Investigating the Role of 53BP1 in Regulating Gene Transcription

by

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SUMMARY

53BP1 is a double strand break repair protein that plays a crucial role in checkpoint activation and DNA repair. 53BP1 is a member of a family of proteins that contain BRCT domains and several of these BRCT domain-containing proteins have been shown to function as both DNA repair proteins and transcriptional regulators. Similarly, in addition to its involvement in the cellular response to double strand breaks, it has been demonstrated that 53BP1 can also function to regulate gene expression. 53BP1 was originally identified in a yeast two-hybrid screen for novel modulators of p53 transcriptional activity and several studies have shown that it acts as a transcriptional co-activator of p53. Despite these studies, the role of 53BP1 in transcriptional regulation remains poorly understood.

To investigate the effect of 53BP1 on cellular transcription, a microarray approach was utilised to study the gene expression patterns in cells treated with and without 53BP1 siRNA, before and after ionising radiation to identify genes regulated by 53BP1. The gene expression profiles were compared at the level of single genes and biological pathways. Microarray analysis identified numerous genes whose expression was regulated by 53BP1 in the absence and presence of overt DNA damage. Interestingly, in the absence of DNA damage, several of these genes encoded proteins involved in TNFR1 signalling pathways, in particular the NF-κB signalling pathway. Consistent with the single gene analysis, analysis of biological pathways also highlighted TNFR1 signalling pathways as being differentially regulated by 53BP1, independently of DNA damage. The potential role of 53BP1 in the NF-κB signalling pathway was further investigated and 53BP1 knockdown studies indicated that 53BP1 may be negatively regulating NF-κB transcriptional activity. In the absence of 53BP1, basal and phosphorylation levels of p65 were elevated in response to TNFα and ionising radiation. Furthermore, phosphorylation of p65 was sustained in 53BP1 depleted cells suggesting that 53BP1 may play a role in the termination of NF-κB response. Co-immunoprecipitation studies revealed that there was no association between 53BP1 and p65 indicating that 53BP1 was regulating NF-κB transcriptional activity through an indirect mechanism.

Data presented in this thesis also demonstrates that 53BP1 can function as a modulator of p53 that can differentially regulate p53 responsive genes. Through cooperating with p53, 53BP1 can induce the expression of PUMA and HDM2 in response to DNA damage. However, in

contrast, 53BP1 can repress p21 expression levels in unstressed cells by repressing p53 transcriptional activity.

In addition, the data presented here shows that 53BP1 can bind and modulate the activity of the transcriptional co-activators CBP/p300. Co-immunoprecipitation and chromatographic studies revealed that 53BP1 and CBP/p300 interact with each other and exist in a large macromolecular complex. Mapping the sites of interaction showed that CBP/p300 bind to 53BP1 via its C-terminal domains whereas 53BP1 binds to multiple regions of CBP/p300. When the transactivation potential of p300 was assessed with a reporter assay, cells over-expressing 53BP1 showed an increase in p300 activity in a dose dependent manner.

In conclusion, the data presented in this thesis indicates that 53BP1 functions as a transcriptional regulator and that this role for 53BP1 may be facilitated by CBP and p300. These findings therefore provide novel insight into our understanding of the cellular role of 53BP1 and may possible have implications for its involvement in suppressing tumourigenesis.

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ABBREVIATIONS

53BP1	p53-binding protein 1
AID	Activation induced deaminase
AIF	Apoptosis inducing factor
Alt-EJ	Alternative end-joining
APC/C	Anaphase promoting complex/cyclosome
ASPP	Apoptosis stimulating protein of p53
A-T	Ataxia-telangiectasia
A-TLD	Ataxia-telangiectasia like disorder
ATM	Ataxia-telangiectasia mutated
ATR	Ataxia-telangiectasia mutated and rad3 related
ATRIP	ATR interacting protein
BARD1	BRCA1 associated ring domain
BAX	Bcl-2-associated protein X
BRCA	Breast cancer susceptibility gene
BRCC	BRCA1/2 containing complex
BRCT	BRCA1 C-terminal
CARM1	Co-activator associated arginine methyltransferase 1
CBP	CREB binding protein
cDNA	Complementary DNA
CDK	Cyclin-dependent kinase
CHK	Checkpoint kinase
CK2	Casein kinase 2
COMMD1	Copper metabolism MURR1 domain containing protein 1
CSR	Class switch recombination
CtIP	CTBP interacting protein
DBD	DNA binding domain
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DNA2	DNA replication helicase 2
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DR	Death receptor
DSB	Double strand break

dsDNA	Double stranded DNA
DUB	Deubiquitylating enzyme
ERCC1	Excision repair cross complementation 1
EYA	Eyes absent
FCS	Fetal calf serum
FADD	Fas-associated death domain
FHA	Forkhead-associated
GADD45	Growth arrest and DNA damage inducible 45
GSEA	Gene set enrichment analysis
GSK3	Glycogen synthase kinase 3
GST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide
H3K79me2	Histone H3 di-methylated lysine 79
H4K20me2	Histone H4 di-methylated lysine 20
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDM2	human double minute 2
HDMX	Human double minute 4
HP1	Heterochromatin protein 1
HR	Homologous recombination
HU	Hydroxyurea
IAP	Inhibitor of apoptosis protein
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
IκB	Inhibitor of NF-κB
IKK	IκB kinase
IL-1	Interleukin 1
IR	Ionising radiation
JMY	Junction-mediating and regulatory protein
JNK	c-jun N-terminal kinase
KAP1	KRAB domain associated protein 1
LPS	Lipopolysaccharide
MCPH1	Microcephalin
MDC1	Mediator of DNA damage checkpoint 1

MERIT40	Mediator of RAP80 interactions and targeting 40kd
MMEJ	Microhomology mediated end-joining
MSK	Mitogen and stress activated kinase
MTC	Multiple testing correction
NAK	NF-κB activating kinase
NBS	Nijmegen breakage syndrome
NBSLD	Nijmegen breakage syndrome like disorder
NEMO	NF-κB essential modifier
NHEJ	Non-homologous end joining
NF-κB	Nuclear factor kappa-light chain enhancer of activated B cells
PALB2	Partner and localizer of BRCA2
PARP	Poly ADP-ribose polymerase
PCR	Polymerase chain reaction
PIAS	Protein inhibitor of activated STAT
PIKK	Phosphatidylinositol 3-kinase-like kinase
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-tween 20
P/CAF	p300/CBP-associated protein
PCNA	Proliferating cell nuclear antigen
PKA	Protein kinase A
PKC	Protein kinase C
Pol	DNA polymerase
PRMT	Protein arginine methyltransferase
PUMA	p53 up-regulated modulator of apoptosis
pRB	Retinoblastoma protein
qRT-PCR	Quantitative reverse transcriptase-PCR
RAG	Recombination activating gene
RAP80	Receptor associated protein 80
RSK1	Ribosomal S6 kinase 1
RIP1	Receptor interacting protein 1
RNA	Ribonucleic acid
RNF	Ring finger protein
rpm	revolutions per minute
ROS	Reactive oxygen species
RPA	Replication protein A

siRNA	Small interfering RNA
SCF$^{\beta TRCP}$	SKIP1-Cul1-F-box ligase containing the F-box protein βTRCP
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SH	Src-homology
SOCS1	Suppressor of cytokine signalling 1
SSA	Single strand annealing
SSB	Single strand break
ssDNA	Single stranded DNA
SDSA	Synthesis-dependent strand annealing
TAB	TAK1 binding protein
TAK1	Transforming growth factor β activated kinase 1
TAD	Transactivation domain
TCR	T-cell receptor
TdT	Terminal deoxynucleotide transferase
TNFα	Tumour necrosis factor α
TNFR	Tumour necrosis factor receptor
TOPBP1	Topoisomerase II binding protein 1
TRADD	TNF receptor-associated protein with a death domain
TRAF	TNF receptor-associated factor
UIM	Ubiquitin interacting motif
UV	Ultraviolet light
v/v	volume/volume
w/v	weight/volume
XLF	XRCC4-like factor
XPF	Xeroderma pigmentosum F
XRCC	X-ray cross complementing
YB1	Y-box protein 1

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CHAPTER 1

CHAPTER 1 GENERAL INTRODUCTION

1.1 GENOMIC INSTABILITY AND CANCER

DNA is constantly being damaged by both exogenous and endogenous sources resulting in the generation of a multitude of DNA lesions such as double strand breaks (DSBs) and single strand breaks (SSBs). To counteract this DNA damage and protect the integrity of the genome, cells have evolved complex mechanisms that detect and signal the presence of the DNA lesion and coordinate a cellular response that involves activation of cell cycle checkpoints and DNA repair. Collectively these mechanisms have been termed the DNA damage response (DDR). Failure to sense and/or repair DNA damage can lead to either senescence, cell death or contribute to genomic instability, the latter of which has recently been described as one of the hallmarks of cancer (Negrini *et al* 2010). One of the major forms of genomic instability is chromosomal instability (CIN), which is characterised by alterations in chromosome number and/or structure due to chromosomal rearrangements such as deletions and translocations. Other forms of genomic instability include the accumulation of mutations and microsatellite instability (MIN), which is caused by the expansion and/or contraction of the number of microsatellite sequences (Aguilera and Gomez-Gonzalez 2008).

It is known the early stage tumours have elevated levels of DNA damage (Bartkova *et al* 2005, Gorgoulis *et al* 2005). Activation of the DDR could normally act as a barrier to tumour progression by inducing cellular senescence or apoptosis of early tumour cells and therefore protecting the integrity of the genome (Bartek *et al* 2007). However, mutations in DDR genes such as *TP53*, *ATM* and *BRCA1* are commonly selected for during tumour development because they allow tumour cells to breach this barrier and proliferate at the expense of elevated levels of DNA damage. This role for DDR as an anti-cancer barrier may explain why mutations in DDR genes occur at a high frequency in human cancers (Greenman *et al* 2007, Halazonetis *et al* 2008, Kastan and Bartek 2004). For example, more than 50% of sporadic cancers harbour *TP53* mutations and germline mutations in *BRCA1* and *BRCA2* account for the majority of familial breast and ovarian cancer (Boulton 2006, Soussi and Lozano 2005).

1.2 DNA DAMAGE RESPONSE

The DDR is primarily mediated by the phosphatidylinositol 3-kinase-like kinase (PIKK) family, which includes ATM (ataxia-telangiectasia mutated), ATR (ataxia-telangiectasia mutated and rad3-related kinase) and DNA-PK (DNA-dependent protein kinase). ATM and DNA-PK are predominantly activated in response to DSBs, which are formed when both strands of the DNA backbone are broken simultaneously. These are the most toxic among DNA lesions with one unrepaired DSB being sufficient to cause cell death (Jeggo and Lobrich 2007, Shiloh 2003). There are a variety of DNA damaging agents that induce DSBs including ionising radiation (IR), metabolic products such as reactive oxygen species (ROS) and chemicals such as those used for chemotherapy. In addition, they can also arise due to replication fork collapse, which occurs when the replication machinery collides with a SSB. Finally, even though DSBs can be lethal to the cell, they are formed in a programmed manner as part of normal cellular processes such as class switch recombination (CSR) and V(D)J recombination, which occur during immune system development (Hiom 2010, Jackson and Bartek 2009). However, unlike ATM, DNA-PK does not primarily function in the DDR signal transduction pathway, but rather coordinates the DSB repair pathway, non-homologous end-joining (NHEJ) (Collis *et al* 2005). In contrast to ATM and DNA-PK, ATR is mainly activated in response to single stranded DNA (ssDNA), which can be generated by bulky adducts, inter-strand crosslinks and replication stress caused by DNA damaging agents such as hydroxyurea (HU), ultraviolet light (UV) and metabolic products such as ROS (Cimprich and Cortez 2008). In addition, ATR can also be activated during DSB repair response since ssDNA is produced as an intermediate during DSB end-resection and is required to initiate homologous recombination (HR).

