating that either the siRNA-dependent depletion of MRE11 is not sufficient to compromise the DDR or that in a manner similar to the HCT116 cells, U2OS cells are not a good system to model MRE11-dependent signalling. However, despite this, loss of the putative CK2 phosphorylation sites on MRE11 again reduced its ability to be phosphorylated on S676 (Figure 4.24).

4.3 Discussion

An increasing number of reports being published identify CK2 as a critical regulator of the cellular DNA damage response (Iles *et al.*, 2007, Loizou *et al.*, 2004, Olsen *et al.*, 2010, Spycher *et al.*, 2008). Previous observations demonstrated that CK2 associates with MDC1 and the MRN complex, and directly phosphorylates MDC1 *in vitro* and *in vivo*. The identification, using large scale mass spectrometry, of three *in vivo* sites on MRE11 that conform to a CK2 consensus phosphorylation site (S649, S688 and S689), in addition to a report demonstrating that at least S649 of MRE11 can be directly modified by this enzyme *in*

vitro (Kim, 2005), suggested that the biochemical link between CK2 and the MRN complex is not just limited to NBS1. Consistent with this premise, *in vitro* CK2 kinase assays demonstrated that CK2 can directly phosphorylate MRE11 (Figure 4.2, 4.3 & 4.10). Although, in contrast to the study by Kim (2005), S649 was not the only target site. The reason for this apparent disparity remains unclear, but it is possible that since the CK2 used by Kim to phosphorylate MRE11 was purified by immunoprecipitation from 293 cells a contaminating kinase maybe responsible for the phosphorylation of MRE11 on this residue. Whilst mutation of the two other serine residues of MRE11 identified as potential CK2 phosphorylation sites (S688/689) did result in a moderate reduction in its phosphorylation, it appeared that a more N-terminal residue (S541), that was only identified by prediction, represented the major site of phosphorylation *in vitro* (Figure 4.10). However, an antibody specific to phosphorylated S688 of MRE11 used on whole cell extracts from cells expressing WT and mutant MRE11, in an MRE11 depleted background, suggested phosphorylation of S688 was constitutive *in vivo* (Figure 4.20).

Despite the identified sites of *in vivo* MRE11 phosphorylation (S649/S688/S689) not representing major targets for CK2 phosphorylation, at least *in vitro*, it is clear that these sites are modified and have not been assigned to regulating any aspect of MRE11 biology. To investigate how loss of phosphorylation on these sites may influence the MRE11-dependent DDR, several cell systems were utilised to model mutant MRE11.

Firstly, isogenic hTERT-immortalised ATLD2 patient derived fibroblasts were used, which express extremely low levels of endogenous MRE11. The reduced level meant that any effect on the DDR caused by expression of the MRE11 mutants would not be influenced by the endogenous protein. Preliminary characterisation carried out on isolated ATLD2 clones expressing the different MRE11 mutants suggested phosphorylation of MRE11 on

S649/688/689 did not affect the capacity of MRE11 to bind to RAD50 and NBS1, its recruitment to IRIF or its ability to mediate repair of IR-induced DNA lesions (Figure 4.5, 4.7 & 4.8). The progressive reduction in the growth in theses cells severely hampered any further analysis. Verification that the cells still expressed hTERT, analysis of different clones at early passage in addition to changing the growth media, failed to identify the cause of the slowed growth. A similar report from another researcher using patient derived fibroblasts from NBS patients suggests that this may be an inherent problem with patient cells with defects in the MRN complex (Dr. Manuel Stucki, personal communication). Further to this problem, significant differences in the expression of DDR proteins in different ATLD2 clones, irrespective of the exogenous MRE11 stably expressed in these cell lines, was observed. Due to these problems, it became apparent an alternative approach was required to address whether modification on these three residues plays any role in modulating MRE11 function.

The second cell system used to model MRE11 mutations took advantage of the HCT116 tumour cell line. This particular cell line has been shown to have a mutation in the *MRE11* gene, which results in the expression of an unstable truncated form of the protein. Moreover, the availability of a derivative, HCT116 Flp-In/T-Rex, would facilitate the production of a relatively homogenous population of cells stably expressing a protein of interest under the control of an inducible promoter. This isogenic cell system would therefore minimise any problems associated with isolating individual clones. Despite generating HCT116 Flp-In/T-Rex cell lines stably expressing either WT MRE11 or various phospho-mutants and getting robust expression of exogenous MRE11, it became apparent that HCT116 cells exhibit very high levels of 53BP1 foci in the absence of any treatment (Figure 4.14). Furthermore, it became apparent that this was not due to underlying mutation in its *MRE11* gene, since re-expression of WT *MRE11* could not complement this defect.

One obvious caveat is that HCT116 cells have mutations in the MMR gene, MLH1, and as a consequence they are genomically unstable, and therefore more likely to acquire somatic mutations in other genes over long periods of *in vitro* culture (Villemure *et al.*, 2003). It is possible that this cell line has accrued mutations in other genes involved in maintaining genome stability. Indeed ATM levels were reduced in this cell line, and from searching the COSMIC database a single heterozygous mutation was identified. Furthermore, interrogating the same database also revealed mutations in additional DDR proteins CHK2, RAD54 and ATRX. The latter has recently been reported to bind to the MRN complex and functions to promote replication fork and restart (Leung *et al.*, 2013). Therefore it is highly likely that the elevated levels of 53BP1 foci could be a consequence of either spontaneous DNA damage due to a defective MMR system or as a result of acquired mutations in DDR proteins.

In an attempt to avoid the problems associated with the HCT116 cells, but to continue using a tumour cell line model to avoid any growth related issues connected with using patient-derived fibroblasts, the Flp-In/T-Rex approach was utilised in U2OS cells. In contrast to the HCT116 cell line, U2OS cells have a WT *MRE11* gene. Therefore prior to carrying out any analysis of the function of mutant MRE11 the endogenous protein was depleted using siRNA. As feasible as this method is, it does present caveats. Firstly, siRNA-mediated depletion of proteins never depletes 100% of the targeted mRNA; secondly, siRNAs can target other mRNAs non-specifically; and thirdly, some siRNA duplexes carry high levels of inherent cytotoxicity. Moreover, using this approach requires the exogenous gene to be made resistant to the siRNA that is used to knockdown the endogenous protein. This can be achieved either by site-directed mutagenesis or by using siRNAs that target UTR sequences. With these limitations in mind, a panel of polyclonal U2OS Flp-In/T-Rex cell lines overexpressing either WT MRE11, an empty vector or various MRE11 mutants were made. A previously published

MRE11 siRNA targeting its 5' UTR was used to knockdown the endogenous MRE11 protein in these cells. Unfortunately, despite achieving greater than good knockdown of the endogenous MRE11 in these cell lines, and robust expression of the exogenous MRE11, which was clearly capable of reinstating the stability of RAD50 and NBS1 caused by the transfection with the siRNA, a complementable defect in DSB repair could not be detected (Figure 4.16 & 4.22). Again using 53BP1 foci as a marker of DNA repair, 24 h post IR, foci persisted in both the vector-only cell line and the WT, suggesting that expression of the exogenous WT MRE11 is not sufficient to complement the defect. This observation is similar to figure 4.8. Here the ATLD vector-only cell line have reduced levels of MRE11, resulting in persistant 53BP1 foci 24 h post low dose IR, compared to the WT ATLD cell lines which are comparable to the 0 h counts. In addition a robust defect in the phosphorylation of known ATM substrates was not detected in cells depleted of MRE11 compared to those in which WT MRE11 had been re-expressed after either low dose IR or CPT treatment (Figure 4.23 & 4.24). Again this would have been expected as ATLD cells show reduced ATM activation (Uziel *et al.*, 2003) It is likely that since the MRN complex is extremely abundant in the cell, despite achieving very high efficiency of endogenous siRNA MRE11 knockdown, the remaining small pool of protein is sufficient to carry out its function with normal effectiveness. In support of this, the low/barely detectable levels of mutant MRE11 protein expressed in ATLD2 cells are sufficient to maintain viability of the cells. In addition, other researchers have screened 8-10 different *MRE11* siRNA sequences, and to achieve a cellular phenotype that is consistent with MRN dysfunction cells must be transfected with a pool of 2-3 different siRNAs, even though many of the siRNAs tested resulted in a >85% knockdown of the protein as judged by Western blotting (Dr. Claus Sorensen, personal communication). Taken together, none of the three cell systems used to address the potential role of MRE11

phosphorylation on putative CK2 target motifs were able to generate a reproducible defect in known MRN-dependent DNA damage responses. Due to this the effect of losing phosphorylation on serines 541,649,688 and 689 and the effect this may have on activation of these pathways could not be effectively studied. Even so, a limited amount of information could be extracted from some of the preliminary observations: the phosphorylation on serines 541,649,688 and 689 is required neither for MRN complex stability, nor for its recruitment to sites of DSBs, or its ability to promote repair of these lesions. Despite these findings it is clear that a different approach is required to carry out a more in depth analysis of MRE11 function that includes its role in activation of the intra-S and G2/M DNA damage checkpoints, replication fork restart, apoptosis, DNA end-resection and ATR activation.

In spite of the problems associated with using siRNA and the U2OS Flp-In/T-Rex cells, it was noted that the DNA damage-induced phosphorylation of MRE11 on serine-676 was reduced when the putative CK2 phosphorylation sites were mutated. S676 is situated between S649 and the double residues S688/S689, and is a SQ motif, which is a putative PIKKs target residue. Based on the observed reduction in damage dependent-phosphorylation of these sites on induction of the MRE11 CK2 mutants, it could be speculated that phosphorylation of S676 is dependent on CK2. Previous studies have demonstrated a mechanism by which constitutive phosphorylation of a CK2 substrate is a prerequisite for the phosphorylation of nearby residues. In one example, phosphorylation by CK2 is required for the damaged induced phosphorylation of a proximal serine on SMC3 by ATM, and this is required for the intra-S-phase checkpoint (Luo *et al.*, 2008). It has been shown that MRE11 is phosphorylated in response to DNA damaging agents, on a number of residues possibly including S676 (Di Virgilio *et al.*, 2009, Mu *et al.*, 2007, Stokes *et al.*, 2007). One report suggested that the phosphorylation of SQ/TQ motifs on MRE11 regulates the disassociation of the MRN

complex from the chromatin in order to facilitate down regulation of the DDR in response to DSBs (Di Virgilio *et al.*, 2009). This implies that phosphorylation on this residue may regulate MRE11 association with chromatin. Yet whether the phosphorylation of this residue is dependent on the modification of proximal motifs by CK2 would require further investigation.

CHAPTER 5

Chapter 5 Final discussion and future perspectives

5.1 Introduction

CK2 is a highly ubiquitous serine/theronine kinase, known to phosphorylate over 300 substrate proteins. The large number of CK2 substrates is reflective of its constitutive activity and its requirement in many cellular processes, including cell survival, proliferation and apoptosis. Unsurprisingly, phosphorylation of proteins by CK2 has been observed during the DDR, both in response to ssDNA breaks and DSBs. In this thesis we have identified a CK2-dependent interaction between the DDR proteins MDC1 and NBS1, in addition to identifying a potential CK2 substrate (MRE11) also involved in the cellular response to DNA damage.

5.2 CK2 is required for retention of NBS1 at damaged chromatin

Based on the work presented in Chapter 3, in collaboration with work from the laboratory of Dr M. Stucki, (Zürich, Switzerland), we have identified a conserved N-terminal cluster of CK2 phosphorylation motifs on the DNA damage mediator protein MDC1. This cluster, which we termed the SDTD domain, was shown to facilitate an interaction between MDC1 and the FHA domain of NBS1, a member of the MRN complex. Furthermore, we have shown that this phosphorylation is constitutive and that the interaction occurs even in the absence of DNA DSBs. These data provide a mechanistic explanation for the previously described interaction between MDC1 and NBS1, even in the absence of DSBs, and also for the requirement of MDC1 to promote MRN IRIF (Goldberg *et al.*, 2003, Lou *et al.*, 2006, Lukas *et al.*, 2004a, Stewart *et al.*, 2003). Disruption of this interaction, by depleting CK2, or by mutation of either the SDTD domain of MDC1 or the FHA domain of NBS1, resulted in the loss of NBS1 foci following IR and resulted in a partial G2/M checkpoint defect. Similar

observations have been reported by several other groups, re-enforcing the biochemical link between MDC1, CK2 and the MRN complex (Chapman & Jackson, 2008, Melander *et al*., 2008, Spycher, *et al*., 2008, Wu *et al*., 2008). Interestingly, Wu *et al*, (2008) demonstrated that the interaction between MDC1 and NBS1 is also required to activate the intra-S phase checkpoint. In addition, these studies revealed that disruption of this interaction did not affect ATM activation, and therefore the CK2-dependent interaction was not required for this aspect of MRN function. In support of this, previous work has shown MDC1 does not seem to be required for the activation of ATM or the DNA end processing activities of MRN (Jazayeri *et al.*, 2006, Lee and Paull, 2005). Therefore, it would seem that the purpose of the interaction between MDC1 and NBS1 is to ensure focal recruitment and retention of the MRN complex at the break, which serves to amplify the DNA damage response and trigger intra-S and G2/M checkpoint activation.