1.2.1 ATM-mediated DNA damage response

DSBs are sensed by the MRE11/RAD50/NBS1 (MRN) complex, which forms a bridge between the broken DNA ends ensuring that the ends remain in close proximity (Moreno-Herrero *et al* 2005). Through an ATM binding motif in NBS1 (Nijmegen breakage syndrome 1), the MRN complex recruits and subsequently activates ATM in the chromatin regions proximal to the DSB (Falck *et al* 2005, Lee and Paull 2004, Uziel *et al* 2003). Activation of ATM involves the dissociation of inactive ATM dimers into active monomers (Bakkenist and Kastan 2003). This process is facilitated by autophosphorylation of ATM on serines 367,

1893, 1981 and 2996 (Bakkenist and Kastan 2003, Kozlov *et al* 2006, Kozlov *et al* 2010), as well as acetylation of ATM on lysine 3016 by the histone acetyltransferase (HAT) Tip60 (Sun *et al* 2005). In response to DSBs, casein kinase 2 (CK2) is recruited to the DNA break where it phosphorylates and displaces heterochromatin protein 1β (HP1) from histone H3 tri-methylated lysine 9 (H3K9me3), thereby enabling Tip60 to interact with H3K9me3. This interaction up-regulates Tip60 acetyltransferase activity leading to acetylation and activation of ATM, as well as acetylation of histones surrounding the DSB (Sun *et al* 2009). Once fully activated, ATM phosphorylates the histone H2A variant H2AX (termed γ-H2AX) on serine 139 surrounding the DNA break triggering relocalisation of DDR proteins to the sites of the break in a highly ordered manner, which can be visualised microscopically as the formation of DNA repair protein foci (Bekker-Jensen *et al* 2006, Burma *et al* 2001). Among those proteins recruited is the DDR mediator protein, mediator of DNA damage checkpoint 1 (MDC1), which interacts with the C-terminal tail of γ-H2AX via its BRCT (BRCA1 C-terminal) domain (Stewart *et al* 2003, Stucki *et al* 2005). However, this interaction is dependent on the dephosphorylation of tyrosine 142 on γ-H2AX by the eyes absent (EYA) tyrosine phosphatases, EYA1 and EYA3 (Cook *et al* 2009, Krishnan *et al* 2009). Once bound to γ-H2AX, MDC1 initiates a positive ATM feedback loop by promoting the stabilisation and further accumulation of the MRN complex and activated ATM, resulting in the spreading of H2AX phosphorylation over an extensive region of chromatin flanking the DSB and amplification of the damage signal (Chapman and Jackson 2008, Lou *et al* 2006, Lukas *et al* 2004a, Melander *et al* 2008, Spycher *et al* 2008). In addition to serving as a molecular scaffold to MRN and ATM, MDC1 also orchestrates the recruitment of other DSB repair/checkpoint proteins to the chromatin surrounding the DNA lesion (Bekker-Jensen *et al* 2006, Goldberg *et al* 2003, Stewart *et al* 2003). This role for MDC1 is achieved, in part, through ATM-dependent phosphorylation of MDC1, which provides a binding site recognised by the forkhead-associated (FHA) domain of the E3 ubiquitin ligase RNF8 (ring finger protein 8) and activates a ubiquitylation cascade (Huen *et al* 2007, Kolas *et al* 2007, Mailand *et al* 2007). RNF8 in conjunction with another E3 ligase, HERC2 (HECT domain and RLD 2) and the E2 conjugating protein, UBC13 catalyse the formation of lysine-63 (K63)-linked polyubiquitin chains on H2A and H2AX proximal to the break (Bekker-Jensen *et al* 2010, Wang and Elledge 2007). These ubiquitylated histones subsequently signal the recruitment of another E3 ubiquitin ligase, RNF168, which binds through its C-terminal MIU2 (motif

interacting with ubiquitin) domain. RNF168, together with UBC13 amplifies the ubiquitin signal by stimulating K63-linked polyubiquitylation (Doil *et al* 2009, Stewart *et al* 2009). Recruitment of RNF168 to the break site is facilitated by the SUMO ligase, protein inhibitor of activated STAT (PIAS) 4 possibly through its ability to stimulate the ubiquitin ligase activity of RNF8 and/or HERC2. (Galanty *et al* 2009, Morris *et al* 2009). Ubiquitylation of histones has been proposed to result in chromatin relaxation surrounding the break site allowing the break to become more accessible to proteins involved in DNA repair and checkpoint activation such as BRCA1 (breast cancer susceptibility gene 1) and 53BP1 (p53-binding protein 1) (Figure 1.1).

In this respect, the K63-linked ubiquitin chains formed on histones are recognised by the ubiquitin-interacting motif (UIM) of RAP80 (receptor associated protein 80), which recruits the BRCA1-A complex through the interaction with the scaffold protein Abraxas (Kim *et al* 2007, Sobhian *et al* 2007, Wang and Elledge 2007, Yan *et al* 2007b). In addition to BRCA1, RAP80 and Abraxas, the BRCA1-A complex also contains BARD1 (BRCA1 associated ring domain 1), which together with BRCA1 forms a heterodimeric E3 ubiquitin ligase, BRCC36 (BRCA1/2 containing complex subunit 36), a deubiquitylating enzyme and the adaptor proteins MERIT40 (mediator of RAP80 interactions and Targeting 40kd) and BRCC45 (BRCA1/2 containing complex subunit 45) (Feng *et al* 2009, Shao *et al* 2009b, Wang *et al* 2009). Subsequently, BRCA1 is sumoylated by the SUMO E3 ligase PIAS1, which stimulates its E3 ubiquitin ligase activity (Galanty *et al* 2009, Morris *et al* 2009). Once recruited BRCA1/BARD1 can initiate activation of cell cycle checkpoints, as well as facilitate HR (Huen *et al* 2010b).

In contrast to other DSB repair proteins, initial recruitment of 53BP1 to the break site does not involve direct interaction with other DSB repair proteins or ubiquitin chains. Instead it has been suggested that RNF8/RNF168-mediated ubiquitylation in conjunction with H2AX phosphorylation and Tip60-mediated acetylation of the histones surrounding the DSB break promotes relaxation of the chromatin (van Attikum and Gasser 2009). This allows exposure of previously hidden histone marks, histone H4 di-methylated lysine 20 (H4K20me2) and possibly histone H3 di-methylated lysine 79 (H3K79me2), which are recognised by the Tudor domain of 53BP1 (Botuyan *et al* 2006, Ng *et al* 2003). Recently, it has been demonstrated that recruitment of 53BP1 to DSBs is facilitated by the SUMO E3 ligases PIAS4, which

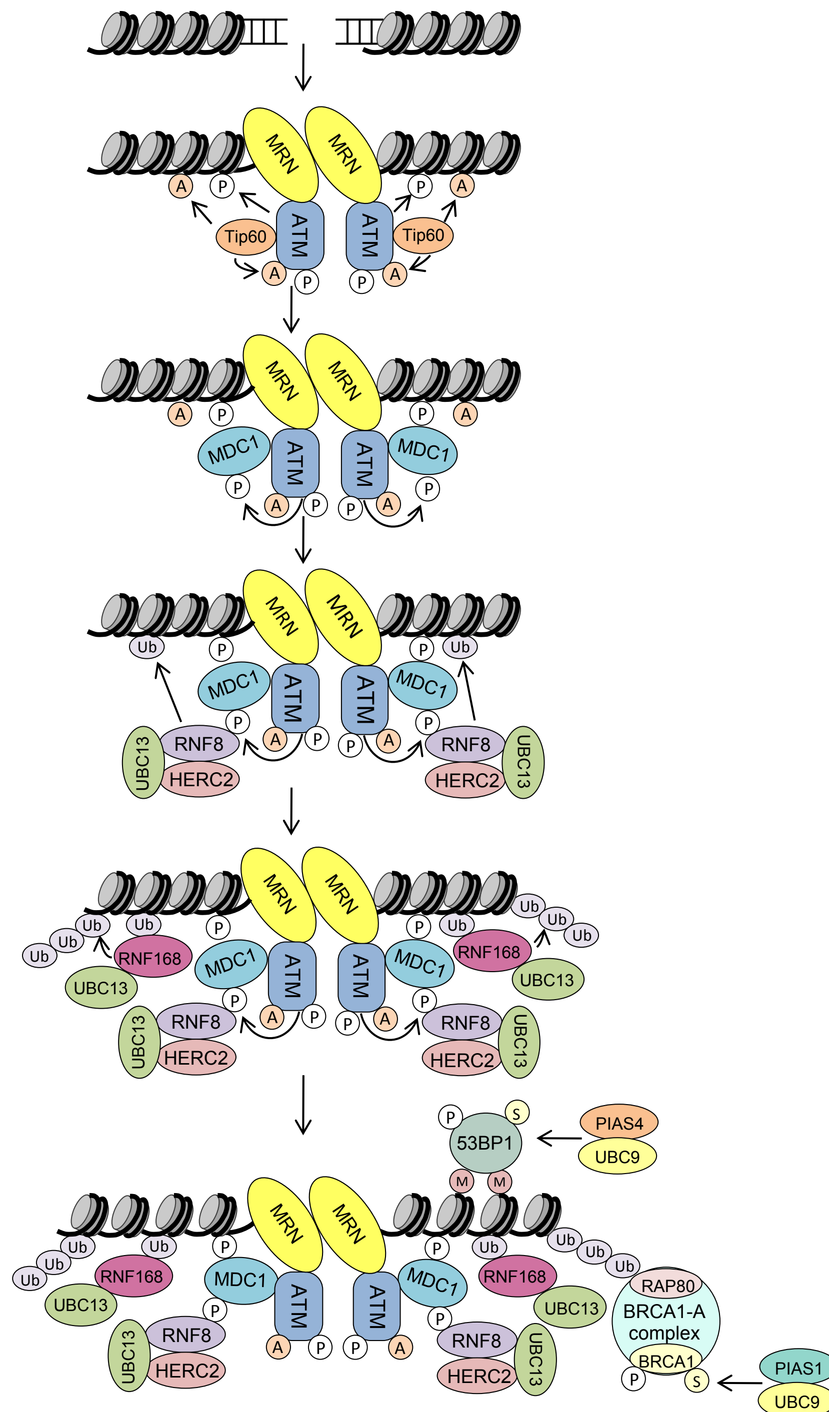


Figure 1.1 ***ATM–mediated DDR pathway****. Induction of a DSB initiates recruitment of the MRN complex, which subsequently recruits ATM. Activation of ATM by MRN and Tip60 leads to phosphorylation of H2AX. γH2AX signals the recruitment of MDC1, which is phosphorylated by ATM triggering its association with RNF8 and HERC2. RNF8/HERC2/UBC13 catalyse the ubiquitylation of H2A-type histones surrounding the break. RNF168 is recruited to ubiquitylated histones where it amplifies the ubiquitylation signal by catalysing polyubiquitylation of H2A histones, resulting in chromatin relaxation, Subsequently, 53BP1 and BRCA1 are recruited to the DSB and are sumoylated by PIAS4 and PIAS1 respectively (Adapted from Ciccia and Elledge 2010).*

together with its E2 conjugating enzyme UBC9 catalyses its sumoylation (Galanty *et al* 2009). Currently little is known about the precise function of 53BP1 in DSB repair, although it has been implicated in the intra-S and G2/M checkpoints (DiTullio *et al* 2002), DNA end-joining (Difilippantonio *et al* 2008) and suppression of DNA end-resection (Bunting *et al* 2010) (Figure 1.1).

Ubiquitylation is a reversible process involving specific proteases called deubiquitylating enzymes (DUBs), which remove ubiquitin from protein substrates. To date three DUBs, namely USP3 (ubiquitin specific protease 3), BRCC36 and OTUB1 (OUT-containing ubiquitin aldehyde-binding protein 1) have been identified that negatively regulate the ubiquitin-dependent DSB signalling cascade. USP3 has been reported to deubiquitylate the histone H2A (Nicassio *et al* 2007). Furthermore, depletion of USP3 resulted in an increase in accumulation of 53BP1 at DSBs, whereas over-expression of USP3 prevented RNF168 accumulation at DSBs, but had no effect on RNF8 foci formation (Doil *et al* 2009, Nicassio *et al* 2007). Together these data indicate that USP3 inhibits RNF8-dependent ubiquitylation events. BRCC36 is a member of the JAMM/MPN$^+$ DUB family and specifically cleaves K63-linked polyubiquitin chains (Cooper *et al* 2009). BRCC36 DUB activity requires interactions with the BRCA1-A complex in particular Abraxas and BRCC45 (Feng *et al* 2010, Patterson-Fortin *et al* 2010). BRCC36 deficient cells display increased levels of ubiquitylated H2A and H2AX and depletion of BRCC36 partially rescues the defect in ubiquitylation associated with loss of RNF8 (Feng *et al* 2010, Shao *et al* 2009a). These results suggest that, like USP3, BRCC36 may be a negative regulator of RNF8-dependent ubiquitylation. RNF168 ubiquitylation has recently been shown to be suppressed by the deubiquitylating enzyme, OTUB1 (Nakada *et al* 2010). Surprisingly, inhibition of RNF168 ubiquitylation by OTUB1 was found to occur independently of its catalytic activity since a catalytically inactive mutant could also inhibit 53BP1 foci formation. This data indicates that unlike the majority of DUBs, the action of OTUB1 did not involve removal of ubiquitin from RNF168. Instead, Nakada *et al* (2010) revealed that OTUB1 directly interacts and inhibits UBC13, thereby preventing formation of RNF168-mediated K63-linked polyubiquitin chains (Nakada *et al* 2010).

The purpose of the DSB-induced ATM signalling pathway is to promote DNA repair and cell cycle arrest. ATM does this by recruiting and activating proteins that facilitate DNA repair through HR or NHEJ, as well as activating proteins required to induce a G1, intra-S or G2/M

cell cycle arrest including p53, BRCA1, NBS1, CtIP (CTBP interacting protein) and CHK2 (checkpoint kinase 2) (Kastan and Lim 2000). One ATM substrate that is important for ATM-dependent DNA repair is the KRAB domain associated protein 1 (KAP1). KAP1 associates with transcriptional repressors such as HP1 and histone deacetylases (HDAC) 1 and 2 forming a transcriptional silencing complex that promotes heterochromatin formation (Craig 2005). ATM-mediated phosphorylation of KAP1 at serine 824 weakens its interaction with the complex resulting in relaxation of the chromatin and increasing the accessibility of DNA repair proteins to the DNA break (Goodarzi *et al* 2008, Ziv *et al* 2006). Interestingly, large scale proteomic studies have highlighted that ATM-mediated signalling pathway may coordinate a much wider variety of cellular processes than originally thought, since ATM can phosphorylate over 700 substrates including proteins involved in metabolism, transcription, chromatin remodelling and RNA splicing (Matsuoka *et al* 2007).

1.2.2 ATR-mediated DNA damage response

Localisation of ATR to sites of DNA damage is dependent on its interacting partner, ATRIP (ATR-interacting protein), which recognises and binds to replication protein A (RPA), a single stranded DNA binding protein complex that coats and stabilises ssDNA (Cortez *et al* 2001, Fanning *et al* 2006, Zou and Elledge 2003). In addition to the ATR-ATRIP complex, ssDNA-RPA complex recruits the RAD9-RAD1-HUS1 (known as 9-1-1) complex, a heterotrimeric ring shaped molecule with homology to PCNA (proliferating cell nuclear antigen), which is loaded onto the DNA by the RAD17-replication C clamp loader (Bermudez *et al* 2003, Parrilla-Castellar *et al* 2004, Zou *et al* 2003). ATR activation is not only dependent on the colocalisation of the ATR-ATRIP complex with the 9-1-1 complex, but also on the recruitment of the ATR activator, TOPBP1 (topoisomerase II binding protein 1) to the DNA damage site. This occurs through an interaction between two of the BRCT domains of TOPBP1 and the phosphorylated C-terminal tail of RAD9 from the 9-1-1 complex. Subsequently, the ATR-activating domain of TOPBP1 interacts with ATR-ATRIP complex stimulating ATR kinase activity (Delacroix *et al* 2007, Kumagai *et al* 2006) (Figure 1.2).

Once activated, ATR can promote cell cycle arrest, DNA repair and replication fork stability through phosphorylation of its substrates such as p53, TOPBP1, RAD9, BRCA1 and CHK1 (checkpoint kinase 1) (Cimprich and Cortez 2008). However, in a similar manner to ATM, the

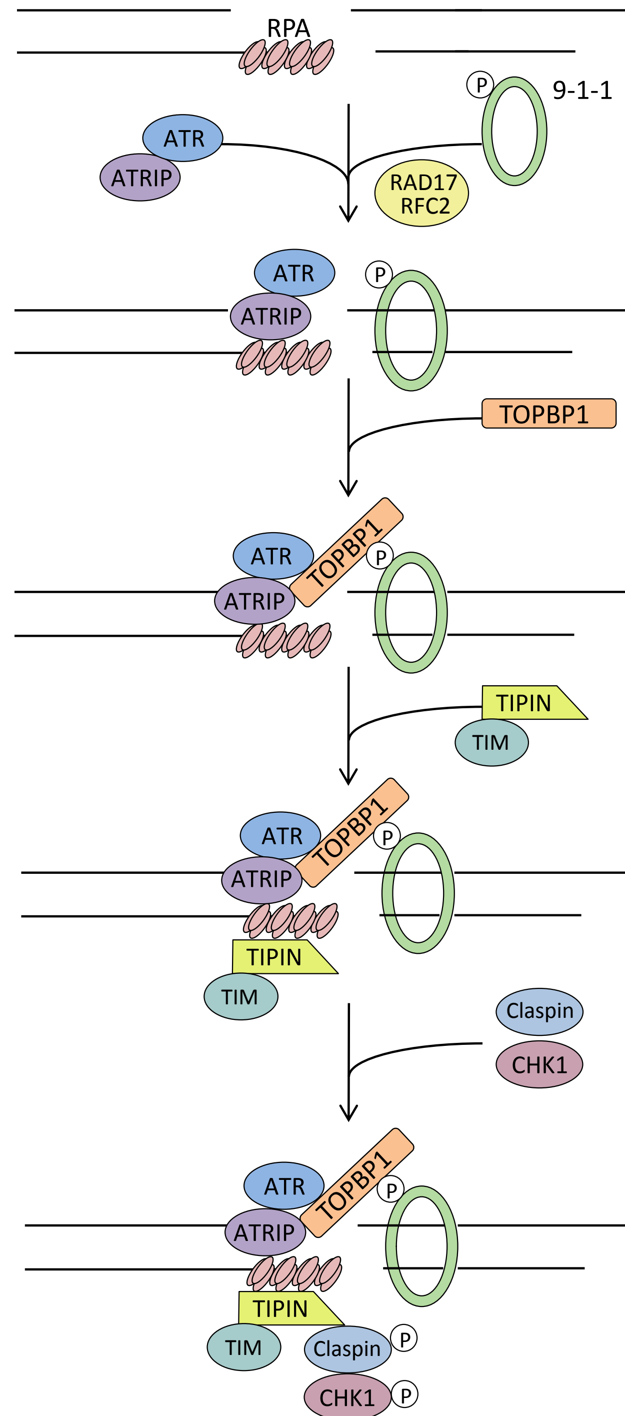


Figure 1.2 ***ATR-mediated DDR pathway***. *In response to DNA damage or replication stress, the generation of ssDNA is recognised by RPA. RPA-coated ssDNA recruits ATR-ATRIP and facilitates the loading of the 9-1-1 complex on to DNA by the RAD17-replication C complex. TOPBP1 is subsequently recruited through its stable association with the C-terminal of RAD9 and stimulates ATR kinase activity. Through an interaction between RPA and Tipin, the Tipin-Timeless complex and then Claspin are able to associate with ATR at sites of DNA damage. ATR phosphorylates Claspin, which both promotes the interaction with CHK1 and facilitates the phosphorylation of CHK1 by ATR (Adapted from Kemp et al 2010).*

list of ATR substrates is rapidly expanding due to large proteomic studies (Matsuoka *et al* 2007, Stokes *et al* 2007). Some of the substrates identified are involved in pathways that have not previously been linked to the DDR such as RNA splicing and metabolic pathways, as well as those invoked by insulin stimulation. Furthermore, many, if not the vast majority of these substrates are also targeted by ATM such as p53, NBS1 and BRCA1 indicating that there is extensive crosstalk between the ATM and ATR pathways (Matsuoka *et al* 2007, Stokes *et al* 2007). Despite this the checkpoint kinase, CHK1 has been the most extensively studied ATR substrate. Interestingly, unlike other ATR substrates, phosphorylation of CHK1 by ATR on serine 317 and serine 345 has been shown to be not only facilitated by TOPBP1, but also by another mediator protein, Claspin (Liu *et al* 2006). Furthermore, CHK1 phosphorylation is also stimulated by the Tipin subunit of the Timeless-Tipin complex, which interacts with the RPA2 subunit of the RPA complex and functions as a molecular scaffold for Claspin and CHK1 to transiently associate with ATR (Kemp *et al* 2010). Phosphorylated CHK1 can then phosphorylate its substrates including CDC25A, CDC25C, Wee1 and p53 leading to cell cycle arrest (Dai and Grant 2010) (Figure 1.2).

1.2.3 Cell cycle checkpoints

Activation of cell cycle checkpoints in response to DNA damage delays cell cycle progression allowing time for the DNA to be repaired. Therefore cell cycle checkpoints are crucial in protecting against genomic instability. The G1/S checkpoint prevents cells entering S-phase with DNA damage, the intra-S-phase checkpoint (also known as the DNA replication checkpoint) suppresses further DNA replication until the DNA damage has been repaired and the G2/M checkpoint delays entry into mitosis, therefore preventing inappropriate segregation of damaged chromosomes during mitosis (Lukas *et al* 2004b). The progression into each phase of the cell cycle is tightly controlled by cyclin-dependent kinases (CDK) and their regulatory subunits known as cyclins (Figure 1.3). Phosphorylation of CDK by the Wee1 and Myt1 protein kinases maintains the CDK-cyclin complexes in an inactive state. When CDK activity becomes required for progression into the next cell cycle phase, the dual specificity CDC25 phosphatases dephosphorylate the CDKs, thereby activating the CDK-cyclin complexes. There are three mammalian CDC25 isoforms: CDC25A, -B and -C, which specifically regulate the activities of CDK1 and CDK2. CDC25A controls progression through S phase by activating CDK2-cyclin E and CDK2-cyclin A complexes, as well as

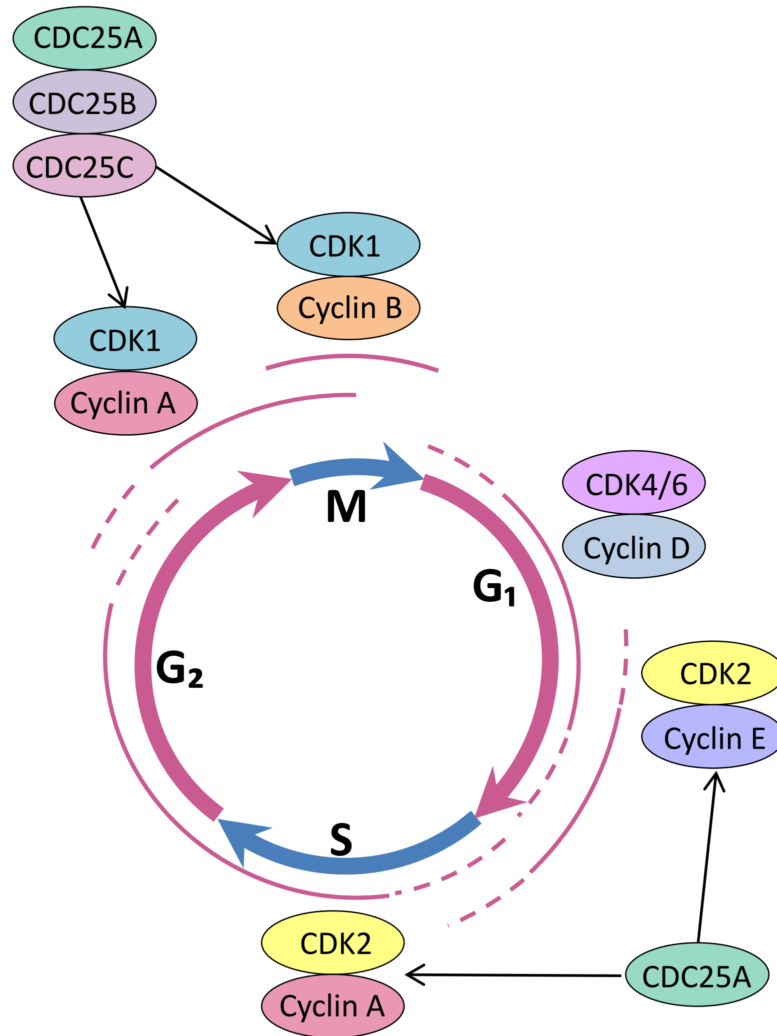


Figure 1.3 ***Cell cycle regulation by CDK-Cyclin complexes****. Entry into the four stages of the cell cycle is regulated by CDK-Cyclin complexes. Activation of specific CDK-Cyclin complexes by CDC25-mediated dephosphorylation drives progression through these cell cycle phases.*

progression into mitosis by activating CDK1-cyclin B complex. CDC25B and CDC25C are primarily involved in activating CDK1-cyclin B complex and therefore control the G2-M transition (Boutros *et al* 2007) (Figure 1.3).

Following DNA damage, CHK1 and to a lesser extent CHK2, -mediated phosphorylation of CDC25A targets it for ubiquitin-dependent proteasomal degradation, which is catalysed by two different ubiquitin ligases, the anaphase promoting complex (APC/C) and the $SCF^{\beta TRCP}$ (SKP1-CUL1-F-box ligase containing the F-box protein βTRCP) complex. APC/C is involved in regulating CDC25A at the exit of mitosis whereas $SCF^{\beta TRCP}$ regulates CDC25A throughout the cell cycle (Donzelli *et al* 2002, Falck *et al* 2001). In contrast, phosphorylation of CDC25C by CHK1 and CHK2 promotes its binding to the 14-3-3 proteins, which export CDC25C from the nucleus subsequently preventing it from activating the CDK1-cyclin B complex (Matsuoka *et al* 1998, Peng *et al* 1997). Failure to dephosphorylate CDK2 and CDK1 leads to activation of the G1/S, intra-S-phase and G2/M checkpoints. Furthermore, CHK1 also phosphorylates Wee1, thereby reinforcing the inhibition of CDK-cyclin complexes (Raleigh and O'Connell 2000). In addition to the 14-3-3 proteins, GADD45 (growth arrest and DNA damage inducible 45) and the CDK inhibitor, p21 have also been shown to be involved in activating the G2/M checkpoint by binding and inhibiting CDK1 activity (Bates *et al* 1998, Medema *et al* 1998, Zhan *et al* 1999). p21 also plays a crucial role in G1/S checkpoint activation through inhibition of CDK4/cyclin D1 and CDK2/cyclin E-mediated phosphorylation of pRb (Retinoblastoma protein) and E2F-dependent transcription, which are important events required for S-phase entry and initiation of DNA replication (Brugarolas *et al* 1999, He *et al* 2005, Lundberg and Weinberg 1998, Massague 2004).