A similar mechanism to that reported for XRCC1 and XRCC4, whose phosphorylation by CK2 permits interaction with several binding partners, may also exist with MDC1. Indeed, two separate studies have shown Aprataxin and TopBP1 interact with the SDTD region of MDC1 (Becherel *et al.*, 2010, Wang *et al.*, 2011). It is also plausible that the NBS1 FHA and BRCT domains, in addition to interacting with MDC1, may associate with other phosphorylated interacting partners. Although, it is currently unclear how disruption of the ability of NBS1 to MDC1 and/or these other potential binding partners contributes to the clinical symptoms exhibited by the NBS^{BRCT} patients identified in this study. Of note, CtIP, a potential CK2-dependent binding partner of NBS1, is mutated in one consanguineous Seckel family (SKL2), which share some of the clinical phenotypes exhibited by the NBS^{BRCT} patients (Borglum *et al.*, 2001, Qvist *et al.*, 2011). Although the microcephaly and growth defects seen in the SKL2 family are mild compared to the NBS^{BRCT} patients, the overlapping

phenotypes could be due to defects in similar pathways. Interestingly the C-terminally truncated form of CtIP expressed in the affected individuals lacks an MRN interaction domain, which is required for DNA end-resection and thus disrupts ATR activation (Qvist *et al.*, 2011, Sartori *et al.*, 2007). To verify these speculations, mass spectrometry techniques could be employed to examine the identification of novel proteins that interact with either the SDTD domain of MDC1 or the FHA/BRCT domains of NBS1. Performing these experiments in the presence or absence of DNA damaging agents could potentially reveal how binding to these regions is regulated, as different proteins may interact depending on the type of DNA lesion induced.

The identification of a homozygous mutation in the BRCT domain of NBS1 in three affected siblings exhibiting a clinical phenotype that overlaps with those of NBS and Seckel Syndrome highlights the importance of phospho-peptide binding mediated by the FHA/BRCT domain during neuronal development. Strikingly, we did not observe any defects in ATM or G2/M activation following the exposure to IR or DSB repair in cells from these patients indicating that phospho-dependent interactions with the BRCT domain of NBS1 are likely to be critical for the cellular response to different DNA lesions. Consistent with this, twins have been identified, compound heterozygous for the common NBS 657del5 mutation and a missense point mutation in the BRCT domain (643C>T (R215W), that exhibit severe microcephaly and growth retardation, but no chromosomal instability or radiosensitivity (Seemanova *et al.*, 2006). Taking this evidence together with the observations from this study, it would seem that the clinical/cellular phenotypes of the NBS^{BRCT} patients may not be attributed to defects in the repair of DNA DSBs. One possible hypothesis is that the patients' symptoms may result from problems during DNA replication or mitosis during neural development, as is thought to be the underlying mechanism for other syndromes that present microcephaly (Kerzendorfer *et*

al., 2013, Mokrani-Benhelli *et al.*, 2012, Stiff *et al.*, 2013). For instance, cells from patients with Seckel syndrome caused by mutations in *ATR* exhibit replication problems and defective cell cycle checkpoint activation (Alderton *et al.*, 2004, Stiff *et al.*, 2005, Stiff *et al.*, 2006). Interestingly, ATR signalling is impaired in NBS cells, which also display problems in recovery following replication stalling (Stiff *et al* 2005). Therefore, to address this hypothesis, the impact of the ΔS118 mutation on ATR activation and signalling should be assessed using a panel of different DNA damaging agents known to cause replication stress and activate ATR, such as HU and UVC. To further examine whether the ΔS118 mutation impacts upon nervous system development *in vivo*, one approach would be to create a knockin transgenic mouse model encoding this mutation, and to use this model to further study neuronal development. Previous studies using mice models have been beneficial in the study of neuropathology. Work by Lee *et al*, 2012, demonstrated by studying Atr loss in the murine nervous system, that its depletion impacts on specific populations of cells, which suggests that it is required to survey genomic integrity selectively during neurogenesis. For instance Atr loss resulted in proliferation arrest, due to replication associated damage in the cerebella, yet the same stress results in apoptosis in ganglionic eminence (responsible for generating diversity of cortical cell types) (Lee *et al.*, 2012). Moreover, a second group showed that by depleting either Nbs, Atr or both in the mouse central nervous system, loss of Nbs1 impacted only on proliferating neuroprogenitor cells, whereas Atr loss impacted on both proliferating and non-proliferating neural cells (Zhou *et al.*, 2012). In light of this evidence, modeling the ΔS118 in a mouse system may reveal that this BRCT domain mutation may disrupt a function of NBS1 required to monitor genetic integrity in proliferating neural cells, that most likely impacts on ATR activity.

5.3 Mre11 and CK2

During the course of this work, it was also investigated whether MRE11 was regulated by CK2-dependent phosphorylation. One residue within MRE11 (S649) had been previously shown to be phosphorylated *in vivo*, although the physiological relevance of this modification was not determined (Kim 2005). Using *in vitro* techniques, it was shown that the residues S541, S688 and S689 were phosphorylated by recombinant CK2, with residue S541 being the major target for CK2 *in vitro*. Furthermore, expressing MRE11 proteins bearing mutations in these sites *in vivo*, revealed that phosphorylation of these sites by CK2 was not required for the focal localisation of MRE11/MRN to IRIF following IR, nor for the repair of these lesions as measured by 53BP1 IRIF persistence. Similarly, the phosphorylation of several ATM substrates in response to DNA DSBs remained unaffected. The study of the cellular consequence(s) of these mutations was attempted in three independent experimental *in vivo* systems, but was hampered by technical issues, preventing significant insight into this aspect of MRE11 function. To definitively study whether these putative CK2 residues have a function *in vivo*, a suitable system would first need to be established in which mutated MRE11 could be expressed. It is possible that the spontaneous immortalized MEFs (Murine Embryonic Fibroblasts) generated from the ATLD2 mouse model may be a more robust cell system with which to study the function of MRE11 phosphorylation, especially since the 4 serine residues that were identified as CK2 targets are conserved in rodents (Figure 4.9). As with using the ATLD2 patient-derived cells, these MEFs would provide an isogenic system with which to express low levels of endogenous mutant protein. However, the only ATLD2 MEFs available to date are immortalised with SV40 Large T antigen, which has been previously shown to interact with, and compromise the function of, the MRN complex (Lanson et al 2000, Wu et al 2004). Furthermore, antibodies recognising mouse DDR

proteins, particularly phospho-specific antibodies, are not readily available. As an alternative to this approach, Dr. Manuel Stucki has recently identified an endometrial tumour cell line (AN3Ca) that expresses extremely low levels of endogenous MRE11, which would render it appropriate for studies requiring the expression of my MRE11 CK2 mutants. However, this cell line has not yet been fully characterised, and thus, in a manner similar to the HCT116 cell line, it is possible that AN3Ca cells may have accumulated other gene mutations rendering it non-permissive for studying the MRE11-dependent DDR.

Despite these caveats, once a suitable robust experimental system is in place, then the role of CK2 phosphorylation on MRE11/MRN function can be thoroughly investigated. Of particular interest, and an avenue of further study, is the impact of CK2 phosphorylation of MRE11 on the phosphorylation of S676, a putative SQ/TQ motif within Mre11, which was induced by DNA damage. Following mutation of the four putative CK2 residues, a reduction of S676 phosphorylation was seen. This observation suggests that the phosphorylation of S676 requires phosphorylation of the CK2 sites to occur, prior to its own damage-dependent activation by ATM/ATR. A study investigating the phosphorylation of several MRE11 SQ/TQ motifs, including S676, showed that mutating these motifs to alanines did not affect MRN complex expression or stability, DNA tethering or ATM activation (Di Virgilio *et al*., 2009). The authors of this study concluded that phosphorylation on these SQ/TQ sites was damage-dependent and was required for the disassociation of MRE11 from the DNA, potentially providing one mechanism to down-regulate the damage signal. More recently ribosomal S6 kinase (Rsk), elevated in a number of cancers has been shown to phosphorylate S676 both *in vitro* and *in vivo* upstream of ATM. In support of earlier observations this is thought to preventing binding of the MRE11 to DSBs (Chen *et al.*, 2013). Moreover the authors go on to suggest that phosphorylation of this residue could function to inhibit

activation of cell cycle checkpoints upstream of ATM, promoting tumor progression. If phosphorylation of S676 requires the phosphorylation of the proximal CK2 sites for its activation, it could be that the association of MRE11 with chromatin is prolonged when these sites are mutated, which may result in excessive MRE11-dependent resection and excessive/uncontrolled homologous recombination. To address this question, it would be interesting to examine the association of MRE11 with chromatin following mutation of the CK2 sites in response to a variety of DNA DSB causing agents, in addition to studying various markers of resection such as RPA IRIF.

Preliminary data from analysis of DNA replication using DNA fibre analysis suggested that U2OS Flp-In/T-Rex cells expressing mutated MRE11, where all four of the putative CK2 sites had been mutated (4A), had DNA replication defects. This was evident by an increase in the proportion of stalled replication forks and defective fork restart following exposure to HU. This data was not included in this thesis due to the inherent caveats identified using this cell system. However, based on these preliminary data it is tempting to speculate that the putative CK2 sites on MRE11 may play some part in regulating the cellular response to replication stress. Investigating whether cells expressing MRE11 harbouring mutations in the CK2 sites have a RDS (Radioresistant DNA synthesis) would be a simple and elegant way to assess whether phosphorylation of MRE11 by CK2 is required during DNA replication.

5.4 CK2 and the DNA damage response: Implications

The work presented in this thesis provides further examples of the involvement of CK2 in the DDR. Based on the large number of DDR proteins that contain potential CK2 phosphorylation residues, including those involved in the sensing, signalling and repair of DNA breaks, it is highly likely that other *in vivo* substrates of CK2 will be identified (Table

5.1). In support of this supposition, cells transfected with CK2 siRNA exhibit a modest prolonged ATM response (Figure 3.6.). Similarly, a separate study demonstrated a reduction in DNA-PKcs autophosphorylation and delayed DSB repair following depletion of the CK2 catalytic subunits by siRNA (Olsen *et al.*, 2010). Moreover, work from the same research group also showed CK2 co-localises with γH2AX after DNA damage, and that loss of CK2 destabilises the interaction between KU80 and DNA-PKcs (Olsen *et al.*, 2012). The phosphorylation of DDR proteins by CK2 likely results in a variety of outcomes including facilitating interactions with phospho-peptide binding domains, priming substrates for damaged-induced phosphorylation by other kinases, stability of complexes and in some cases altering the localisation of proteins (Iles *et al.*, 2007, Kubota *et al.*, 2009, Luo *et al.*, 2008, Parsons *et al.*, 2010, Yang *et al.*, 2009). Many of the substrates of CK2 appear to be constitutively phosphorylated, contrasting to the function of other cellular kinases whose activity is often regulated in the response to a particular stimulus. This poses the question: why have a modification, that to all intents and purposes is permanently active? One simple explanation could be that phosphorylation by CK2 allows the substrate to carry out its function rapidly when required, omitting any need for intermediate regulatory steps. Equally, phosphorylation by CK2 could be extremely transient, with the phosphate being removed by phosphatases as quickly as it is added. Therefore substrates appear to be constitutively phosphorylated when this may not be the case.

The role CK2 plays in the DDR response, in addition to its role in cellular survival and proliferation, has attracted attention in the field of cancer research, since defects in the cellular response to DNA damage may be a precursor to tumourgenesis. Equally, kinases are often tempting therapeutic targets in the treatment of human diseases, since the catalytic activity of these enzymes can be targeted using specific inhibitors, which can impact upon downstream

pathways in which the kinase is required. A number of p38 mitogen-activated protein kinases (p38 MAPK), activated during cellular stress, inhibitors have been developed to treat inflammatory diseases such as rheumatoid arthritis (Kumar *et al.*, 2003, Thalhamer *et al.*, 2008). Moreover, targeting the kinome is also used in the treatment of cancers. For instance the first to do so, Herceptin (trastuzumab), targets the receptor tyrosine kinase ErbB2, which is overexpressed in a large number of breasts cancers (Hudis, 2007, Valabrega *et al.*, 2007). It has been known for some time that CK2 expression and activity is elevated in a number of human cancers, and for this reason has highlighted its potential as a target for pharmacological inhibition (Gapany *et al.*, 1995, Hamacher *et al.*, 2007, Landesman-Bollag *et al.*, 2001). The major benefit of CK2 as a potential target in cancer therapy is that, unlike canonical kinases, CK2, due to its constitutive activity, functions laterally in many pathways, as opposed to residing at the top of these cascades. Therefore inhibition is likely to impact on signalling networks at a variety of levels, although this could be at the detriment to selectivity.

A number of kinases are frequently mutated in carcinogenesis, producing oncogenic kinases that become permanently activated, and driving the transformation of the cell, such as fibroblast growth factor receptor 2 (FGFR2) mutated in some breast cancers (Reintjes *et al.*, 2013). Interestingly, CK2 does not seem to be a significant target for such somatic mutations, in that no mutations have as yet been identified that account for the gain of CK2 activity observed in tumours (Ruzzene *et al.*, 2010). Instead, evidence suggests that CK2 does not act as driver of tumourgenesis *per se*, but that once a cell has become transformed, elevated CK2 levels potentiate its survival by suppressing anti-apoptotic signals. For instance, the caspase inhibitor, activity-regulated cytoskeleton-associated protein (ARC) is activated following phosphorylation by CK2 (Li *et al.*, 2002d). The first CK2 inhibitor to be described was DRB (5, 6-di-chloro-1-(β-$_D$-ribofuranosyl)-benzimida-zole) (Zandomeni *et al.*, 1986). However,

since its discovery, other inhibitors have been discovered that exhibit greater specificity, namely TBB and DMAT (2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole) (Pagano *et al.*, 2004, Sarno *et al.*, 2001). Both these inhibitors have been reported to be effective at inducing cell death in cancer cell lines. For example, DMAT has been shown to kill human breast cancer cells resistant to anti-oestrogens (Yde *et al.*, 2007). Inhibiting such a prolific kinase though would presumably have caveats due to the number of putative CK2 substrates within the cell. Despite the inhibitors disrupting the targeted pathway, other cellular processes dependent on CK2 would also be affected, potentially resulting in unwanted toxicity. Though this may be the case, encouragingly *in vivo* work in mice studying an involvement of CK2 in retinal angiogenesis, using several CK2 inhibitors showed no additional toxicity (Kramerov *et al.*, 2006, Ljubimov *et al.*, 2004). In terms of treating cancer with CK2 inhibitors, it could be postulated that as CK2 levels are elevated in tumours, disrupting activity to a minimal extent would have an impact on the malignancy without significantly impacting on normal tissues or cells. In context of the role of CK2 in the DDR, recently, a potent CK2 inhibitor (CX-4945) was used in combination with either the chemotherapy treatments cisplatin or gemacitabine, which are both DNA damaging agents, to investigate their combined therapeutic potential (Siddiqui-Jain *et al.*, 2012). This study was based on the growing role of CK2 in the repair of DNA damage, and aimed to investigate whether CK2 inhibition could enhance the activity of DNA-damaging chemotherapeutics. Indeed, the study showed that treatment with the CK2 inhibitor increased the anti-proliferative effect of these agents on ovarian cancer cells (Siddiqui-Jain *et al*., 2012). This observation opens the potential for the use of CK2 inhibitors in combination with DNA-targeting chemotherapy drugs to improve treatment of those cancers with partial resistance to these particular drugs.

In conclusion, the continued study of how CK2-dependent phosphorylation regulates the cellular DDR will be essential for understanding if CK2 inhibitors could be used clinically to hyper-sensitise tumours cells to chemotherapeutic agents.

Protein	Putative CK2 sites
ATRX	S978, S1291, S1292
BRCA1	S1218
Claspin	T639, S642, S744, S1012, S1067
ERCC6	S367, T377, S489
EXO1	S659
FANCD2	S1412
FANCJ	T1123
HDAC1	S393, S421
PARP2	T218
PIAS1	S468
PIAS2	S477, S478
PIAS4	S467, S476
RIF1	S1557
RPA1	S1429
SENP3	S75
SETX	S1489
TIPIN	T40
TOP2A	S1337, T1343
TOP2B	S1613
TRIP12	S394, S1322, S1376
XPC	S129
XRCC1	T523
XRCC4	S232, T233

Table 5.1 ***A selection of proteins associated with the DNA damage response and putative CK2 sites, as predicted by a web-based search tool (http://scansite.mit.edu/).***

APPENDICES

Constitutive phosphorylation of MDC1 physically links the MRE11–RAD50–NBS1 complex to damaged chromatin

Christoph Spycher,[1] Edward S. Miller,[3] Kelly Townsend,[3] Lucijana Pavic,[1] Nicholas A. Morrice,[4] Pavel Janscak,[2] Grant S. Stewart,[3] and Manuel Stucki[1]

[1]Institute of Veterinary Biochemistry and Molecular Biology and [2]Institute of Molecular Cancer Research, University of Zürich, 8057 Zürich, Switzerland
[3]Cancer Research UK Institute for Cancer Studies, Birmingham University, Birmingham B15 2TT, England, UK
[4]Medical Research Council Protein Phosphorylation Unit, School of Life Sciences, University of Dundee, Dundee DD1 5EH, Scotland, UK

The MRE11–RAD50–Nijmegen breakage syndrome 1 (NBS1 [MRN]) complex accumulates at sites of DNA double-strand breaks (DSBs) in microscopically discernible nuclear foci. Focus formation by the MRN complex is dependent on MDC1, a large nuclear protein that directly interacts with phosphorylated H2AX. In this study, we identified a region in MDC1 that is essential for the focal accumulation of the MRN complex at sites of DNA damage. This region contains multiple conserved acidic sequence motifs that are constitutively phosphorylated in vivo. We show that these motifs are efficiently phosphorylated by caseine kinase 2 (CK2) in vitro and directly interact with the N-terminal forkhead-associated domain of NBS1 in a phosphorylation-dependent manner. Mutation of these conserved motifs in MDC1 or depletion of CK2 by small interfering RNA disrupts the interaction between MDC1 and NBS1 and abrogates accumulation of the MRN complex at sites of DNA DSBs in vivo. Thus, our data reveal the mechanism by which MDC1 physically couples the MRN complex to damaged chromatin.

Introduction

Eukaryotic cells are equipped with sophisticated mechanisms to detect, signal the presence of, and repair DNA damage. Of particular importance are the pathways that deal with DNA double-strand breaks (DSBs): highly toxic lesions that, if unrepaired or repaired incorrectly, can cause cell death, mutations, and chromosomal translocations and can lead to diseases such as cancer. Cells react to DSBs by rapidly deploying a host of proteins to the damaged chromatin regions. Some of these factors engage in DNA repair, whereas others trigger a signaling pathway (called the DNA damage checkpoint) that provokes delays in cell cycle progression and coordinates the repair process; together, these events comprise the so-called DNA damage response (DDR; Zhou and Elledge, 2000).

Among the first proteins that accumulate at sites of DSBs in eukaryotic cells is the MRE11–RAD50–Nijmegen breakage syndrome 1 (NBS1 [MRN]) complex, a conserved and essential DDR factor that functions in a multitude of cellular processes involving DSBs, including DSB repair, checkpoint signaling, DNA replication, meiotic recombination, and induction of apoptosis (Stracker et al., 2004, 2007; Difilippantonio et al., 2007). The MRN complex consists of three subunits. The first is the structure-specific nuclease MRE11, which is most likely involved in nucleolytic processing of DNA ends to allow homologous recombination repair (Jazayeri et al., 2006), and the second is the ATPase and adenylate kinase subunit RAD50, which, together with MRE11, appears to facilitate tethering of DNA molecules to promote DSB repair (Costanzo et al., 2004; Bhaskara et al., 2007). The third subunit of the MRN complex, NBS1, does not exhibit any catalytic activities. Instead, its domain composition suggests that it belongs to the family of adaptor/mediator proteins of the DDR, a group of recently emerging factors that integrate, coordinate, and enhance the various cellular responses to DNA damage by promoting protein–protein interactions (D'Amours and Jackson, 2002). Consistent with this notion, NBS1 features both forkhead-associated (FHA) and BRCA1 C-terminal (BRCT) domains at its N terminus, which are protein interaction modules that specifically mediate the interaction with

Correspondence to Manuel Stucki: m.stucki@vetbio.uzh.ch

Abbreviations used in this paper: ATM, ataxia telangiectasia mutated; BRCT, BRCA1 C terminal; CK, caseine kinase; DDR, DNA damage response; DSB, double-strand break; FHA, forkhead associated; IR, ionizing radiation; MEF, mouse embryonic fibroblast; MR, MRE11 and RAD50; MRN, MRE11–RAD50–NBS1; NBS, Nijmegen breakage syndrome; PIKK, phosphoinositide-3-kinase–related protein kinase; PNK, polynucleotide kinase; pSDpT, phosphorylated SDT peptide; PST, Pro-Ser-Thr; SDT, Ser-Asp-Thr; TBB, tetrabromo-2-azabenzimidazole.

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phosphorylated proteins (Durocher and Jackson, 2002; Glover et al., 2004). Moreover, several NBS1 interaction partners have been described; most prominent among these is the ataxia telangiectasia mutated (ATM) kinase, the key upstream component of DSB signaling (Falck et al., 2005). Mutations in the *NBS1* gene leads to NBS in humans, and cells derived from NBS patients display a DSB repair and signaling deficiency, including radiosensitivity, chromosomal instability, and checkpoint defects (D'Amours and Jackson, 2002). Mouse models in which the native mouse *NBS1* allele was exchanged with hypomorphic mutant alleles recapitulate many features of NBS in the mouse, including developmental defects, chromosomal instability, and checkpoint deficiency (Difilippantonio et al., 2005, 2007).

Accumulation of the MRN complex at sites of DSBs is manifested by the formation of microscopically discernible subnuclear structures, so-called nuclear foci that represent large chromatin regions containing one or several unrepaired DSBs (Maser et al., 1997). The key regulator of nuclear foci formation in higher eukaryotes is the histone variant H2AX, an integral component of the nucleosome core structure that comprises 10–15% of total cellular H2A in higher organisms (Fernandez-Capetillo et al., 2004). H2AX is phosphorylated extensively on a conserved Ser residue at its C terminus in chromatin regions bearing DSBs, and this is mediated mainly by the ATM kinase, a member of the phosphoinositide-3-kinase–related protein kinase (PIKK) family (Burma et al., 2001).

Although it has been previously suggested that MRN accumulation at sites of DSBs occurs through interaction between the FHA/BRCT region of NBS1 and phosphorylated H2AX (γ-H2AX; Kobayashi et al., 2002), recent evidence suggests that the interaction between NBS1 and γ-H2AX is not direct but is mediated by MDC1, a large nuclear factor that interacts with the MRN complex and also features the criteria of a DDR mediator/adaptor protein, including the presence of FHA and BRCT domains (for review see Stucki and Jackson, 2004). First, it was shown that MDC1 exists in a complex with MRN in extracts of undamaged cells (Goldberg et al., 2003). This complex dissociates upon modification of the N-terminal FHA domain of NBS1, suggesting that the NBS1 FHA domain may be participating in the interaction between the two factors (Lukas et al., 2004). Second, MDC1 directly and specifically interacts with the phosphorylated H2AX C terminus through its tandem BRCT domains (Stucki et al., 2005). The x-ray structure of the MDC1–γ-H2AX complex suggests that the MDC1 BRCT domains are uniquely tailored to interact with the γ-H2AX chromatin mark (Stucki et al., 2005). Third, a phosphopeptide derived from the H2AX C terminus interacted with the MRN complex only in the presence of MDC1 (Lukas et al., 2004). Fourth, experimental disruption of the interaction between MDC1 and γ-H2AX (Stucki et al., 2005) or loss of MDC1 expression by genetic manipulation and/or siRNA-mediated depletion leads to a complete abrogation of MRN foci formation (Goldberg et al., 2003; Stewart et al., 2003; Lukas et al., 2004; Lou et al., 2006). Finally, mutations in the N-terminal FHA/BRCT region of NBS1 also interfere with MRN accumulation at sites of DSBs (Kobayashi et al., 2002; Zhao et al., 2002; Cerosaletti and Concannon, 2003; Lee et al., 2003; Horejsi et al., 2004).

MDC1 is composed of several distinct sequence domains. Besides an FHA domain at its N terminus and the γ-H2AX–interacting BRCT tandem domain at the C terminus, MDC1 also features a unique repeat region in the middle of the protein (Pro-Ser-Thr [PST] repeat) and contains several highly conserved putative PIKK target sites, some of which are phosphorylated in response to DNA damage in vivo (Matsuoka et al., 2007). In addition, MDC1 is also phosphorylated in the absence of DNA damage: several recent large-scale phosphorylation site screens of the human and mouse proteome revealed that MDC1 is constitutively phosphorylated on a significant number of Ser and Thr residues in vivo (Beausoleil et al., 2004; Olsen et al., 2006; Villen et al., 2007).

Although it is well established that MDC1 mediates the accumulation of many DDR factors in damaged chromatin regions (including the MRN complex, 53BP1, BRCA1, and ATM; Stucki and Jackson, 2006), it is still unclear how MDC1 mediates MRN recruitment. The observation that MDC1 exists in a complex with MRN in extracts from undamaged cells suggests that MDC1 may recruit MRN to γ-H2AX–containing chromatin in a simple "piggyback ride" mechanism whereby the physical interface between the two factors is made up by the NBS1 N-terminal FHA/BRCT region and by one or several phosphoepitopes on MDC1.

To put such a mechanism to the test, we set up an in vivo complementation system for MDC1 to search for a region in MDC1 that is responsible to mediate MRN foci formation. Through this approach, we identified a new region in MDC1 that is composed of several acidic repeats featuring a unique sequence motif, the Ser-Asp-Thr (SDT) motif. Furthermore, we provide evidence that these SDT repeats are constitutively phosphorylated by caseine kinase 2 (CK2) and that the phosphorylated form of these motifs constitute the phosphoepitope that the NBS1 FHA domain is binding to. These findings allow us to draw a model of the mechanism by which MDC1 physically links the MRN complex to damaged chromatin.