1.3 DOUBLE STRAND BREAK REPAIR

Repair of DSBs can be carried out by various pathways, but the two main pathways are non-homologous end-joining (NHEJ) and homologous recombination (HR). Repair by NHEJ is error-prone because it involves religating the DNA ends at the break using small regions of sequence homology that can potentially lead to the loss of DNA bases immediately adjacent to the break. In contrast, HR is regarded as an error-free mechanism since it uses a homologous template to replace the DNA bases across the break. As the sister chromatid is the preferred template, HR predominantly operates in the late S and G2 phases of the cell

cycle. However, HR can also use homologous sequences within the genome as a template, so can occur in G1, although this can potentially lead to loss of heterozygosity, deletions and chromosomal translocations. Therefore, NHEJ is the preferred method of repair in G1, although it can also occur in S and G2. In addition to sister chromatid availability, the choice between HR and NHEJ is also dependent on the activity of CDKs and their ability to promote DNA end-resection. DNA end-resection is fundamental for HR, so in the presence of high levels of CDK activity HR is the favoured pathway (Branzei and Foiani 2008).

1.3.1 Homologous recombination

HR is initiated by DNA end-resection, which involves the degradation of the 5′ ends of either side of the DSB to produce 3′ ssDNA overhangs. The MRN complex together with CtIP is responsible for the formation of these ssDNA overhangs at DSBs (Limbo *et al* 2007, Sartori *et al* 2007, Yun and Hiom 2009). The function of CtIP is highly regulated by post-translational modifications to ensure that DNA end-resection and HR occur at the appropriate stages of the cell cycle. This requires the kinase activity of CDKs, which phosphorylate CtIP on serine 327 and threonine 847 during S and G2 (Huertas and Jackson 2009). Phosphorylation at both these sites is essential for DSB resection. However, only phosphorylation at serine 327 is required for CtIP to interact with BRCA1, which subsequently ubiquitylates CtIP facilitating its association with damage sites (Yu and Chen 2004, Yu *et al* 2006). Recruitment of CtIP is also dependent on the MRN complex and this is thought to stimulate the nuclease activity of MRE11, which is important for DNA end-resection, but not for the role of MRN in DSB sensing or ATM recruitment and activation (Buis *et al* 2008, Chen *et al* 2008, Sartori *et al* 2007, Williams *et al* 2008). Finally, ATM is important for DSB resection, although its precise role is currently unknown (Adams *et al* 2006, Cuadrado *et al* 2006, Jazayeri *et al* 2006, Myers and Cortez 2006). Recently, it has been suggested that ATM kinase activity may promote the recruitment of CtIP to DSBs. However, whether this is dependent on ATM phosphorylation of CtIP is unknown (You *et al* 2009).

DNA end-resection is currently thought to proceed through a two step mechanism based initially on studies in yeast (Mimitou and Symington 2008, Zhu *et al* 2008). First, the initial end processing is mediated through the nuclease activity of the MRN complex in a CtIP-dependent manner. Following this priming step, the resulting partially resected DNA is further processed by the action of the exonucleases EXO1 (exonuclease 1) and DNA2 (DNA

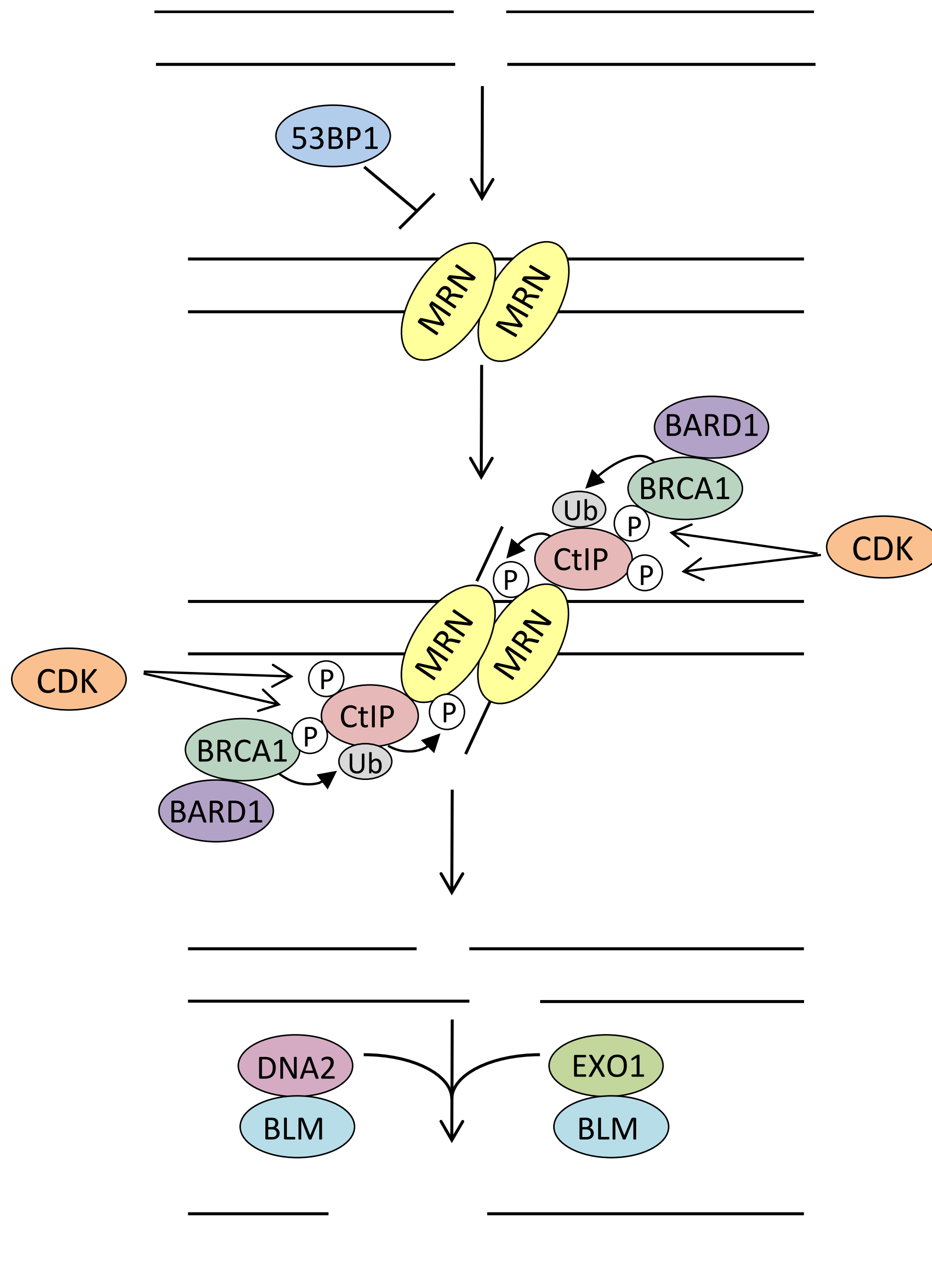


Figure 1.4 ***DNA end-resection***. *The MRN complex in conjunction with CtIP catalyses limited nucleolytic processing of the DNA ends. This process is dependent on CDK-mediated phosphorylation of CtIP and is facilitated by BRCA1, which binds to phosphorylated serine 327 and ubiquitylates CtIP. The action of MRN and CtIP primes the DNA ends for resection by nucleases such as EXO1 and DNA2. This activity is facilitated by the helicase, BLM. 53BP1 is thought to suppress DNA end-resection (Adapted from Ciccia and Elledge 2010).*

replication helicase 2) together with BLM (Bloom syndrome protein) in human cells (Gravel *et al* 2008, Nimonkar *et al* 2008, Nimonkar *et al* 2011, Zhu *et al* 2008) (Figure 1.4). Initially, the 3′ ssDNA ends generated by DNA end-resection become coated with RPA triggering activation of ATR and subsequent phosphorylation of CHK1. However, RPA is soon displaced by RAD51 in conjunction with RAD52, BRCA2 (breast cancer susceptibility gene 2) and PALB2 (partner and localizer of BRCA2) to form a RAD51 nucleoprotein filament (Chen *et al* 1998b, Sugiyama and Kowalczykowski 2002, Zhang *et al* 2009a). The RAD51 nucleoprotein filament together with RAD54 then invades the homologous double-stranded DNA template and promotes exchange of the DNA strands resulting in the formation a heteroduplex structure known as a D-loop (Mazin *et al* 2000, Petukhova *et al* 1998). DNA synthesis is then performed by DNA polymerases, which use the homologous DNA strand as a template to replace the sequence where the DSB was located on the damaged strand. Initially, the DNA polymerases, Polε and Polη were thought to mediate HR DNA synthesis. However, a recent study has suggested that DNA polymerase δ (Polδ) may be the preferred enzyme (Maloisel *et al* 2008).

At this point the newly synthesised strand can be displaced from the D-loop and annealed to the ssDNA tail created by resection at the other end of the break in a process known as synthesis-dependent strand-annealing (SDSA). Alternatively, the second resected strand can be annealed to the displaced strand in a process that is mediated by RAD52 and is referred to as second end capture (Nimonkar *et al* 2009). DNA synthesis at both sides of the structure and branch migration produces a double Holliday junction. Resolution of the Holliday structure is then required in order to separate the covalently bound sister chromatids. This can be carried out by endonucleases complexes such as MUS81/EME1, SLX1/SLX4 and GEN1 which cleave the Holliday junction generating two products (Ciccia *et al* 2003, Constantinou *et al* 2002, Fekairi *et al* 2009, Ip *et al* 2008, Svendsen *et al* 2009). If there is no exchange of the DNA sequences flanking the original DSB, then non-crossover products are produced. However, if DNA is exchanged between the two chromosomes then crossover products are created. Alternatively, Holliday junctions can be dissolved by the Topoisomerase IIIα/BLM complex through convergent branch migration, which exclusively yields non-crossover products (Plank *et al* 2006, Wu and Hickson 2003) (Figure 1.5).

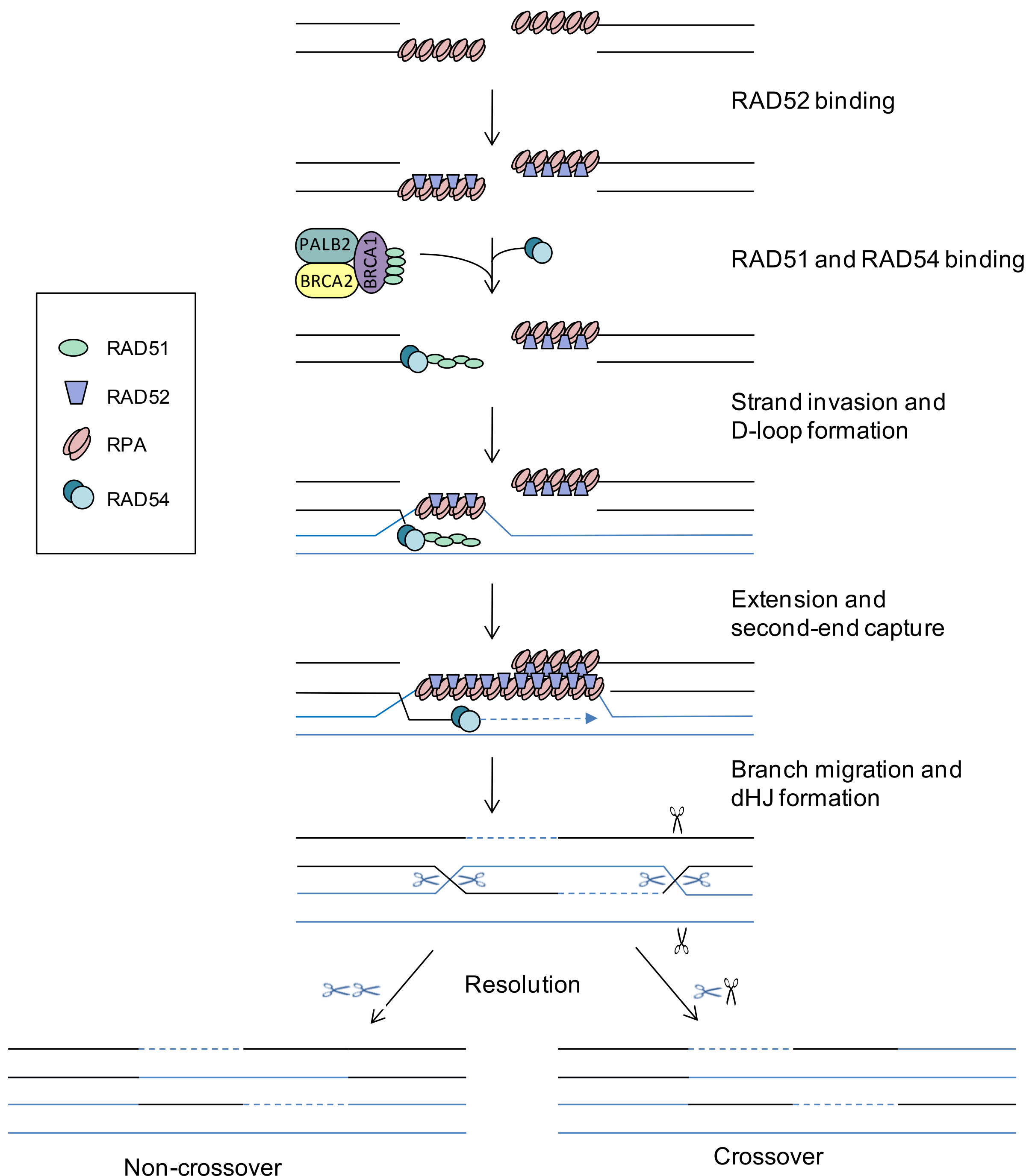


Figure 1.5 ***Repair of DSBs by HR****. RPA binds to the ssDNA tails generated by DNA end-resection where it is subsequently displaced by RAD52. Together with BRCA1, BRCA2 and PALB2, RAD52 facilitates the formation of the RAD51 nucleoprotein filament. The filament then invades the homologous DNA with the aid of RAD54 to form a D-loop structure. The other 3′ end of the DSB is captured, a process that is mediated by RAD52. DNA synthesis followed by branch migration and ligation of the nicks generates double Holliday junctions (dHJ). Resolution of the recombination intermediates to produce crossovers and non-crossovers completes DSB repair (Adapted from Nimonkar et al 2008).*

1.3.2 Non-homologous end-joining

In the absence of high levels of CDK activity, the default repair pathway is NHEJ. DSBs are recognised by the KU70/KU80 heterodimer, a ring shaped complex that has high affinity for DNA ends (Walker *et al* 2001). Once loaded onto the DNA ends, the KU complex then functions as a scaffold for the assembly of other NHEJ proteins. The first protein to be recruited is the serine/threonine kinase, DNA-dependent protein kinase catalytic subunit (DNA-PKcs). The complex that forms through the association of KU70/80 and DNA-PKcs at the DNA ends is generally referred to as DNA-PK and localises within seconds of the DSB being generated (Uematsu *et al* 2007). Once the complex is assembled at either side of the DSB, the two DNA-PKcs subunits tether the DNA ends together forming a synaptic complex (DeFazio *et al* 2002). The kinase activity of DNA-PKcs can then be activated allowing DNA-PKcs to phosphorylate its targets, some of which are components of the NHEJ pathway including XRCC4 (X-ray cross complementing 4), Artemis and KU70/80 (Burma and Chen 2004). Even though it is unclear what the biological relevance of these phosphorylation events are on NHEJ, it is well established that autophosphorylation of itself is important for efficient progression of the NHEJ (Chan *et al* 2002, Ding *et al* 2003). The unphosphorylated form of DNA-PKcs protects the ends from degradation or premature and incorrect ligation (Block *et al* 2004, Weterings *et al* 2003). However, once the synaptic complex has formed, trans-autophosphorylation of DNA-PKcs induces a conformational change, which allows the DNA ends to become accessible to processing enzymes and ligases (Meek *et al* 2007).

The synaptic complex acts as a scaffold for the religation of the DNA ends. However, very few DSBs produced have blunt ends that can be directly religated together. In the majority of cases, the two DNA ends will not be directly compatible because at least one of them will possess a 3′ or 5′ single strand overhang. In order to ligate the ends back together, the overhangs need to either be filled in by DNA synthesis using the overhang as a template or be removed by nucleotide trimming. Several enzymes have been implicated in the processing of DNA ends in preparation for ligation including the error prone polymerase such as terminal deoxynucleotidyltransferase (TdT), DNA polymerase μ (Polμ) and DNA polymerase λ (Polλ), as well as the nuclease Artemis (Ma *et al* 2002, Mahajan *et al* 1999, Mahajan *et al* 2002). Artemis has intrinsic 5′- 3′ exonuclease activity. However, its association with DNA-PKcs allows it to acquire the endonuclease activity needed to process the overhangs (Ma *et al*

2002). It has been proposed that phosphorylation of Artemis by DNA-PK is also key for its endonuclease function (Niewolik *et al* 2006). In contrast, another report suggested that the kinase activity of DNA-PK is dispensable for Artemis endonuclease activation and instead DNA-PK autophosphorylation is important (Goodarzi *et al* 2006). Once processed the DNA ends can be ligated together by the DNA ligase IV/XRCC4 complex, which is thought to be recruited to the synaptic complex via an interaction with KU70/80 (Costantini *et al* 2007, Nick McElhinny *et al* 2000). Despite the fact that this complex can ligate DNA *in vitro*, association with the XRCC4-like factor (Cernunnos/XLF) has been shown to further potentiate this activity (Ahnesorg *et al* 2006, Hentges *et al* 2006, Lu *et al* 2007). Furthermore, the addition of XLF to this complex facilitates the ligation of non-compatible ends (Gu *et al* 2007, Tsai *et al* 2007) (Figure 1.6).

1.3.3 Alternative DSB repair pathways

In addition to the principal DSB repair pathways, HR and NHEJ, two other DSB repair pathways have recently been identified, which are single strand annealing (SSA) and alternative end-joining (Alt-EJ). Both these pathways are error-prone and result in chromosomal abnormalities such as deletions and chromosomal translocations.

1.3.3.1 Single strand annealing

SSA can either occur if the DSB has been formed between two repetitive sequences or if components of HR are lost such as RAD51 and BRCA2 (Stark *et al* 2004). As in HR, the first step in SSA is DNA end-resection, which is performed using the same set of proteins. When the homologous single stranded regions of the two repeats are exposed they can anneal to one another in a process facilitated by RAD52 (Singleton *et al* 2002). The displaced non-homologous 3′ ssDNA tails are removed by the flap endonucleases, excision repair cross complementation 1 (ERCC1)/xeroderma pigmentosum F (XPF) and the two strands are then ligated together. (Ahmad *et al* 2008, Al-Minawi *et al* 2008). SSA results in large deletions because efficient SSA depends on the length of the homologous sequence flanking the DSB being >30bp (Sugawara *et al* 2000).

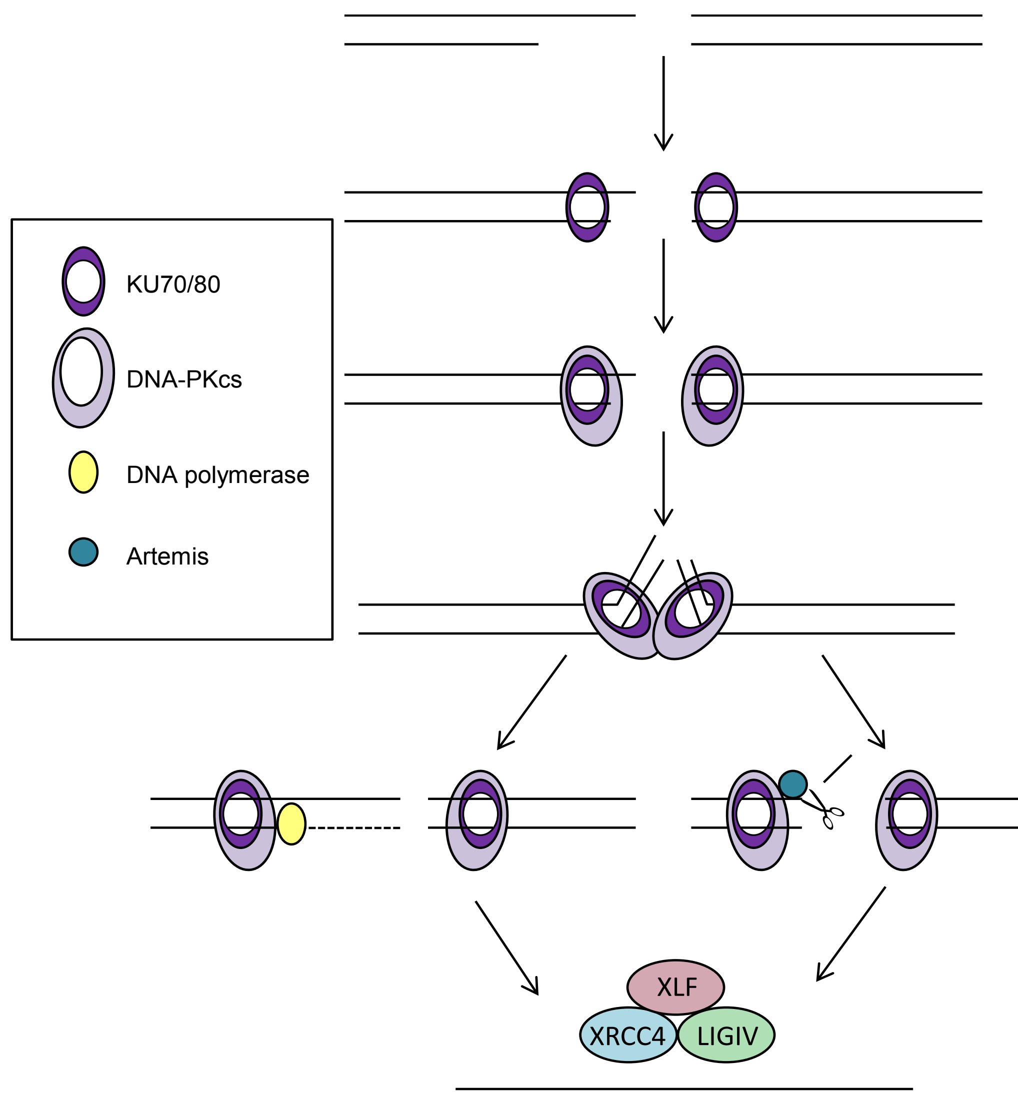


Figure 1.6 ***Repair of DSBs by NHEJ****. The KU70/80 heterodimer associates with the two ends of the DSB and recruits DNA-PKcs. DNA-PKcs tethers the DNA ends together to form a synaptic complex. Once formed, DNA-PKcs undergoes autophosphorylation, which makes the DNA ends accessible to NHEJ processing enzymes. Non-compatible DNA ends are processed either by nucleases such as Artemis, which remove the unwanted nucleotides or by polymerase, which fill in the gap. Subsequently, the blunt DNA ends are ligated together by the LigIV/XRCC4/XLF complex (Adapted from Wetterings and Chen 2008).*

1.3.3.2 Alternative end-joining

Alt-EJ has been described as a variation of classical end-joining that can repair DSBs independently of the core components of NHEJ namely, KU70, KU80, DNA ligase IV, XLF and XRCC4 (Boulton and Jackson 1996, Liang and Jasin 1996, Wang *et al* 2003a). Instead, Alt-EJ utilises proteins from HR, mismatch repair and base excision repair and is considered a backup end-joining pathway since it mainly operates when core NHEJ components are absent. The majority of Alt-EJ events require sequences of extensive microhomology (5-25bp) to anneal the DNA ends and therefore, Alt-EJ is sometimes referred to as microhomology mediated end-joining (MMEJ) (McVey and Lee 2008). The regions of microhomology flanking the break site are exposed as a result of limited MRN and CtIP- dependent DNA end-resection (Bennardo *et al* 2008, Rass *et al* 2009, Xie *et al* 2009, Yun and Hiom 2009). However, unlike HR, DNA end-resection in Alt-EJ is carried out in a BRCA1-independent manner (Yun and Hiom 2009). Annealing at the two microhomology regions is thought to produce 3′ ssDNA tails containing the non-complementary DNA, which are removed by ERCC1/XPF (Ahmad *et al* 2008). In addition, the ends are also processed by error-prone polymerase such as Polμ and Polλ and polynucleotide kinase (PNK) (Audebert *et al* 2006, Gu *et al* 2007, Simsek and Jasin 2010). Finally, ligation of the ends is independent of DNA ligase IV, but is facilitated by DNA ligase I and the DNA ligase III/XRCC1 complex (Liang *et al* 2008, Wang *et al* 2005). Recruitment of DNA ligase III/XRCC1 to DNA ends relies on PARP1, which can interact with both XRCC1 and DNA ligase III (Audebert *et al* 2006). Furthermore, PNK has been shown to be co-recruited with the DNA ligase III/XRCC1 complex to DNA ends where it is activated by XRCC1 (Whitehouse *et al* 2001).

1.3.4 Repair of programmed DSBs

DSBs occur as normal intermediates during the immunological processes, V(D)J recombination and CSR. These pathways are fundamental for generating a diverse antigen receptor repertoire, which is important for the immune system to be able to respond to a wide variety of antigens.