Results

An acidic region near the N terminus of MDC1 is essential for NBS1 foci formation

Efficient accumulation of the MRN complex in foci at sites of DSBs is critically dependent on MDC1 (Goldberg et al., 2003; Lukas et al., 2004). To determine the region of MDC1 that mediates MRN foci formation, we transfected MDC1$^{-/-}$ mouse embryonic fibroblasts (MEFs; Lou et al., 2006) with a series of N-terminal deletion mutants of mouse MDC1 and assessed MRN accumulation by indirect immunofluorescence using an antibody specific for mouse NBS1 (Celeste et al., 2003). Consistent with published data (Lou et al., 2006), MDC1$^{-/-}$ MEFs were completely defective for NBS1 accumulation (unpublished data), but transient transfection of these cells with HA-tagged full-length mouse MDC1 readily restored NBS1 foci formation in response to 5 Gy of ionizing radiation (IR; Fig. 1 B, WT). Deletion of 153 and 295 N-terminal amino acids of MDC1 did not result in any detectable reduction of NBS1 foci (Fig. 1 B, ΔN1 and ΔN2), but deletion of 452 and 645 amino acids led to a complete loss of NBS1 accumulation (Fig. 1 B, ΔN3 and ΔN4). This indicated that the region in MDC1 essential for mediating NBS1 accumulation is located somewhere between amino acids 295 and 452. Indeed, internal deletion of this 157–amino acid region completely abolished

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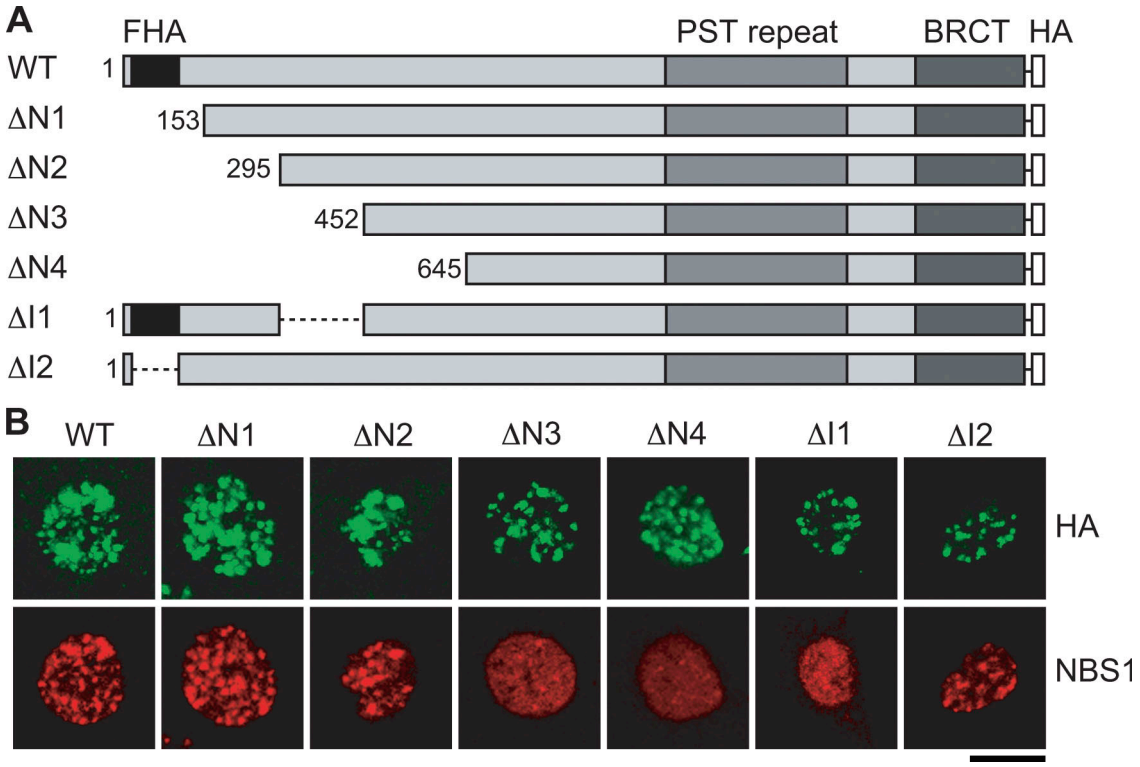

Figure 1. **An acidic domain near the N terminus of MDC1 is essential for NBS1 foci formation.** (A) Schematic representation of HA-tagged full-length mouse MDC1 and various deletion mutants. (B) MDC1$^{-/-}$ MEFs were transiently transfected with wild-type (WT) MDC1 and the individual deletion constructs. 48 h later, cells were irradiated, fixed with methanol, and stained with antibodies specific for HA and mouse NBS1. Nuclear foci were assessed by confocal microscopy. Bar, 10 μm.

the ability of MDC1 to mediate NBS1 foci formation (Fig. 1 B, ΔI1). Preliminary sequence analysis of this region revealed a significant abundance of acidic amino acids as compared with other regions of MDC1 (Fig. 2 A).

Interestingly, deletion of a region between amino acids 51 and 107 of mouse MDC1 (containing the FHA domain) did not have any effect on NBS1 accumulation (Fig. 1, ΔI2) even though overexpression of the FHA domain was reported to trigger a dominant-negative effect on NBS1 foci formation (Goldberg et al., 2003). In summary, these results suggest that an acidic region near the N terminus of MDC1 mediates MRN accumulation at sites of DSBs.

Phosphorylation-dependent interaction between the MDC1 N terminus and the MRN complex

To understand how the region identified by the systematic deletion analysis mediates MRN foci formation, we carefully analyzed the sequence between amino acids 295 and 452 of mouse MDC1. As mentioned in the previous section, this region of MDC1 is highly acidic with a calculated isoelectric point of 4.04. To detect conserved sequence motifs within this region, we compared the mouse sequence to MDC1 sequences from other vertebrate species (including human, dog, swine, and zebrafish). This analysis revealed several conserved patches of 8–10 amino acids interspersed with less conserved regions of variable length (Fig. 2 A; patches are highlighted by horizontal bars). The conserved patches feature a repeated sequence motif: Ser and Thr residues are embedded in an acidic sequence environment. A single amino acid (typically Asp) sits in between the highly conserved Ser and Thr residues. Thus, we hereafter refer to this motif as the SDT motif. The motif also contains two to three consecutive acidic amino acids (usually Glu) that are five residues C terminal to the initial Ser. Mammalian MDC1 contains a total of six SDT motifs (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200709008/DC1): five are located within the region of MDC1, whose deletion abrogates MRN foci (Fig. 2 A), and one is located ∼80 amino acids N terminal to this region (not depicted). Zebrafish MDC1 features a total of eight SDT motifs, and even honey bee MDC1 (the only clear nonvertebrate MDC1 orthologue identified to date) comprises a very similar motif (Fig. S1), indicating that the SDT motif is conserved in all known MDC1 orthologues.

In untreated mammalian cells, MDC1 is a phosphoprotein that becomes rapidly hyperphosphorylated in response to DNA damage in a PIKK-dependent manner (Goldberg et al., 2003; Stewart et al., 2003). A large number of constitutive and DNA damage–induced phosphorylation sites have recently been identified in MDC1 by means of several large-scale mass spectrometry screens of the human and mouse proteome (Beausoleil et al., 2004; Olsen et al., 2006; Matsuoka et al., 2007; Villen et al., 2007). Interestingly, many of the constitutive phosphorylation sites of MDC1 are located within the SDT motifs (Fig. 2 A). In summary, it appears that in human cells, a population of MDC1 molecules is phosphorylated on any Ser and Thr residue in at least four of the six SDT motifs in vivo (Beausoleil et al., 2004; Olsen et al., 2006). In addition, mouse MDC1 appears to be phosphorylated in at least one SDT motif (Villen et al., 2007).

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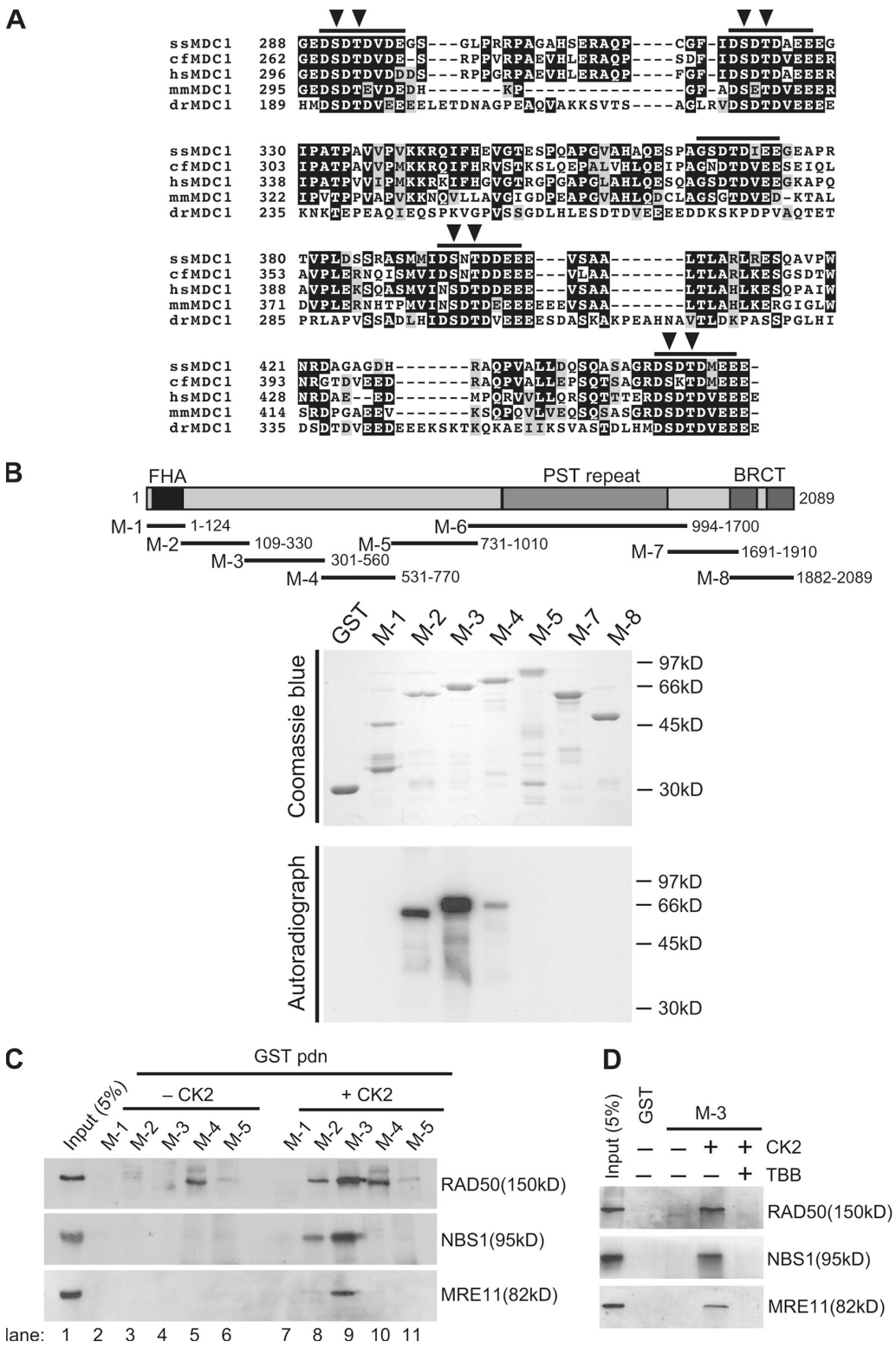

Figure 2. **Phosphorylation-dependent interaction between the MDC1 N terminus and the MRN complex.** (A) Sequence alignment of the region in MDC1 that is essential for MRN foci. The conserved acidic motifs are highlighted by horizontal bars. Phosphorylated residues identified by in vivo phosphorylation site mapping are highlighted by arrowheads (Beausoleil et al., 2004; Olsen et al., 2006; Villen et al., 2007). (B, top) Representation of human MDC1 and the overlapping GST fragments. (bottom) Two fragments at the N terminus of MDC1 are phosphorylated by CK2 in vitro. Purified GST-MDC1 fragments were incubated with purified recombinant CK2 in the presence of radioactive ATP. Proteins were separated by SDS-PAGE, and dried gels were subjected to autoradiography. A Coomassie blue–stained gel of the purified GST fragments is shown on top of the autoradiograph. Note that fragment M-6 (PST repeat region) was not expressed in bacteria. (C) Purified GST-MDC1 fragments (M-1–5) were preincubated with and without recombinant CK2 in the presence of ATP. (D) Purified GST-MDC1 fragment M-3 was preincubated with CK2 either in the presence or absence of the CK2 inhibitor TBB. (C and D) The fragments were then used to pull down proteins from HeLa nuclear extract. Bound proteins were separated on SDS-polyacrylamide gels followed by immunoblotting. The blots were probed with antibodies against RAD50, NBS1, and MRE11.

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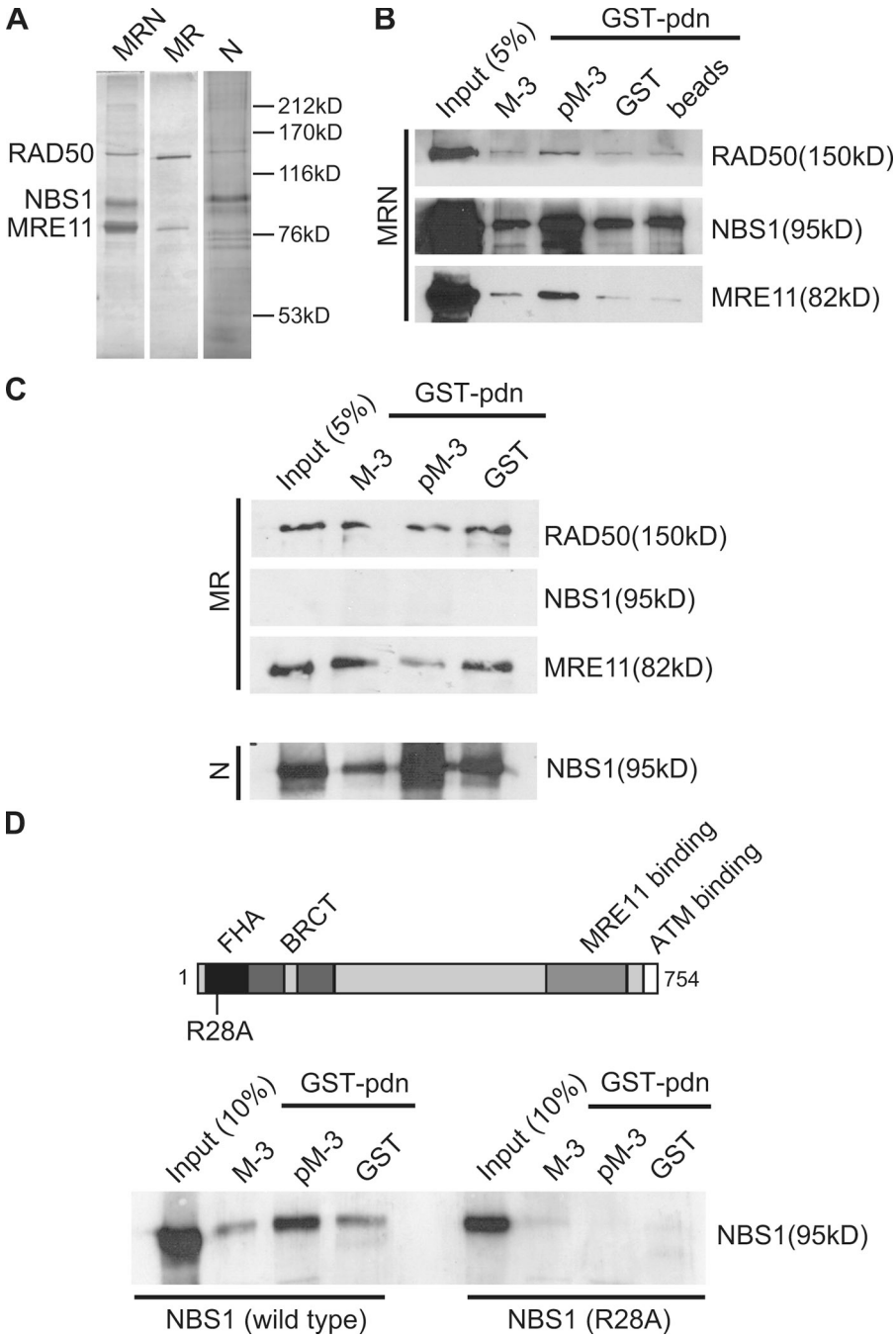