1.3.4.1 V(D)J recombination

The variable regions of antigen receptors are important for recognising and binding specific antigens. These regions are generated by randomly recombining dispersed variable (V), diversity (D) and joining (J) gene segments through a process known as V(D)J recombination. The T cell receptor (TCR) α and δ chains and the immunoglobulin heavy chain (IgH) variable regions are formed from V, D and J segments whilst the TCR β and γ chains and the Ig light chain (IgL) variable region only consists of the V and J segments. In the case of the IgH, the D and J segments are recombined first, followed by the joining of the DJ assembly with a V segment. The initial stages of V(D)J recombination are carried out by the lymphoid specific recombination-activating genes, RAG1 and RAG2, which form an endonuclease complex. The RAG1/RAG2 complex binds to recombination signal (RS) sequences flanking each V, D and J gene segments and introduces a single strand nick between the coding and RS sequences. A direct transesterification reaction then follows, in which the free hydroxyl group at the 3′ end of the coding sequence attacks the phosphodiester bond on the opposite strand of DNA forming a DSB. This cleavage generates covalently sealed hairpin coding ends and blunt 5′ phosphorylated signal ends (McBlane *et al* 1995, van Gent *et al* 1996). The four ends remain associated with the RAG1 and RAG2 in the stable synaptic complex, which serves as a scaffold to recruit proteins that facilitate repair (Yarnell Schultz *et al* 2001).

Completion of V(D)J recombination occurs through the classical NHEJ pathway. The hairpin coding ends are bound by KU70/80, which recruits DNA-PKcs, which in turn recruits and activates Artemis enabling it to open the hairpin ends through its acquired endonucleolytic activity (Ma *et al* 2002). The DNA end-processing step that follows is different between classical, repair-associated NHEJ and VDJ recombination. Unlike during classical NHEJ where the ends undergo limited processing to protect the integrity of the genome, the exposed coding ends in V(D)J recombination are subject to extensive processing. Firstly, the ends are trimmed by nucleases, then gap filling is performed by DNA synthesis (template-dependent) using DNA polymerases such as Polμ and Polλ and finally the template-independent polymerase TdT inserts random nucleotides to the end of the DNA. These modifications contribute to creating diversity in the variable regions of the antigen receptor, which are important for antigen specificity (Bertocci *et al* 2006). The two processed coding ends are then ligated together by the DNA ligase IV/XRCC4/XLF complex. In addition, ligation of the

two blunt signal ends results in the formation of a circular double stranded episome containing the unwanted intervening DNA sequence between the coding gene segments (Arnal and Roth 2007). Assembly of the IgH and IgL variable regions leads to expression of IgM, which is displayed on the cell surface of a B cell.

1.3.4.2 Class switch recombination

Following stimulation by antigens, the Ig receptors formed during V(D)J recombination undergo further genetic alterations through CSR. This process involves exchanging the initially expressed IgH constant region gene, Cμ with one of the downstream IgH constant region genes such as Cγ, Cα or Cε, resulting in a switching of Ig isotype from IgM to IgG, IgE or IgA respectively. Therefore, CSR allows the expression of antigen receptors that have the same antigen specificity, but a different effector function. Even though CSR is a deletional recombination reaction similar to V(D)J recombination, the method in which DSBs are formed is different between these two processes (Chaudhuri and Alt 2004). Unlike during V(D)J recombination, where DSBs are induced directly by the RAG endonuclease complex in a sequence specific manner, DSBs in CSR are created in a region specific manner involving the B cell specific enzyme activation-induced cytidine deaminase (AID) (Muramatsu *et al* 2000, Revy *et al* 2000). AID deaminates cytosine to uracil in switch (S) regions, which are repetitive sequences that precede the constant region gene segments. Prior to deamination the S regions are transcribed generating secondary structures such as R loops that provide the ssDNA required for AID activity (Chaudhuri *et al* 2003, Shinkura *et al* 2003, Sohail *et al* 2003). Following deamination, the inappropriate uracil is recognised and excised by uracil DNA glycosylase (UNG) creating an abasic site that is converted into a SSB by the endonucleases, APE1 and APE2 (Guikema *et al* 2007, Krokan *et al* 2002). Staggered DSBs can be generated through the close proximity of two SSBs on opposite strands of the DNA. Alternatively, the uracil-guanine mismatches can be recognised and processed by components of the mismatch repair pathway resulting in the formation of a DSB (Stavnezer and Schrader 2006). The staggered DNA ends are then processed to generate blunt ends DSBs or overhangs that are short enough for efficient ligation. This could be achieved by error prone polymerases such as Polη, which fill in the overhangs by DNA synthesis or by endonucleases such as the ERCC1/XPF complex, which remove the overhangs (Stavnezer *et al* 2008). Subsequent recombination between the two breaks located within these switch regions results in exchange

of the heavy constant region with deletion of the intervening sequences, including Cμ, which is released as an episomal circle.

Finally, ligation of the DNA ends involves proteins from the classical NHEJ including DNA-PKcs, KU70/80 and DNA ligase IV/XRCC4/XLF (Casellas *et al* 1998, Franco *et al* 2008, Manis *et al* 1998, Yan *et al* 2007a). Initially, Artemis was considered to be dispensable for end-joining in CSR (Rooney *et al* 2005). However, recent reports indicate that it may also function in CSR (Du *et al* 2008, Franco *et al* 2008, Rivera-Munoz *et al* 2009). Interestingly, CSR is not totally abrogated in the absence of these proteins. A recent study revealed that CSR in cells deficient in the core components of classical NHEJ could occur through two different Alt-EJ pathways depending on which NHEJ component was missing. The pathway that operates in the absence of XRCC4 or DNA ligase IV utilises the upstream classical NHEJ proteins such as KU and substitutes the DNA ligase for either DNA ligase 1 or DNA ligase III. Furthermore, end-joining in this pathway is mediated by regions of microhomology and therefore is likely to require DNA end-resection by MRN and CtIP (Boboila *et al* 2010, Dinkelmann *et al* 2009, Lee-Theilen *et al* 2011). The other Alt-EJ pathway functions in the absence of both KU70 and DNA ligase IV and also utilises microhomology to ligate ends. However, in contrast to XRCC4 or DNA ligase IV deficient cells, this pathway also generates a number of direct joins indicating that the proteins involved in the two pathways are different (Boboila *et al* 2010). Consistent with this data, Mansour *et al* (2010) demonstrated that KU deficient cells did not necessarily require microhomology for efficient ligation (Mansour *et al* 2010). These data support the notion that not all Alt-EJ events are reliant on microhomology (McVey and Lee 2008, Zha *et al* 2009).

In addition to the NHEJ machinery, CSR has also been shown to be affected by components of the DDR as illustrated by the observed CSR defects in cells deficient in ATM (Lumsden *et al* 2004, Pan *et al* 2002, Reina-San-Martin *et al* 2004), MRN (Dinkelmann *et al* 2009), H2AX (Reina-San-Martin *et al* 2003), MDC1 (Lou *et al* 2006), RNF8 (Li *et al* 2010, Santos *et al* 2010) and 53BP1 (Manis *et al* 2004, Ward *et al* 2004). These observations highlight the essential requirement for the ubiquitin-dependent DDR pathway in immune system development.

1.3.5 Human DSB repair syndromes

Repair of DSBs is crucial for maintaining the integrity of the genome and for normal developmental processes, in particular immune system development. Mutations in DSB repair genes result in the inability to properly repair DNA damage and are the underlying cause for a number of human autosomal recessive genetic syndromes.

1.3.5.1 Ataxia-Telangiectasia

The first human DSB repair disorder to be described was Ataxia-Telangiectasia (A-T), which is an early onset disease caused by mutations in the *ATM* gene. Hallmark clinical features of A-T include progressive cerebellar neurodegeneration that causes ataxia (lack of balance) and dysarthria (speech impediment), telangiectasia (diluted blood vessels) mainly of the eyes, face and ears, immunodeficiency that affects both the cellular and humoral components of the immune system, profound radiosensitivity and a elevated predisposition to the development of cancer, in particular leukemia and lymphoma (Lavin and Shiloh 1997, Taylor *et al* 1975).

1.3.5.2 Syndromes affecting the MRN complex

Nijmegen Breakage Syndrome (NBS) is caused by mutations in the *NBS1* gene. Unlike A-T, NBS patients primarily present with mild to moderate microcephaly, mental retardation, growth retardation and ‘bird-like’ facial features. However, similar to A-T, NBS patients exhibit a hypersensitivity to IR, immunodeficiency affecting both the humoral and cellular components of the immune system and have increased predisposition to lymphoid malignancies (Digweed and Sperling 2004).

Initially, it was thought that mutations in *MRE11* and *RAD50* genes caused the hereditary disorders, Ataxia-Telangeictasia-Like-Disorder (A-TLD) and Nijmegen Breakage Syndrome-Like Disorder (NBSLD) respectively (Stewart *et al* 1999, Taylor *et al* 2004, Waltes *et al* 2009). However, recently, two patients have been identified that have mutations in *MRE11*, but have NBSLD and not A-TLD indicating that *MRE11* mutations can give rise to a wider range of clinical features than was previously thought (Matsumoto *et al* 2011). As the name suggests A-TLD patients have a clinical phenotype resembling that of A-T, whereas patients with NBSLD exhibit clinical symptoms mirroring those of NBS. Interestingly, unlike A-T and NBS patients, A-TLD and NBSLD patients are not immunodeficient despite the involvement of MRN complex in both V(D)J recombination and CSR. Furthermore, despite the low

numbers of patients identified with these disorders, a predisposition to lymphoid malignancies has not been observed, although two A-TLD patients did develop lung cancer (Uchisaka *et al* 2009).

1.3.5.3 RIDDLE syndrome

RIDDLE (Radiosensitivity, Immunodeficency, Dysmorphic features and Learning Difficulties) syndrome is a newly described human DSB repair syndrome that is caused by mutations in the *RNF168* gene. To date only two patients have been identified with RIDDLE syndrome whose clinical features include short stature, mild hypersensitivity to IR, dysmorphic facial features, learning difficulties and immunodeficiency. The immunodeficiency in these patients is presented as abnormal immunoglobulin levels indicating that it is likely to be caused by a CSR rather than V(D)J recombination defect (Devgan *et al* 2011, Stewart *et al* 2007). In contrast to the first patient, the second patient also exhibited ataxia and telangiectasia (Devgan *et al* 2011).

1.3.5.4 Seckel syndrome

Seckel syndrome (SCKL) is characterised by growth retardation, mental retardation, microcephaly and 'bird-like' facial features. In addition, some Seckel patients have also been reported to have lymphoma (Alderton *et al* 2004). Unlike other DSB repair disorders, Seckel syndrome demonstrates genetic heterogeneity. Genetic mapping studies have revealed four Seckel syndrome causative genomic loci, namely *Skl 1-4,* however to date only two genetic defects have been identified in this disorder (Borglum *et al* 2001, Faivre *et al* 2002, Goodship *et al* 2000, Kilinc *et al* 2003). Some individuals with Seckel syndrome have mutations in ATR and have ATR-Seckel syndrome (also referred to as SCKL1), whereas other individuals have been reported that have mutations in the gene encoding pericentrin and have PCNT-Seckel syndrome (also known as SCKL4) (Griffith *et al* 2008, O'Driscoll *et al* 2003).

1.3.5.5 Ligase IV syndrome

DNA ligase IV was the first component of the core NHEJ machinery to be found mutated in a human syndrome. All the patients diagnosed so far exhibit hypersensitivity to IR and with the exception of the first patient, some form of immunodeficiency, typically pancytopenia (Riballo *et al* 1999). This is likely to be due to the involvement of DNA ligase IV in V(D)J recombination and consequently these patients are all predisposed to lymphomas.

Furthermore, the vast majority of patients also exhibit clinical features that overlap with NBS including microcephaly, developmental delay, severely delayed growth and 'bird-like' facial features. Some additional clinical features also overlap with those observed in A-T patients such as the presence of telangiectasia and type 2 diabetes (Ben-Omran *et al* 2005, Buck *et al* 2006b, Enders *et al* 2006, O'Driscoll *et al* 2001).

1.3.5.6 Radiosensitive Severe Combined Immunodeficiency (RS-SCID)

To date there are two forms of RS-SCID, which are Artemis-dependent RS-SCID and XLF-dependent RS-SCID caused by mutations in *ARTEMIS* and *XLF* genes respectively. Complete loss of Artemis protein is associated with the most severe form the disease that typically presents with $T^{-}B^{-}NK^{+}$ SCID, as well as a hypersensitivity to IR (Moshous *et al* 2001). Patients with hypomorphic mutations in *ARTEMIS* are less severely affected, as they do develop T and B lymphocytes, but at reduced levels (Moshous *et al* 2003). The severity of the immunodeficiency in patients with Artemis-dependent RS-SCID highlights the critical role of Artemis in NHEJ DSB repair during V(D)J recombination (Rooney *et al* 2003). Furthermore, patients with hypomorphic mutations also have hypogammaglobulinaemia affecting IgG and IgA indicating a role for Artemis during the repair of CSR associated DSBs (Du *et al* 2008, Moshous *et al* 2003). Unsurprisingly, these patients are predisposed to develop lymphoid malignancies (Moshous *et al* 2003).