Figure 3. **Direct interaction between the phosphorylated MDC1 N terminus and the MRN complex is mediated by the NBS1 FHA domain.** (A) MRN proteins were purified as described in Materials and methods. Proteins were separated on SDS-polyacrylamide gels and stained with silver. MR, MRE11–RAD50 subcomplex; N, partially purified NBS1. (B and C) Purified GST-MDC1 fragment M-3 comprising part of the SDT region was preincubated with CK2 and ATP. The fragment was incubated with purified MRN complex (B), purified MR subcomplex (C, top), and partially purified NBS1 (C, bottom) followed by GST pull-down analysis. Bound proteins were separated on SDS-polyacrylamide gels followed by immunoblotting. The blots were probed with antibodies against RAD50, NBS1, and MRE11. (D, top) Schematic representation of NBS1 with its functional domains. (bottom) Purified GST-MDC1 fragment M-3 was preincubated with CK2 and ATP. The fragment was incubated with purified MRN complex where the NBS1 subunit was either wild type or contained a point mutation in the FHA domain (R28A). Bound proteins were separated on SDS-polyacrylamide gels followed by immunoblotting. The blots were probed with a polyclonal antibody against NBS1.

The acidic nature of the SDT motif suggests that it may be targeted by acidophilic kinases such as CK1 and 2. Indeed, analysis of the MDC1 SDT domain by Scansite (Yaffe et al., 2001) revealed that the sequence encompassing the Ser and Thr residues within the SDT motifs conform to consensus CK2 phosphorylation sites. To test whether CK2 would specifically phosphorylate MDC1 in the SDT region, we generated eight overlapping random fragments of the human MDC1 cDNA and expressed them in *Escherichia coli* as GST fusion proteins. Seven fragments were expressed well and were purified (Fig. 2 B, top), whereas one fragment (M-6) comprising the MDC1 PST repeat region was not expressed in bacteria. The purified fragments were subjected to an in vitro kinase assay using recombinant CK2. CK2 efficiently phosphorylated fragment M-2 (amino acids 109–330) and M-3 (amino acids 301–560) but none of the other five fragments. Fragment M-2 contains two SDT motifs, and fragment M-3 contains the other four SDT motifs.

MDC1 exists in a complex with MRN in extracts derived from undamaged cells, indicating that these proteins interact constitutively (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200709008/DC1). However, the mechanism of this interaction has not yet been elucidated. Significantly, GST pull-down analysis with our GST-MDC1 fragments revealed that fragment M-2 and M-3 only pulled down significant quantities of the MRN complex from HeLa nuclear extract when preincubated with recombinant CK2 and ATP (Fig. 2 C, compare lanes

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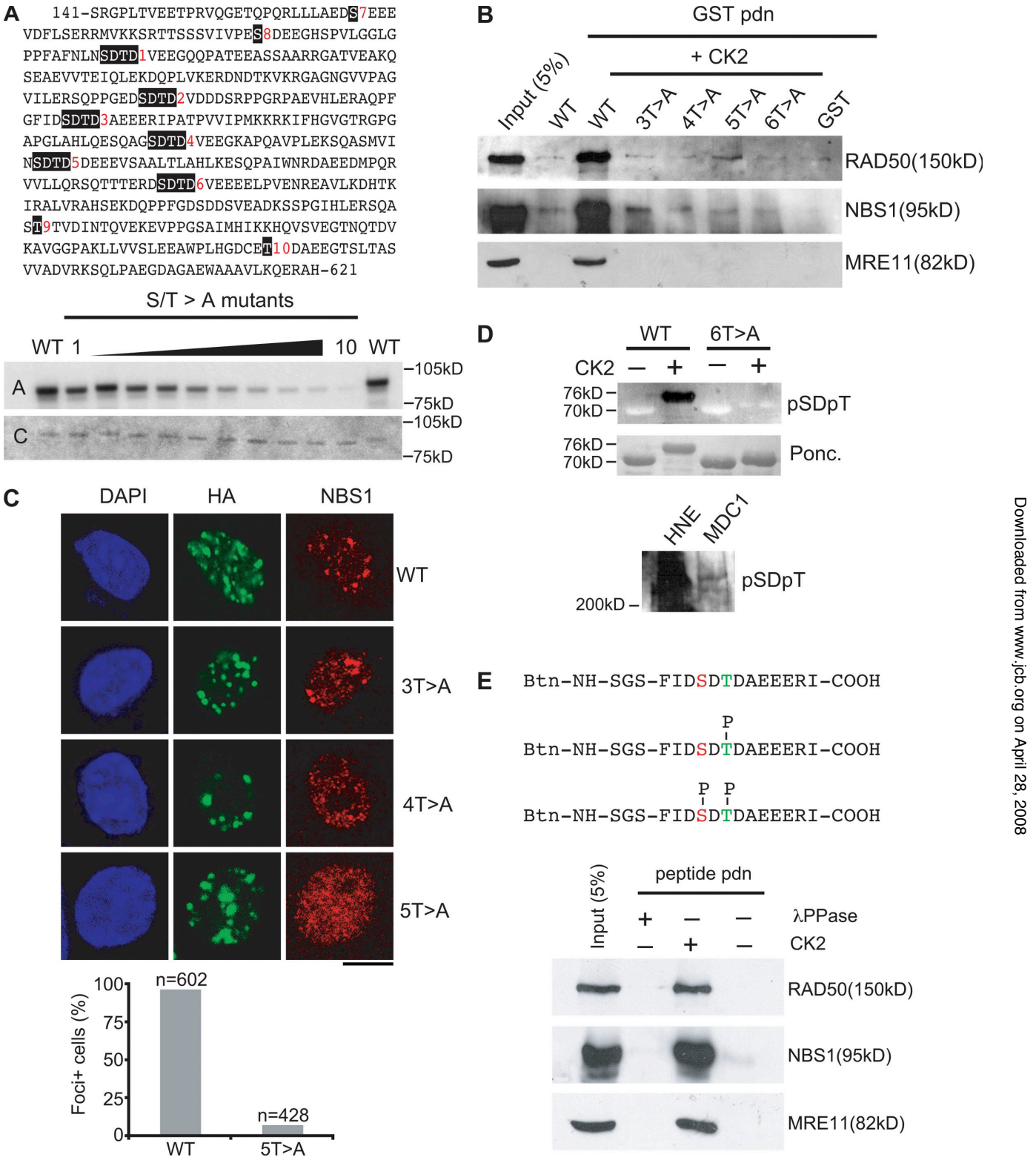

Figure 4. **A repeated phosphorylated motif in MDC1 mediates the interaction between MDC1 and NBS1.** (A) In vitro mapping of the CK2 phosphorylation sites in MDC1. (top) Sequence of the human MDC1 fragment comprising the conserved CK2 consensus sites (highlighted in black). The CK2 consensus sites are numbered from 1 to 10. (bottom) All of the putative CK2 phosphoacceptor Ser and Thr residues in the above MDC1 fragment (amino acids 141–621) were mutated to Ala, and the mutants were expressed as GST fusion proteins in *E. coli*. The GST fragments were incubated with purified recombinant CK2 in the presence of radioactive ATP. Proteins were separated by SDS-PAGE, and dried gels were subjected to autoradiography. A, autoradiograph; C, Coomassie blue–stained gel. (B) The SDT repeats are essential for the interaction between MDC1 and NBS1 in vitro. Several SDT motifs in a fragment derived from mouse MDC1 (amino acids 221–456) were mutated to SDA (3T>A: T362A, T387A, and T444A; 4T>A: T315A, T362A, T387A, and T444A; 5T>A: T300A, T315A, T362A, T387A, and T444A; 6T>A: T222A, T300A, T315A, T362A, T387A, and T444A) and were expressed as GST fusion proteins in *E. coli*. The purified fragments were preincubated with recombinant CK2 in the presence of ATP. The fragments were then used to pull down proteins from HeLa nuclear extract. Bound proteins were separated on SDS-polyacrylamide gels followed by immunoblotting. The blots were

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Table I. **Tryptic MDC1 phosphopeptides**

MDC1 tryptic peptide	CK2 phosphorylation site
162-LLLAEDpSEEEVDFLSER-179	pS168
186-TTSSSVIVPESDEEGHSPVLGGLGPPFAFNLN(pSDpT)DVEEGQQPATEEASSAAR-238	$+2PO_4$
247-QSEAEVVTEIQLEKDQPLVK-266	NA
292-SQPPGEDpSDpTDVDDDSRPPGRPAEVHLERAQPFGFIDpSDpTDAEEERIPATPVVIPMK-348	pS299, pT301, pS329, pT331
361-PGAPGLAHLQESQAGpSDpTDVEEGKAPQAVPLEKSQASMVINpSDpTDDEEEVSAALTLAHLK-420	pS376, pT378, pS402, pT404
445-SQTTTERDpSDpTDVEEEELPVENR-467	pS453, pT455

NA, not applicable.

3 and 4 with lanes 8 and 9; note that the bands in lanes 5 and 10 in the top panel result from a cross-reactivity of the RAD50 antibody with a contaminating bacterial protein in the GST–M-4 fraction). In the absence of CK2, no interaction could be observed under these conditions (Fig. 2 C, lanes 2–6). In the presence of the specific CK2 inhibitor tetrabromo-2-azabenzimidazole (TBB), recombinant CK2 did not transform fragment M-3 into a form capable of pulling down the MRN complex from HeLa nuclear extract, indicating that CK2 activity is required for this process (Fig. 2 D). Together, these data suggest that the phosphorylated N-terminal region in MDC1 mediates the interaction with the MRN complex in vitro.

Direct interaction between the phosphorylated MDC1 N terminus and the MRN complex is mediated by the NBS1 FHA domain

To determine whether the MRN complex interacts directly with the phosphorylated MDC1 N terminus, we coexpressed all three MRN subunits and a subcomplex consisting of MRE11 and RAD50 (MR) in Sf9 cells by means of recombinant baculovirus infection followed by purification of the recombinant proteins to near homogeneity (Fig. 3 A, MRN and MR). We also isolated partially purified NBS1 alone (Fig. 3 A, N). Interaction studies with purified recombinant MRN, MR, and NBS1 were complicated by the fact that these proteins exhibited a significant unspecific binding activity toward the glutathione–Sepharose beads used in this analysis (Fig. 3 B, beads alone). Nevertheless, we consistently observed a significant enrichment of purified MRN and partially purified NBS1 when we used CK2-phosphorylated fragment M-3 in the pull-down assay (Fig. 3, B and C; bottom). Untreated M-3 or GST alone did not result in such enrichment. Similarly, neither phosphorylated nor unphosphorylated M-3 was capable of efficiently binding to MRE11/RAD50 in the absence of NBS1 (Fig. 3 C, top). In summary, these results indicate that the NBS1 subunit of the MRN complex directly associates with the phosphorylated M-3 fragment of MDC1 and mediates the interaction between MDC1 and the MRN complex.

NBS1 features two well-established phosphopeptide recognition modules at its N terminus: an FHA domain and a tandem BRCT domain (Fig. 3 D, top). It was previously shown that introduction of a point mutation in the FHA domain that changes a conserved Arg residue to Ala (R28A) disrupted the interaction between MDC1 and the MRN complex in vitro (Lukas et al., 2004) and abolished MRN accumulation at sites of DSBs in vivo (Cerosaletti and Concannon, 2003; Lee et al., 2003; Horejsi et al., 2004; Lukas et al., 2004). Thus, we isolated the MRN complex harboring the same mutation in NBS1 from baculovirus-infected Sf9 cells and compared its binding activity toward the phosphorylated M-3 fragment to the wild-type complex. Significantly, although the wild-type NBS1 readily interacted with phosphorylated M-3, no interaction was detected with the R28A mutant (Fig. 3 D). This indicates that an intact NBS1 FHA domain is essential for the interaction between the phosphorylated N-terminal region of MDC1 and the MRN complex.

A repeated phosphorylated motif in MDC1 mediates the interaction between MDC1 and NBS1

To map the phosphorylation sites within fragments M-2 and M-3 of MDC1, we isolated a new GST fragment comprising amino acids 141–621 of human MDC1 (M-SDT). The purified fragment was phosphorylated in vitro by recombinant CK2 followed by digestion with trypsin. The tryptic peptides were then analyzed by mass spectrometry. Thus, several phosphorylation sites were mapped (Table I), most notably all six of the conserved SDT motifs that were targeted by CK2 on both Ser and Thr residues. Next, all of the putative CK2 target Ser and Thr residues in the M-SDT fragment were mutated to Ala and expressed in *E. coli* (Fig. 4 A, top; the SDT motifs and other putative CK2 target sites are highlighted in black and numbered from 1–10). The purified mutants were subjected to in vitro phosphorylation by re-

probed with antibodies against RAD50, NBS1, and MRE11. (C) Mutation of a subset of the conserved SDT motifs abrogates NBS1 foci formation. (top) $MDC1^{-/-}$ MEFs were transiently transfected with various forms of HA-tagged full-length mouse MDC1, including wild-type (WT) and SDT-deficient (3T>A: T362A, T387A, and T444A; 4T>A: T315A, T362A, T387A, and T444A; 5T>A: T300A, T315A, T362A, T387A, and T444A) variants. 48 h later, cells were irradiated, fixed with methanol, and stained with antibodies against HA and mouse NBS1. (bottom) HA-positive cells were scored for NBS1 foci in wild-type and 5T>A-transfected populations (results were consistent in two independent datasets). (D) The SDT in MDC1 is phosphorylated in vivo. (top) A phosphospecific antibody raised against a doubly phosphorylated SDT peptide (pSDpT) was tested on CK2-phosphorylated GST-SDT. WT, mouse MDC1 (amino acids 221–456); 6T>A, all six SDT motifs were mutated to SDA. (bottom) Immunoblot analysis of phosphorylated MDC1 isolated from HeLa nuclear extract by a H2AX phosphopeptide using the pSDpT phosphospecific antibody. (E) A synthetic biotinylated peptide comprised of the SDT sequence motif and phosphorylated on the Thr residue was either left untreated or was preincubated with λ−PPase and CK2, respectively. (top) This leads to the three indicated products: unphosphorylated peptide, singly phosphorylated peptide (Thr), and doubly phosphorylated peptide (Ser and Thr). The peptides were then used to pull down proteins from HeLa nuclear extract. Proteins were detected as in B. Bar, 10 μm.

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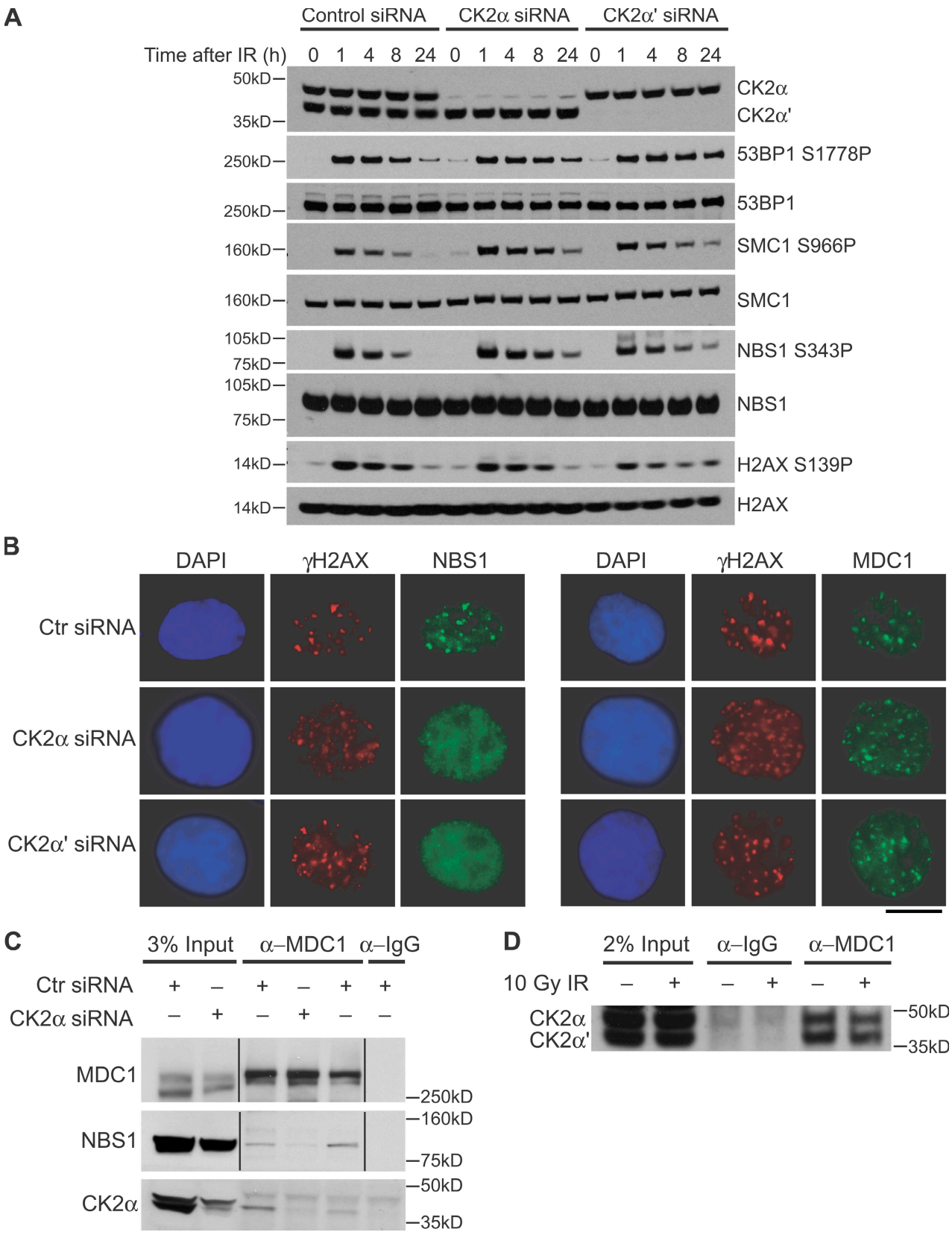

Figure 5. **CK2 is essential for the interaction between MDC1 and NBS1 and for the accumulation of NBS1 at sites of DSBs in vivo.** (A) Down-regulation of CK2α and CK2α′ by siRNA triggers a prolonged DDR. 72 h after transfection with siRNA duplexes, cells were irradiated and harvested at the indicated time points. Extracts were prepared and resolved by SDS-PAGE followed by immunoblotting. The blots were probed with the indicated antibodies. (B) Down-regulation of CK2α and CK2α′ by siRNA abrogates NBS1 accumulation at sites of DSBs. 72 h after transfection with siRNA duplexes, cells were irradiated, fixed with methanol, and stained with antibodies against γ-H2AX and NBS1. (C) Down-regulation of CK2α by siRNA disrupts the interaction between MDC1 and NBS1 in vivo. 72 h after transfection with siRNA duplexes, cells were lysed, and immunoprecipitation was performed using the

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combinant CK2, and radioactive phosphate incorporation was measured by autoradiography (Fig. 4 A). This analysis revealed that all of the SDT motifs and other putative CK2 target sites were efficiently phosphorylated in vitro.

To test whether the highly conserved SDT repeats were necessary for the interaction with the MRN complex, we changed the conserved Thr residues to Ala within several SDT motifs and phosphorylated the isolated GST fusion proteins by CK2 in vitro. Significantly, GST pull-down analysis with the mutant fragments revealed that only the phosphorylated wild type was capable of pulling down significant quantities of the MRN complex from HeLa nuclear extract, whereas alteration of the three C-terminal SDT motifs (Fig. 4 A, 4–6) already reduced the interaction to almost background level (Fig. 4 B).

To test whether the SDT repeats of MDC1 mediate NBS1 accumulation at sites of DSBs in vivo, we introduced the same SDT mutations as before (Fig. 4 B) in the tagged full-length mouse MDC1 cDNA followed by transient transfection of MDC1$^{-/-}$ MEFs with those mutants and assessment of MDC1 and NBS1 foci formation by indirect immunofluorescence. Surprisingly, alteration of the last three C-terminal SDT motifs (Fig. 4 A, 4–6) did not trigger any significant reduction in NBS1 foci formation (Fig. 4 C, 3T>A) even though the corresponding GST fusion protein only weakly interacted with the MRN complex in vitro (Fig. 4 B, 3T>A). However, mutation of four SDT motifs (Fig. 4 A, 3–6) led to a detectable reduction in NBS1 accumulation even though small NBS1 foci were still detectable in a subset of the transfected cells (Fig. 4 C, 4T>A). Altering five of the six SDT motifs (Fig. 4 A, 2–6) completely abrogated NBS1 foci formation in all MDC1-positive cells (Fig. 4 C, 5T>A). Significantly, although the 5T>A mutant was unable to mediate NBS1 focal accumulation in response to IR, it still localizes to foci itself, indicating that an intact SDT region is not required for MDC1–γ-H2AX interaction.

Collectively, these results show that the SDT motifs are essential for the interaction between MDC1 and the MRN complex in vitro and for NBS1 accumulation at sites of DSBs in vivo, suggesting that the SDT motif may define a novel phospho-specific MRN-interacting element within MDC1. To test this directly, we first sought to ascertain that the SDT motifs of MDC1 are constitutively phosphorylated in vivo. To this end, we generated two phosphospecific antibodies against doubly phosphorylated SDT peptides (pSDpTs) derived from the human SDT repeat region (see Materials and methods for details). These antibodies were affinity purified and tested against the purified CK2-phosphorylated GST-SDT fragment and its 6T>A mutated derivative. Although one of the two antibodies did not recognize the CK2-phosphorylated GST-SDT fragment (not depicted), the other antibody specifically recognized GST-SDT only when it was preincubated with CK2 and ATP (Fig. 4 D, top). Significantly, the 6T>A mutant was not recognized at all by this antibody, indicating that it is specific for doubly phosphorylated SDT repeats. We next used this antibody to detect MDC1 in extracts from undamaged and irradiated mammalian cells. Unfortunately, the pSDpT antibody unspecifically cross-reacted with many proteins in whole cell and nuclear extracts, and, thus, we were unable to assess the MDC1 phosphorylation status in crude cell extracts (Fig. 4 D, bottom; first lane). However, MDC1 can be isolated along with the MRN complex from HeLa nuclear extract to near homogeneity by a phosphopeptide pull-down strategy using a phosphopeptide derived from the H2AX C terminus (Fig. S2; Stucki et al., 2005). When we probed the isolated MDC1–MRN complex with the pSDpT antibody, we observed two bands at the position where MDC1 runs on SDS gels, indicating that it is indeed phosphorylated on at least a subset of the SDT motifs in vivo (Fig. 4 D, bottom; second lane).

To clarify whether the MRN complex interacts with a single doubly phosphorylated SDT motif, we designed a biotinylated synthetic phosphopeptide comprising the sequence of one of the SDT motifs (Fig. 4 A, 3), which was phosphorylated on the Thr residue. This peptide was either left untreated or was treated with λ phosphatase and CK2. Mass spectrometry and in vitro CK2 assays revealed that such treatment resulted in singly phosphorylated SDpT peptide, unphosphorylated SDT peptide, and doubly phosphorylated pSDpT peptide (Fig. 4 E, top; and not depicted). Significantly, only the doubly phosphorylated peptide, in which both Ser and Thr residues are being phosphorylated, retrieved the MRN complex from HeLa nuclear extract (Fig. 4 E). Collectively, these results suggest that doubly phosphorylated SDT motifs interact with MRN and determine MRN accumulation in vivo.

CK2 is essential for the interaction between MDC1 and NBS1 and for the accumulation of NBS1 at sites of DSBs in vivo

Because the SDT repeats are efficiently targeted by CK2 in vitro (Fig. 4), we next sought to investigate whether CK2 activity was required for NBS1 accumulation at sites of DSBs in vivo. Several commercial small-molecule CK2 inhibitors are available, and two of them were tested on human and mouse cells (TBB and DMAT). Surprisingly, neither of the two inhibitors triggered a detectable reduction in NBS1 foci formation in cultured human and mouse cells (unpublished data). Thus, we speculated that there may either exist additional acidophilic kinases that are capable of efficiently phosphorylating the SDT repeats in MDC1 or that the inhibitors are not potent enough to completely eliminate SDT phosphorylation in our experimental settings.

To test the latter possibility, we took an siRNA approach to down-regulate the two catalytic subunits of CK2 (CK2α and CK2α′) in U2OS cells. 72 h after siRNA transfection, CK2α and CK2α′ expression reached background levels (Fig. 5 A). At the same time, we observed massive cell death and severe mitotic defects, corroborating the essential role of CK2 in the cellular metabolism and life cycle. Interestingly, we also

indicated antibodies. Proteins were separated by SDS-PAGE followed by immunoblotting. The blots were probed with antibodies against MDC1, NBS1, and CK2α. Black lines indicate that intervening lanes have been spliced out. (D) MDC1 is associated with CK2 in vivo. HeLa cell extracts were used to immunoprecipitate proteins with the indicated antibodies. The immunocomplexes were separated by SDS-PAGE followed by immunoblotting. The blot was probed with antibodies against CK2α and CK2α′. Bar, 10 μm.