The immunodeficiency observed in XLF-dependent RS-SCID is similar to that observed in the less severely affected Artemis-dependent RS-SCID patients, with most patients exhibiting a hypersensitivity to IR, severe lymphopenia involving both B and T lymphocytes and reduced or absent IgG and IgA. These characteristics support a role for XLF in V(D)J recombination and CSR. Unlike Artemis-dependent RS-SCID, these patients also present with non-immunological clinical features including microcephaly, growth retardation and dysmorphic facial features. This highlights the importance of XLF in the repair of DSBs outside of the immune system (Buck *et al* 2006a, Dutrannoy *et al* 2010).

1.3.6 Mediators

Mediators are proteins that act downstream of the ATM and ATR kinases and are important for transducing the DNA damage signal to effector proteins. These proteins facilitate the interactions between ATM/ATR and their substrates and/or act as scaffolds to aid recruitment

of other DDR proteins to the damage sites. MDC1, PTIP, BRCA1, Microcephalin (MCPH1) and 53BP1 are mainly involved in the ATM pathway whereas TOPBP1 and Claspin have been proposed to co-regulate the ATR pathway (Harper and Elledge 2007). All these proteins contain protein-protein interaction modules such as BRCT domains and/or FHA domains. Furthermore, apart from the E3 ubiquitin ligase activity of BRCA1, these proteins have no characterised intrinsic enzymatic activity (Mohammad and Yaffe 2009). Here I will focus on MDC1, BRCA1 and 53BP1, proteins which are orthologues of the budding yeast *Saccharomyces cerevisiae (S.cerevisiae)* RAD9 and the fission yeast *Schizosaccharomyces pombe* (*S.pombe)* Crb2 (FitzGerald *et al* 2009).

1.3.6.1 MDC1

MDC1 is a large nuclear protein that is composed of several distinct sequence domains. Besides a N-terminal FHA domain and two BRCT domains at its C-terminus, MDC1 also features a unique repeat region in the middle of the protein known as the proline, serine and threonine (PST) region, a cluster of TQXF motifs and a SDTD repeat region. Localisation of MDC1 to DSBs is dependent on γH2AX, as MDC1 failed to form foci in $H2AX^{-/-}$ MEFs following IR. Phosphorylated serine 139 of H2AX produces a binding site recognised by the BRCT domains of MDC1 (Stucki *et al* 2005). However, H2AX is constitutively phosphorylated on tyrosine 142 by the kinase WSTF (Williams syndrome transcription factor), which prevents MDC1 from being recruited. Consequently, the binding of MDC1 to γH2AX was shown to be dependent on tyrosine 142 dephosphorylation by the tyrosine phosphatases EYA1/3 (Cook *et al* 2009, Krishnan *et al* 2009). Once bound to γH2AX, MDC1 plays a crucial role as a core scaffold in DDR where it recruits several key DDR proteins to the DSB. MDC1 interacts with NBS1 through multiple SDTD motifs that are constitutively phosphorylated by CK2 stabilising the MRN complex at the DNA ends (Lukas *et al* 2004a, Melander *et al* 2008, Spycher *et al* 2008). In addition, MDC1 also associates with activated ATM via its FHA domain facilitating the retention of ATM at sites of DSBs. These interactions result in propagation of γH2AX along the chromatin through a MDC1-dependent positive feedback loop (Lou *et al* 2006). Furthermore, phosphorylation of MDC1 on its TQXF motifs by ATM aids recruitment of RNF8, ultimately triggering the ubiquitin dependent component of the ATM cascade (Huen *et al* 2007, Kolas *et al* 2007, Mailand *et al* 2007). In the absence of MDC1, many proteins involved in DNA repair and checkpoint activation such

as 53BP1, BRCA1 and the MRN complex fail to accumulate at sites of DNA damage. As a consequence, these cells have defective ATM signalling and fail to activate the intra-S phase and G2/M phase checkpoints (Goldberg *et al* 2003, Stewart *et al* 2003). Therefore, MDC1 plays an important role in amplification of the damage signal.

MDC1 has also been shown to function in DNA DSB repair. siRNA studies demonstrated that MDC1 promotes HR by directly interacting with RAD51 and is required for the recruitment of RAD51 to ionising radiation-induced foci (IRIF) and for maintaining RAD51 protein stability (Zhang *et al* 2005). In addition, MDC1 increases the rate of NHEJ at dysfunctional telomeres, which may be due to its ability to interact and regulate DNA-PK autophosphorylation, although currently this is unknown (Dimitrova and de Lange 2006, Lou *et al* 2004). In support of its importance in the DDR, MDC1 knockout mice are growth retarded, sensitive to IR, have a mild CSR defect and have gross genomic instability in response to IR (Lou *et al* 2006).

More recently, MDC1 has been reported to be involved in mitotic progression, independent of DNA damage. MDC1 regulates the metaphase to anaphase transition through its ability to interact directly with the APC/C and modulates its E3 ubiquitin ligase activity. As a result, mitotic cells deficient in MDC1 have reduced APC/C activity and arrest in metaphase (Townsend *et al* 2009).

1.3.6.2 BRCA1

BRCA1 is a tumour suppressor protein that is important in maintaining genome stability. Germ line heterozygous mutations in *BRCA1* lead to an enhanced predisposition to both breast and ovarian cancer. In addition to its two C-terminal BRCT domains, BRCA1 also contains an N-terminal RING domain that enables it to interact with E2 conjugating enzymes. BRCA1 has been implicated in a multitude of cellular processes including checkpoint activation, DNA repair and transcriptional regulation (Boulton 2006, Huen *et al* 2010b).

The RING domain of BRCA1 mediates its stable association with another RING/BRCT domain containing protein, BARD1 (Brzovic *et al* 2001). This heterodimer serves as an E3 ubiquitin ligase at sites of DSBs and facilitates the formation of lysine-6 (K6)-linked ubiquitin chains (Morris and Solomon 2004). Recently, it has been reported that conjugation of SUMO-1 on BRCA1 by PIAS1 together with its E2 conjugating enzyme, UBC9 stimulates this E3 ubiquitin ligase activity (Morris *et al* 2009). Despite, the function of the K6-linked ubiquitin

chains being unknown, they are essential for BRCA1-mediated suppression of genomic instability (Morris *et al* 2006, Ruffner *et al* 2001).

In response to DSBs, BRCA1-BARD1 heterodimers form a number of protein super-complexes. One of these complexes, BRAC1-A consists of RAP80, Abraxas, BRCC36, BRCC45 and MERIT40 and is important for the localisation of BRCA1 to sites of DSBs (Feng *et al* 2009, Kim *et al* 2007, Shao *et al* 2009b, Sobhian *et al* 2007, Wang *et al* 2007, Wang *et al* 2009). Abraxas interacts with the BRCT domains of BRCA1 and links it to RAP80, which targets BRCA1 to the DSB by binding to K63-linked ubiquitin chains through its UIM domains. Cells depleted of RAP80 and Abraxas exhibit impaired accumulation of BRCA1 at sites of DSBs.

Once recruited to DSBs by this complex, BRCA1 plays a role in G2/M checkpoint activation. This is partly by regulating the expression, phosphorylation and localisation of CHK1, a known regulator of the G2/M checkpoint (Yarden *et al* 2002). However, prior phosphorylation of BRCA1 on serine 1423 by ATM is required for activation of the G2/M checkpoint (Xu *et al* 2001a). The importance of BRCA1 in G2/M checkpoint activation is illustrated by the fact that cells lacking functional BRCA1 exhibit severe defects in checkpoint activation (Xu *et al* 1999b).

In addition to the G2/M checkpoint, BRCA1 has also been shown to be involved in the intra-S-phase checkpoint that is activated in response to replication stress such as stalled replication forks (Xu *et al* 2001a). This requires ATM-dependent phosphorylation of BRCA1 on serine 1387 and involves interactions between BRCA1 with BACH1 and TOPBP1, which together form the BRCA1-B complex (Greenberg *et al* 2006, Xu *et al* 2002). Absence of any of these three proteins results in the failure of cells to reduce the rate of DNA replication when irradiated, a phenomenon known as radioresistant DNA synthesis (RDS) and a consequence of a defective intra-S-phase checkpoint (Greenberg *et al* 2006).

The involvement of BRCA1 in HR-mediated DNA repair has long been known, but is poorly understood. BRCA1 facilitates the recruitment of RAD51 to DSBs through its association with BRCA2 (Bekker-Jensen *et al* 2006, Chen *et al* 1998a, Greenberg *et al* 2006, Scully *et al* 1997b). In the absence of BRCA1, RAD51 fail to form foci and consequently cells lacking functional BRCA1 are deficient in homology-mediated repair (Greenberg *et al* 2006, Moynahan *et al* 1999, Scully *et al* 1999). Furthermore, this interaction between BRCA1 and

BRCA2 is mediated by PALB2. PALB2 directly associates with BRCA1 and promotes the accumulation of BRCA2 and RAD51 to DSBs (Sy *et al* 2009, Zhang *et al* 2009b). The interaction between PALB2 and BRCA1 is crucial for HR since mutations in the PALB2 binding site in BRCA1 result in compromised HR (Zhang *et al* 2009b). Despite these studies, the mechanism by which BRCA1 promotes HR remains unclear. More recently, BRCA1 has been shown to be involved in DNA end-resection through its ability to interact with components of the BRCA1-C complex, CtIP and the MRN complex (Chen *et al* 2008). In support of a role for BRCA1 in DNA end-resection, accumulation of RPA at sites of DNA damage was found to be impaired in BRCA1 defective cells (Schlegel *et al* 2006). Interestingly, DNA end-resection is a prerequisite for RAD51 nucleoprotein filament formation, therefore the reason behind the lack of RAD51 foci formation may be due to an inability of BRCA1 deficient cells to efficiently process the ends of DSBs.

Further to its roles in checkpoint activation and DNA repair, BRCA1 also functions in transcription regulation. BRCA1 has been shown to be a component of the general transcription machinery as it interacts with the RNA polymerase II holoenzyme complex, in part through binding to RNA helicase A (Anderson *et al* 1998, Scully *et al* 1997a). Although BRCA1 has not been demonstrated to bind DNA in a sequence specific manner, it has been established that it can interact with various transcription factors and function as a co-regulator. In response to DNA damage, BRCA1 cooperates with p53 to stimulate the expression of some of its target genes, in particular those involved in cell cycle arrest and DNA repair such as p21 and GADD45 (Harkin *et al* 1999, MacLachlan *et al* 2002, Ouchi *et al* 1998, Zhang *et al* 1998). Furthermore, it has been reported that BRCA1 can also induce the expression of p21 and GADD45 in a p53-independent manner (Harkin *et al* 1999, Somasundaram *et al* 1997). In the case of GADD45, this is mediated through interactions between BRCA1 and the transcription factors, Oct1 and NF-Y (Fan *et al* 2002). In addition to p53, BRCA1 also acts as a co-activator for STAT1 (Ouchi *et al* 2000), NF-κB (Benezra *et al* 2003), ATF1 (Houvras *et al* 2000) and androgen receptor-dependent transcription (Park *et al* 2000, Yeh *et al* 2000). Moreover, BRCA1 has also been shown to function as a co-repressor of transcription, as it inhibits the transcriptional activity of the estrogen receptor (Fan *et al* 2001) and c-myc (Wang *et al* 1998). Finally, through its BRCT domains, BRCA1 interacts with various transcriptional regulatory proteins, which may contribute to its ability to function as a transcriptional co-regulator. These include the HATs, CBP (CREB binding protein) and

p300, which are general transcriptional co-activators (Pao *et al* 2000), as well as HDAC1/2 and the retinoblastoma susceptibility protein, RB1 (Yarden and Brody 1999). The biological consequences of many of these interactions still remain to be elucidated. However, a recent study revealed that the BRCT domains of BRCA1 preferentially bound to methylated p300. Interestingly, methylation of p300 by the co-activator associated arginine methyltransferase 1 (CARM1) has been demonstrated to be increased in response to DNA damage suggesting that the damage-inducible interaction of BRCA1 with co-activators may potentiate its role in regulating transcription (Lee *et al* 2011).