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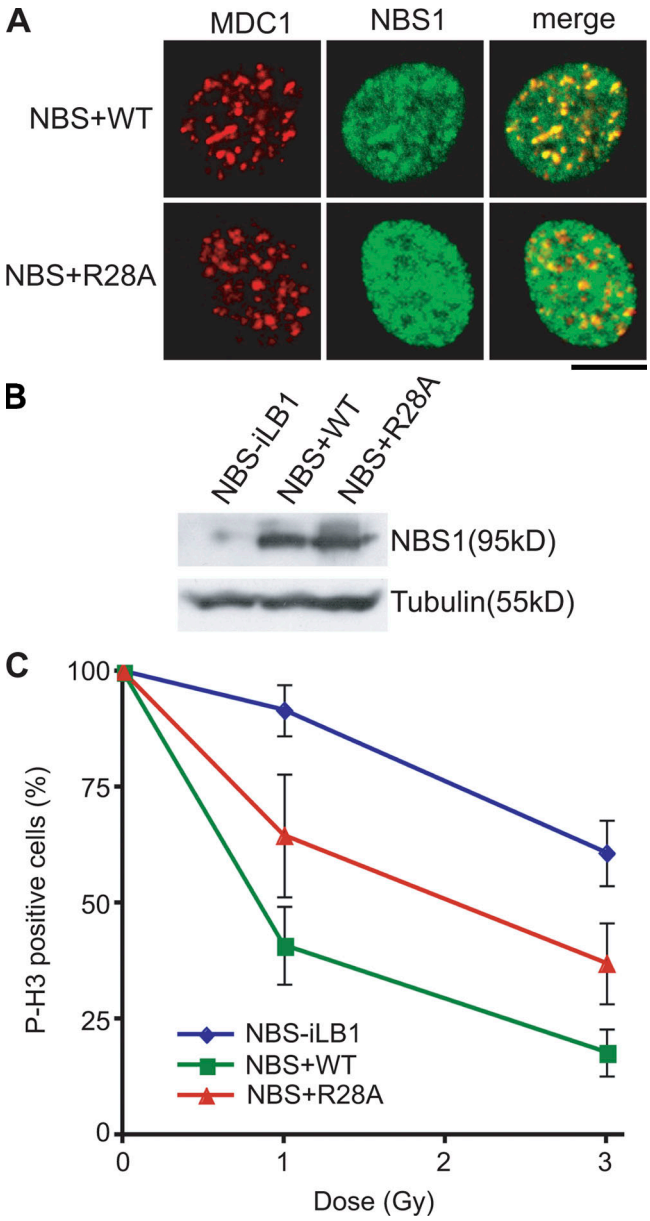

Figure 6. **Disruption of the MDC1–NBS1 interaction triggers a partial G2/M checkpoint defect.** (A) NBS-iLB1 fibroblasts and NBS-iLB1 fibroblasts stably transduced with wild-type NBS1 and R28A mutant NBS1 were irradiated, fixed with methanol, and stained with antibodies against MDC1 and NBS1. (B) Whole cell extracts of NBS-iLB1 fibroblasts and NBS-iLB1 fibroblasts stably transduced with wild-type (WT) NBS1 and R28A mutant NBS1 were resolved by SDS-PAGE followed by immunoblotting. The blot was probed with antibodies against NBS1 and tubulin. (C) NBS-iLB1 fibroblasts and NBS-iLB1 fibroblasts stably transduced with wild-type NBS1 and R28A mutant NBS1 were left untreated or irradiated with 1 Gy and 3 Gy, respectively. Cells were harvested 1 h after irradiation, fixed with methanol, and stained with an antibody against phosphorylated H3 (P-H3) and propidium iodine. The percentage of phosphorylated H3–positive cells was determined by FACS analysis. Error bars represent SD. Bar, 10 μm.

observed a prolonged phosphorylation of ATM substrates in response to IR (Fig. 5 A), suggesting that down-regulation of CK2 by siRNA may cause a repair defect.

Significantly, down-regulation of either CK2α or CK2α′ led to a marked defect in NBS1 foci formation in response to IR (Fig. 5 B, left). At the same time, MDC1 accumulation at sites of DSBs was not significantly reduced (Fig. 5 B, right) even though the foci appeared smaller as compared with the foci in control siRNA-treated cells. This indicates that CK2 is essential for the accumulation of NBS1 at sites of DSBs, whereas it is dispensable for the interaction between MDC1 and γ-H2AX.

Next, we tested whether CK2 was required for the constitutive interaction between MDC1 and NBS1. To this end, MDC1 antibodies were used to coimmunoprecipitate NBS1 from extracts derived from siRNA-treated human cells. No NBS1 coimmunoprecipitated with MDC1 from cells that were transfected with CK2α siRNA, whereas a small but significant amount of NBS1 coimmunoprecipitated with MDC1 from cell extracts that were prepared from control siRNA-transfected cells (Fig. 5 C). The membrane was also probed with an antibody against CK2α to monitor the efficiency of CK2α depletion (Fig. 5 C, bottom; note that CK2α corresponds to the faster migrating band). Interestingly, we noticed that CK2α also coimmunoprecipitated with MDC1 (Fig. 5 C, bottom; third and fifth lanes), indicating that CK2 may exist in a complex with MDC1 in vivo. Indeed, antibodies raised against MDC1 efficiently coimmunoprecipitated both CK2α and CK2α′ from HeLa cell extracts (Fig. 5 D). Notably, the interaction does not change upon the introduction of DNA damage by IR (Fig. 5 D, compare the last two lanes), indicating that it is constitutive and not induced by DNA damage.

Collectively, these data reveal that CK2 is essential for NBS1 foci formation and for the interaction between MDC1 and NBS1. In addition, CK2 exists in a complex with MDC1 and, thus, may be the primary kinase to target the SDT repeats in vivo.

Disruption of the MDC1–NBS1 interaction triggers a partial G2/M checkpoint defect

As shown in Fig. 3 D, the R28A mutation in the NBS1 FHA domain triggers a severe defect in the association of NBS1 with the CK2-phosphorylated MDC1 SDT region in vitro (Fig. 3 D). Consistent with previous studies (Cerosaletti and Concannon, 2003; Lee et al., 2003; Horejsi et al., 2004), we also observed that this mutant is unable to accumulate in foci in stably transduced NBS fibroblasts, whereas MDC1 accumulation is not affected (Fig. 6 A). It was previously shown that the R28A mutant was not capable of rescuing the radiation sensitivity phenotype of NBS cells, whereas it did fully rescue the intra–S-phase checkpoint defect, at least at higher doses of irradiation (Lee et al., 2003). In contrast, primary B cells derived from a humanized mouse model in which another key amino acid at the phosphopeptide recognition interface of the NBS1 FHA domain had been mutated to Ala (H45A) showed partial G2/M and intra–S-phase checkpoint defects specifically at lower doses of irradiation (Difilippantonio et al., 2007). To test whether the R28A mutation causes a similar checkpoint defect, we measured alterations in the mitotic index in response to low (sublethal) doses of irradiation (1–3 Gy) in NBS fibroblasts stably transduced with full-length wild-type and R28A NBS1. Consistent with previous findings (Falck et al., 2005), NBS-iLB1 fibroblasts displayed a clear G2/M checkpoint defect in this dose range (Fig. 6 C). Stable transduction with wild-type NBS1 fully rescued the G2/M checkpoint arrest in response to 1–3 Gy of IR. However, stable

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transduction with R28A mutant NBS1 only partially restored the G2/M checkpoint, which is similar to the situation in the mouse B cells expressing the H45A mutant (Fig. 6 C; Difilippantonio et al., 2007). Notably, this checkpoint defect was not caused by lower expression levels of the mutant transgene as compared with the wild type (Fig. 6 B). Collectively, these results suggest that the constitutive CK2-dependent association of the MRN complex with MDC1 plays an important role in eliciting a full cell cycle checkpoint arrest.

Discussion

Previously, we and others have shown that MDC1 mediates accumulation of the MRN complex at sites of DSBs (Goldberg et al., 2003; Stewart et al., 2003; Lukas et al., 2004; Stucki et al., 2005). We also presented evidence that MDC1 exists in a complex with MRN in extracts from undamaged cells and that an intact NBS1 FHA domain is essential for the stability of this interaction and for efficient retention of the MRN complex in γ-H2AX–containing damaged chromatin regions (Goldberg et al., 2003; Lukas et al., 2004; Stucki et al., 2005). However, the precise mechanism by which MDC1 regulates the MRN complex remained unknown.

In this study, we identify a region in MDC1 that is essential for efficient accumulation of the MRN complex at sites of DSBs. This region of MDC1 features a repeated acidic sequence motif (the SDT motif), and our data, in combination with the accompanying study by Melander et al. (see p. 213 of this issue) and several recently published large-scale phosphorylation site screens of the human and mouse proteome (Beausoleil et al., 2004; Olsen et al., 2006; Villen et al., 2007), suggest that at least a subset of the SDT motifs in MDC1 are constitutively phosphorylated by the acidophilic kinase CK2 on highly conserved Ser and Thr residues. Furthermore, we present unexpected evidence that the doubly phosphorylated SDT motifs regulate accumulation and retention of the MRN complex in the DSB-flanking chromatin compartment via a mechanism that involves direct interaction with the NBS1 N-terminal FHA domain. Finally, we show that CK2 is essential for NBS1 accumulation in damaged chromatin and that depletion of CK2 disrupts the MDC1–MRN complex in vivo. Thus, our data successfully integrate and explain two observations whose interrelation has previously not been appreciated: first, we provide a mechanistic explanation as to why MDC1 and the MRN complex exist in a complex even before DNA damage; and second, our findings also put into perspective the previous observation that an intact FHA domain of NBS1 is critical for efficient accumulation of the MRN complex at sites of DSBs (Kobayashi et al., 2002; Zhao et al., 2002; Cerosaletti and Concannon, 2003; Lee et al., 2003; Horejsi et al., 2004; Lukas et al., 2004) and for an intact DDR in living organisms (Difilippantonio et al., 2005, 2007).

However, it is essential to appreciate that not all MRN functions seem to require MDC1. For instance, DNA end processing activities of MRN do not appear to be dependent on MDC1 (Jazayeri et al., 2006). Furthermore, there are no indications that MRN's role in mediating ATM activation would require MDC1 (Lee and Paull, 2005). Finally, MRN seems to occupy two distinct compartments at sites of DSBs: it accumulates on single-stranded DNA regions generated by enzymatic resection of DSBs (Jazayeri et al., 2006) as well as in large chromatin domains flanking DSBs that often span several thousand base pairs (Bekker-Jensen et al., 2006). Although the accumulation of MRN on single-stranded DNA does not require MDC1 (Bekker-Jensen et al., 2006), accumulation and retention of MRN in γ-H2AX–containing chromatin are critically dependent on MDC1 (Goldberg et al., 2003; Stewart et al., 2003; Lukas et al., 2004; Stucki et al., 2005) and on CK2-dependent phosphorylation of its SDT repeats (Melander et al., 2008; this study).

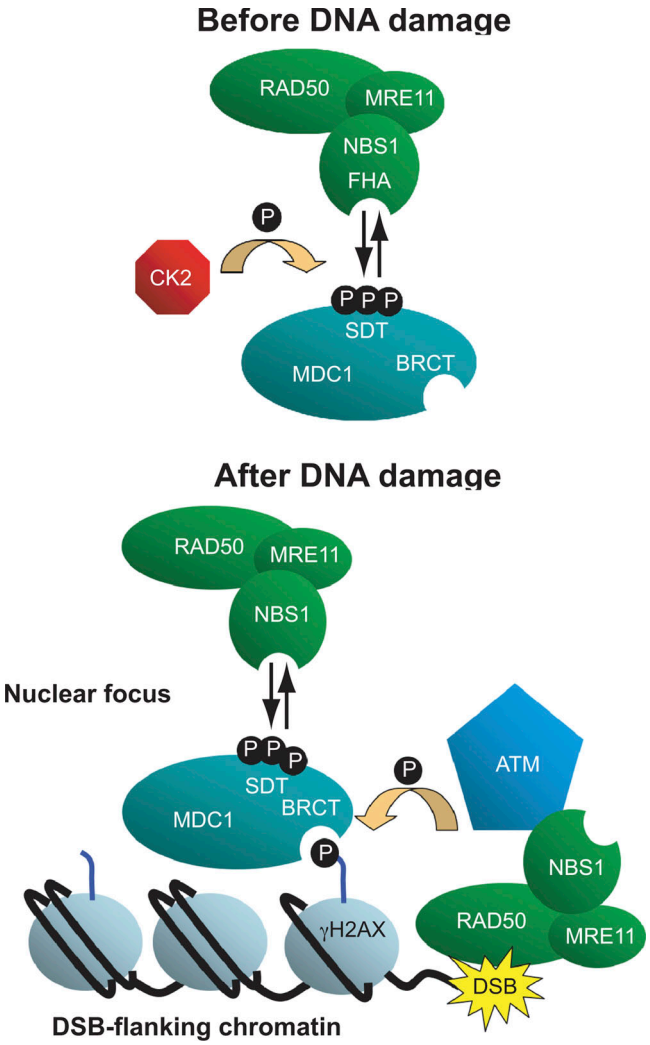

Figure 7. **Model of the mechanism of the MDC1–MRN interaction before and after DNA damage.** See Discussion for details.

Based on these novel findings and on previously published observations, we propose the following model of the events that occur before and after a cell has suffered genotoxic stress that generates DSBs (Fig. 7): in the absence of DNA damage, MDC1 is phosphorylated on multiple sites by CK2 and perhaps other constitutively active kinases. The MRN complex associates with MDC1 through direct interaction between the N-terminal FHA domain of the NBS1 subunit and the CK2-phosphorylated SDT repeat region of MDC1. Upon induction of DSBs in the genome, a fraction of MRN (that is probably not associated with MDC1) is rapidly deployed to the free DNA ends. Once bound to the

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DNA, MRN participates in a multitude of events that include DNA end processing and tethering of DNA molecules that may facilitate accurate repair as well as activation of the ATM signaling cascade (for review see Williams et al., 2007). Although the precise mechanisms of these processes are still not understood in detail, there is no experimental indication that the MRN complex would require MDC1 for these functions. However, in a second step, after the ATM signaling cascade has been initiated, the MDC1-bound fraction of MRN enters the stage; once activated, ATM phosphorylates a vast variety of targets. Among these targets are H2AX molecules in nucleosomes that are located close to the break site. Phosphorylated H2AX is recognized by MDC1 through its C-terminal BRCT domains (Stucki et al., 2005). MDC1, along with the MDC1-bound fraction of MRN, forms a tight complex with phosphorylated H2AX, thus recruiting more MRN to the chromatin compartments flanking DSBs (Stucki et al., 2005; Lou et al., 2006). This process is manifested by the formation of microscopically discernible nuclear foci containing both MDC1 and the MRN complex.

Although such a model of MDC1–MRN interplay is intriguing, there are several outstanding issues that need to be discussed. It is clear that the interaction between MDC1 and the MRN complex is constitutive, but it is still dependent on the phosphorylation of MDC1 by CK2. A very similar mechanism has recently been described in two different aspects of the mammalian DDR, namely single-strand DNA break repair and DSB repair by nonhomologous end joining (Koch et al., 2004; Loizou et al., 2004). CK2 phosphorylates XRCC1, an adaptor protein that recruits several repair factors to single-stranded break sites (Loizou et al., 2004). In this case, the CK2 phosphorylation mark creates a binding site for the FHA domain–containing proteins polynucleotide kinase (PNK), aparataxin, and Xip1 (Loizou et al., 2004; Luo et al., 2004; Bekker-Jensen et al., 2007). In addition, PNK recruitment to sites of DSBs by XRCC4 is also mediated by the CK2-dependent phosphorylation of XRCC4 (Koch et al., 2004). Thus, the mechanisms of PNK, aparataxin, and Xip1 recruitment by XRCC1 and PNK recruitment by XRCC4 closely resemble MRN chromatin retention by MDC1. However, although the recruitment of PNK by XRCC1 was abolished by the CK2 inhibitor TBB (Loizou et al., 2004), we were unable to abolish NBS1 recruitment to damaged chromatin by using the same inhibitor and other (even more potent) small-molecule CK2 inhibitors. One possible explanation for this discrepancy may lie in the existence of one or more CK2-related acidophilic kinases that are capable of targeting the MDC1 SDT repeats. However, the fact that down-regulation of CK2 by siRNA completely abolishes NBS1 recruitment to damaged chromatin argues against this possibility but rather suggests that the CK2 inhibitors were not potent enough to reduce CK2 activity sufficiently to produce an effectual reduction in MDC1 phosphorylation. Indeed, in vivo phosphorylation analysis of MDC1 by Melander et al. (2008) reveals that treatment of cells with CK2 inhibitors maximally reduced SDT phosphorylation levels to 50%. This may not be enough for a visually discernible defect in NBS1 foci formation.

With the discovery of the mechanism by which MDC1 mediates accumulation and retention of the MRN complex in γ-H2AX–containing damaged chromatin regions, an important step in the hierarchy of events that lead to the formation of nuclear foci at sites of DSBs has been resolved. Although we do not yet understand the function of MRN in the DSB-flanking chromatin compartment, one intriguing possibility is that MDC1-bound MRN may act as a mediator of downstream phosphorylation events of the DDR. In this case, the MDC1–MRN complex may enhance the DSB-induced signal by means of a positive feedback loop: MDC1–MRN accumulates as the γ-H2AX mark spreads into more distal chromatin regions, thus helping to trigger a global DDR even in the presence of very low numbers of DSBs. Consistent with such a scenario is the observation that cells expressing NBS1 with a mutated FHA domain display a partial G2/M checkpoint defect at low doses of irradiation (Difilippantonio et al., 2007; this study), whereas the checkpoint seems to be normal at higher doses (Difilippantonio et al., 2007). This suggests that the prevalent role of the MDC1–MRN complex in checkpoint activation may not constitute the initiation of the signal but rather its amplification. The discovery of the SDT region of MDC1 and its specific role in MRN localization in response to DNA damage will greatly facilitate the investigation of functional aspects of MRN in the DSB-flanking chromatin compartment.

Materials and methods

Cell culture and gene transfer

MDC1$^{-/-}$ and MDC1$^{+/+}$ MEFs were gifts from J. Chen (Yale University, New Haven, CT). NBS-iLB1 cells stably transduced with wild-type NBS1 and R28A mutant NBS1 were gifts from S. Jackson (University of Cambridge, Cambridge, UK) and K. Cerosaletti (University of Washington, Seattle, WA), respectively. MEFs, HeLa, U2OS, and NBS-iLB1 cells were cultured in DME (Invitrogen) supplemented with 10% FCS. Transfection of plasmids was performed using either Lipofectamine 2000 (Invitrogen) or calcium phosphate. Sf9 cells were cultured in Grace's insect medium (Invitrogen) supplemented with 10% FCS. Recombinant MRE11, RAD50, and NBS1 baculoviruses were gifts from V. Bohr (National Institute on Aging, Baltimore, MD). The Bac-To-Bac Baculovirus Expression System (Invitrogen) was used to generate and amplify recombinant baculoviruses. All steps were performed according to the manufacturer's protocols. CK2 inhibitors TBB and DMAT were purchased from EMD. Irradiation of MEFs was performed in an x-ray cabinet (Faxitron) at 5–10 Gy/min.

Plasmids

The full-length mouse MDC1 cDNA was a gift from A. Nussenzweig (National Institutes of Health, Bethesda, MD) and was HA tagged at the C terminus by PCR and cloned into pcDNA3.1(+) mammalian expression vector (Invitrogen). Human MDC1-GST constructs were generated by PCR amplification of the human MDC1 cDNA followed by cloning into pGEX4T3 bacterial expression vector (GE Healthcare). Myc-NBS1 (Falck et al., 2005) was subcloned into pFastBac transfer vector (Invitrogen) to generate recombinant NBS1 baculoviruses. Deletion mutants were generated by a standard PCR-based method, and point mutations were introduced using the QuikChange Site-Directed Mutagenesis kit (Stratagene).

siRNA and transfections

The siRNA duplexes were 21 bp with a two-base deoxynucleotide overhang (Dharmacon Research). The sequences of the CK2α and CK2α′ siRNA oligonucleotides used were GAUGACUACCAGCUGGUUCdTdT and CAGUCUGAGGAGCCGCGAGdTdT, respectively. The control siRNA used was CGUACGCGGAAUACUUCGAdTdT. Cells were transfected with siRNA duplexes by using Oligofectamine (Invitrogen) according to the manufacturer's instructions. Cells were routinely harvested 72 h after siRNA transfection.

Cell extraction and protein purification

HeLa nuclear extract was purchased from Cilbiotech. Cell extraction for immunoblot analysis was described previously (Stewart et al., 2003).

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MDC1-GST fragments were affinity purified on glutathione–Sepharose beads (GE Healthcare). Sf9 cells expressing recombinant MRN, MR, and N were lysed by sonication in buffer A (50 mM sodium phosphate, pH 7.0, 0.3 M NaCl, 10% glycerol, and 0.5 mM PMSF) containing 20 mM imidazol followed by centrifugation. The supernatants were loaded on HiTrap chelating (Ni^{2+}) columns (GE Healthcare) equilibrated with buffer A. The columns were washed with 50 ml buffer A/20 mM imidazol and with 50 ml buffer A/50 mM imidazol. Proteins were eluted with a 50-ml linear concentration gradient of 50–350 mM imidazole in buffer A. MRN-containing fractions were pooled and either used directly for analysis after dialysis against buffer B (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, and 1 mM DTT) or loaded on 1-ml HiTrapQ columns (GE Healthcare) for further purification. The HiTrapQ columns were eluted with a 10-ml linear concentration gradient of 50–500 mM NaCl.

Antibodies and immunological techniques

Mouse monoclonal HA antibodies were purchased from Covance Research Products. The anti–mouse NBS1 antibody was a gift from A. Nussenzweig. The antibodies used against human Nbs1 were obtained from Genetex, Novus, and Millipore. Antibodies against phospho-Nbs1 Ser-343 and anti-phospho-H2AX were obtained from Genetex and Millipore, respectively. Anti-SMC1, phospho-SMC1 Ser-966, and H2AX antibodies were purchased from Bethyl, and the antiphospho-53BP1 and anti-53BP1 antibodies were obtained from Cell Signaling Technology. Sheep polyclonal antibodies against human MDC1, MRE11, and RAD50 have been described previously (Goldberg et al., 2003). Rabbit polyclonal antibodies to MDC1 have been described previously (Stewart et al., 2003). The anti-CK2α and -CK2α′ antibodies were purchased from Santa Cruz Biotechnology, Inc. Phosphospecific MDC1 antibodies were raised in rabbits to the MDC1 phosphopeptides GFIDS(P)DT(P)DA and TERDS(P)DT(P)DV and were affinity purified using the phosphorylated and nonphosphorylated peptides (Eurogentec).

For immunoprecipitation, HeLa cells were lysed for 30 min in NETN lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 mM $MgCl_2$, 1% NP-40 supplemented with protease inhibitors [Roche], and benzonase [EMD]). The clarified extract was precleared with the appropriate IgG (Dako) and protein A or G beads (GE Healthcare) for 1 h at 4°C. 5 µg of immunoprecipitating antibody was added with protein A or G beads to the precleared supernatant and incubated for 3 h at 4°C. The immunocomplexes were washed four times in NETN lysis buffer (containing 0.5% NP-40), boiled in SDS sample buffer, and loaded on an SDS-polyacrylamide gel. Proteins were analyzed by immunoblotting using standard methods.

For immunofluorescence staining, cells were grown on glass coverslips, fixed in ice-cold methanol, and stained with the indicated antibodies for 1 h at room temperature. Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (FITC and rhodamine) and Invitrogen (AlexaFluor488 and -596). Images were captured at room temperature on a confocal microscope (SP2; Leica) with a 40× NA 1.25 oil immersion objective (Leica; Figs. 1, 4, and 6) and on a microscope (Eclipse E600; Nikon) with a 60× oil immersion objective (Nikon; Fig. 5).

Biochemical analysis

For GST pull-down assays, purified 5 µg GST fusion proteins were mixed with 200 µg HeLa nuclear extract or with 5 µg of purified MRN. Where indicated, GST fusion proteins were pretreated with 100 U CK2 (New England BioLabs, Inc.). The mixture was incubated at 4°C for 30 min to allow binding. Glutathione–Sepharose beads were then added, and the suspension was incubated at 4°C for a further 60 min. The beads were washed three times with wash buffer (50 mM Tris, pH 7.5, 120 mM NaCl, 1 mM DTT, and 0.2% NP-40), resuspended in SDS loading buffer, and analyzed by SDS-PAGE and immunoblotting. For peptide pull-down analysis, the biotinylated synthetic peptide Btn-NH-SGSFIDSD[pT]DAEEERI-COOH (Eurogentec) was used. Where indicated, 25 nmol of the peptide was incubated with 500 U of recombinant CK2 (New England Biolabs, Inc.) at 30°C for 45 min and with 100 U λ phosphatase (New England Biolabs, Inc.) at 30°C for 20 min. Peptide pull-down analysis was performed as described previously (Stucki et al., 2005).

CK2 in vitro kinase assays for phosphorylation site mapping was performed by adding 100 ng of recombinant CK2α (Millipore) to 1 µg GST-MDC1 (amino acids 141–621) in CK2 kinase buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 37.5 mM $MgCl_2$, 1 mM DTT, 100 µM ATP, and 10 µCi γ-[^{32}P]ATP) and incubating for 10 min at 30°C. Kinase reactions were inactivated by boiling in SDS sample buffer and were run on an SDS-polyacrylamide gel. Gels were stained with Coomassie blue and either dried and subjected to autoradiography, or the GST-MDC1 band was excised and processed for mass spectrometric phospho–amino acid analysis (Jowsey et al., 2007). Mutation of putative CK2 phosphorylation sites in the GST-MDC1 fragment was performed using site-directed mutagenesis (Stratagene).

Checkpoint analysis

G2/M checkpoint analysis was performed as described previously (Falck et al., 2005). In brief, cells were irradiated with the indicated doses during the exponential growth phase. 1 h later, cells were harvested, fixed with 70% ethanol/PBS, and incubated overnight at −20°C. Fixed cells were washed with PBS and permeabilized with 0.25% Triton X-100/PBS on ice for 10 min. Cells were stained with an antiphosphohistone H3 antibody (Millipore) and propidium iodide. Data were acquired with a flow cytometer (FC500; Becton Dickinson).

Phosphorylation site analysis by mass spectrometry

MDC1 samples that had been incubated with CK2 with or without ATP were subjected to electrophoresis on a 4–12% polyacrylamide gel that was stained with colloidal Coomassie blue. MDC1 bands were excised from the gel, washed with 0.1% NH_4HCO_3 and 50% acetonitrile/50 mM NH_4HCO_3, reduced with 10 mM DTT in 0.1 M NH_4HCO_3 for 45 min at 65°C, and alkylated by the addition of 50 mM iodoacetamide for 30 min at room temperature. Gel pieces were then washed in 0.1% NH_4HCO_3 and 50% acetonitrile/50 mM NH_4HCO_3, dried, and incubated with 25 mM triethylammonium bicarbonate with 5 µg/ml trypsin for 16 h at 30°C. For identification of phosphorylation sites, the extracted tryptic peptides were analyzed by liquid chromatography mass spectrometry on a spectrometer (4000 Q-TRAP; Applied Biosystems) with precursor ion scanning as described previously (Williamson et al., 2006). The tandem mass spectrometry spectra were searched against a local database containing the GST-MDC1 sequence using the Mascot search algorithm (www.matrixscience.com) run on a local server. Sites of phosphorylation were validated by manual inspection of the mass spectrometry spectra using Analyst 1.4.1 software (MDS Sciex).

Online supplemental material

Fig. S1 shows a sequence alignement of the SDT repeats in human, mouse, zebrafish, and honey bee MDC1. Fig. S2 shows the purified MDC1–MRN complex isolated from HeLa nuclear extract. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200709008/DC1.

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CHAPTER 6

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