1.3.6.3 53BP1

53BP1 is comprised of 1972 amino acids and contains several domains including two tandem BRCT domains at the C-terminus and a tandem Tudor domain that recognises methylated histones (Callebaut and Mornon 1997, Charier *et al* 2004). Theses domains are also present in *S.cerevisiae* RAD9 and *S.pombe* Crb2. Therefore, even though MDC1 and BRCA1 share some functional similarities to RAD9 and Crb2, 53BP1 is considered to be the most closely related orthologue (FitzGerald *et al* 2009). 53BP1 also contains a GAR domain (glycine/arginine rich region) that is methylated by the protein arginine methyltransferase 1 (PRMT1) and is required for the DNA binding activity of 53BP1 (Boisvert *et al* 2005). 53BP1 also possess several PIKK S/TQ (Serine/Threonine-Glutamine) consensus sites, which are phosphorylated in a PIKK-dependent manner following IR and UV (Jowsey *et al* 2007) (Figure 1.7). 53BP1 has also been shown to be a substrate for CDKs, as mapping of phosphorylation networks revealed that 53BP1 contains multiple CDK-dependent phosphorylation sites (Linding *et al* 2007). The function of many of these phosphorylation sites is currently unknown, although the roles of some of these sites have been characterised. ATM-dependent phosphorylation of 53BP1 on serine 25 is required for the interaction between 53BP1 and PTIP. Abrogation of this interaction results in a defective cellular response to DNA damage (Munoz *et al* 2007). Additionally, phosphorylation of 53BP1 on serine 1219 by ATM has been reported to function in G2/M cell cycle arrest following IR (Lee *et al* 2009). However, ATM-mediated phosphorylation of 53BP1 is dispensable for the recruitment of 53BP1 to sites of DSBs (Schultz *et al* 2000)

The Tudor domain of 53BP1 is important for its localisation to sites of DNA DSBs through its ability to interact with methylated histones. In yeast it has been shown that *S. cerevisiae*

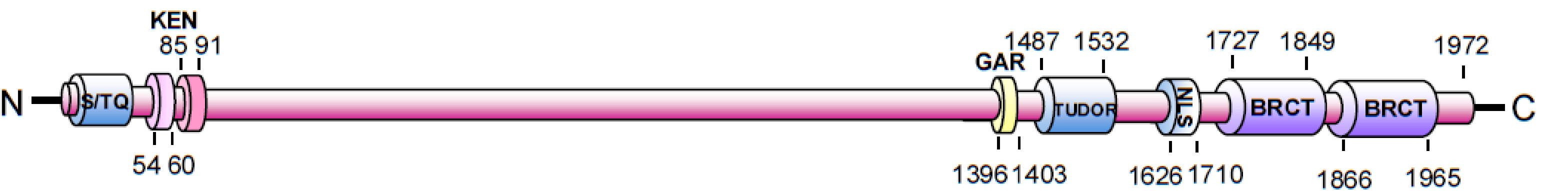


Figure 1.7 ***Schematic representation of the 53BP1 protein.*** *53BP1 is 1972 amino acids long and contains several functional domains including two C-terminal BRCT domains, a Tudor domain and a GAR domain.*

RAD9 is recruited to DNA DSBs via binding of its Tudor domain to H3K79me2 (Grenon *et al* 2007, Wysocki *et al* 2005), whereas *S.pombe* Crb2 is recruited via binding to H4K20me2 (Greeson *et al* 2008, Sanders *et al* 2004). Both these modifications have been implicated in the recruitment of 53BP1 to chromatin. The involvement of H3K79me2 in the recruitment of 53BP1 was first reported by Huyen *et al* (2004), who showed that the Tudor domain of 53BP1 binds to H3K79me2 both *in vitro* and *in vivo* (Huyen *et al* 2004). Point mutations in the Tudor domain abolished this interaction and impaired 53BP1 foci formation. Furthermore, depletion of hDot1/KMT4, the histone methyltransferase that di-methylates H3K79 also reduced foci formation indicating that methylation of H3K79 by hDot1/KMT4 was important for 53BP1 recruitment (Huyen *et al* 2004). However, subsequent reports suggested a predominant role for H4K20me2 in recruitment of 53BP1 to foci. 53BP1 was found to bind to H4K20me2 with greater affinity than to H3K79. In addition, reduction of H4K20 methylation levels through knockdown of the H4K20 histone methyltransferases, MMSET, Set8/KMT5A, Suv4-20h1/KMT5B and Suv4-20h2/KMT5C decreased 53BP1 foci formation (Botuyan *et al* 2006, Pei *et al* 2011, Yang *et al* 2008a). It is still unclear why 53BP1 requires two methylation marks for its recruitment to chromatin. However, it has been suggested that these two methylation marks may function redundantly in humans (FitzGerald *et al* 2009). These methylation marks are present on chromatin independently of DNA damage and their levels are not altered following DNA damage. Furthermore, they are normally found buried within the chromatin. Initially, it was postulated that DNA damage induced passive relaxation of the chromatin structure surrounding the DSB, thereby allowing 53BP1 to bind to H3K79me2/H4K20me2 (Huyen *et al* 2004, Sanders *et al* 2004). However, more recently, it has been demonstrated that ubiquitylation of histones by RNF8/RNF168, phosphorylation of H2AX and acetylation of histones by Tip60 facilitate the recruitment of 53BP1 to DSBs by allowing H3K79me2/H4K20me2 to become accessible to 53BP1 through the formation of a more open chromatin structure (Doil *et al* 2009, Murr *et al* 2006). Even though recruitment of 53BP1 to DSBs is not dependent on it directly interacting with other DDR proteins, its retention at DSBs is mediated through interacting with γH2AX (Celeste *et al* 2003, Fernandez-Capetillo *et al* 2002, Ward *et al* 2003a). Currently it is unknown what the functional significance of this interaction is. However, two recent studies revealed that the interaction between the BRCT domains of *S.pombe* Crb2 and γH2AX is important to maintain cell cycle arrest mediated by the G2/M DNA damage checkpoint (Sanders *et al* 2010, Sofueva

et al 2010). Furthermore, sumoylation of 53BP1 by PIAS4 has been shown to be necessary for the accumulation of 53BP1 at DSBs, although the biological relevance of this modification is currently unknown (Galanty *et al* 2009).

53BP1 has been implicated in cell cycle checkpoint activation and maintenance following IR. Human cells depleted of 53BP1 by siRNA and mouse *53BP1*$^{-/-}$ cells have a mild G2/M checkpoint defect. This was only observed after exposure to low, but not high doses of IR (DiTullio *et al* 2002, Fernandez-Capetillo *et al* 2002, Wang *et al* 2002). However, in contrast to these results, other groups have reported no defects in G2/M checkpoint activation in the absence of 53BP1 (Morales *et al* 2003, Shibata *et al* 2010, Ward *et al* 2003b). Moreover, a recent study has indicated that 53BP1 may not be required for G2/M checkpoint activation, but rather may play a role in maintaining the IR-induced G2/M checkpoint arrest (Shibata *et al* 2010). Partial intra-S-phase checkpoint defects have also been observed in cells lacking 53BP1 following IR (Wang *et al* 2002). These defects may be due to the requirement of 53BP1 in the recruitment of the intra-S-phase checkpoint protein, Rif1 to sites of DSBs (Silverman *et al* 2004).

One of the most recent functions of 53BP1 to be identified is that it is involved in limiting or suppressing DNA end-resection. Mice homozygous for exon 11 deletion (Δ11) isoform of BRCA1 (*BRCA1*$^{\Delta11/\Delta11}$) die during embryonic development. However, this embryonic lethality can be rescued by loss of one or both copies of p53, but as a consequence, the viable mice develop multiple types of tumours and age prematurely (Xu *et al* 2001c). It is likely that the increased genome instability and tumour predisposition observed in the *BRCA1*$^{\Delta11/\Delta11}$ /p53$^{+/-}$ mice is due to reduced HR caused by defective DNA end-resection. Interestingly, loss of 53BP1 also rescues the embryonic lethality of the *BRCA1*$^{\Delta11/\Delta11}$ mice, as well as the tumour susceptibility and premature aging (Cao *et al* 2009). Moreover, the reduced HR caused by loss of BRCA1 is significantly enhanced in the absence of 53BP1 as a consequence of elevated levels of DNA end-resection (Bunting *et al* 2010). This suggests that a function of 53BP1 may be to limit HR by controlling the level of processing at DNA ends. This observation is supported by further investigations conducted in 53BP1 depleted *BRCA1* null MEFs and embryonic stem cells (ES) which showed restored RAD51 foci formation and partial, although not complete, restoration of HR compared to conditional *BRCA1*-deleted cells (Bouwman *et al* 2010). However, the ability of *BRCA1*$^{\Delta11/\Delta11}$ *53BP1*$^{-/-}$ cells to perform

DNA end-resection is dependent on ATM and CtIP, as was shown from the failure of *BRCA1*$^{\Delta11/\Delta11}$ *53BP1*$^{-/-}$ cells to restore RPA phosphorylation in response to IR when ATM was inhibited or CtIP was depleted (Bunting *et al* 2010). In light of these observations, it has been postulated that 53BP1 acts as a barrier to DNA end-resection and that this activity of 53BP1 may be important for preventing HR-mediated DNA repair of breaks that should be repaired by NHEJ (Boulton 2010).

One of the key functions of 53BP1 was discovered from 53BP1 knockout (*53BP1*$^{-/-}$) mouse models. 53BP1$^{-/-}$ mice are severely impaired in performing CSR and consequently are immunodeficient (Manis *et al* 2004, Ward *et al* 2004). The CSR defect observed in these mice is more severe than that exhibited by mice null for H2AX, MDC1 and RNF8, highlighting the importance of 53BP1 during immune system development. In *53BP1*$^{-/-}$ cells there is almost a complete loss of long-range CSR and a concomitant increase in the frequency of short-range intra-switch recombination (Manis *et al* 2004, Reina-San-Martin *et al* 2007, Ward *et al* 2004). Currently, two roles for 53BP1 in CSR have been proposed. One of these roles is that 53BP1 facilitates end-joining through promoting and/or maintaining synapsis of two distal switch regions (Reina-San-Martin *et al* 2007). Alternatively, it has suggested that 53BP1 promotes long-range CSR by protecting the DNA ends from unwanted resection. Conversely, loss of 53BP1 results in an ATM-dependent increase in resection leading to short-range joining between regions of microhomology within the switch regions by Alt-EJ (Bothmer *et al* 2010). However, as discussed by Bothmer *et al* (2010), it is unlikely that a switch from C-NHEJ to Alt-EJ can solely account for the profound defects in CSR in *53BP1*$^{-/-}$ B cells. Therefore, it has been suggested that both these functions of 53BP1 are required to explain the dominant effect of 53BP1 on CSR (Bothmer *et al* 2010).

In contrast to its importance in CSR, 53BP1 has been described as being dispensable for V(D)J recombination (Manis *et al* 2004, Ward *et al* 2004). However, a recent report has demonstrated that 53BP1 does play a role in V(D)J recombination by facilitating long-range DNA end-joining. Loss of 53BP1 resulted in a decrease in V(D)J recombination between distal gene segments (Difilippantonio *et al* 2008). Interestingly, 53BP1 has been shown to promote the fusion of deprotected telomeres by increasing chromatin mobility and thereby increasing the chance that the two telomere ends come within sufficient proximity to allow the NHEJ machinery to bind (Dimitrova *et al* 2008). Therefore, it has been suggested that by increasing the mobility of the local chromatin surrounding the break site, 53BP1 is able to

facilitate NHEJ by bringing together distally located DNA ends, such as those DNA ends created by DSBs during V(D)J recombination and CSR, that otherwise would have a low probability of encountering each other (Difilippantonio *et al* 2008, Dimitrova *et al* 2008).

Taken together the involvement of 53BP1 in cell cycle checkpoint control and DNA repair indicates that 53BP1 plays an integral role in maintaining genomic stability and suppressing tumourigenesis. In support of this, *53BP1*$^{+/-}$ and *53BP1*$^{-/-}$ mice exhibit chromosomal aberrations, which are indicative of a defect in DNA repair and are prone to developing tumours, in particular lymphoma, that could be enhanced when combined with a loss of p53 (Morales *et al* 2003, Morales *et al* 2006, Ward *et al* 2003b, Ward *et al* 2005). Loss of one or both alleles of *53BP1* greatly accelerated tumour development in a *p53* null background. Interestingly in human tumours, loss of 53BP1 has been shown to correlate with cancer progression (Gorgoulis *et al* 2005). Moreover, loss of 53BP1 has been found to be associated with triple-negative breast cancer and *BRCA1* and *BRCA2*-mutated breast cancer (Bouwman *et al* 2010).

53BP1 was originally identified as a p53 interacting protein in a yeast two-hybrid screen. The core DNA binding region of p53 was found to interact with the BRCT domains of 53BP1. Furthermore, the conformation of p53 appears to be crucial for this interaction because 53BP1 failed to bind to mutant p53 (R175H) (Iwabuchi *et al* 1994). In a subsequent report, 53BP1 was proposed to function as a transcriptional co-activator of p53 based on its ability to enhance p53 transcriptional activity in a reporter assay (Iwabuchi *et al* 1998). However, this function of 53BP1 appeared unlikely when the crystal structure of the 53BP1-p53 interaction was solved. These studies revealed that the p53 residues involved in binding to 53BP1 were the same as those required for p53 to bind to DNA demonstrating that it is sterically impossible for p53 to bind simultaneously to 53BP1 and the p53 DNA binding sites present in the promoters of p53 target genes (Derbyshire *et al* 2002, Joo *et al* 2002). Interestingly, in contrast to previous data, recent reports have shown that the interaction between 53BP1 and p53 is dependent on lysine methylation and involves the Tudor domain of 53BP1 (Huang *et al* 2007a, Kachirskaia *et al* 2008). Huang *et al* (2007) revealed that the interaction between 53BP1 and p53 required di-methylation of lysine 370 of p53 and this association resulted in an increase in p53 transactivation, thereby supporting a role for 53BP1 in transcriptional regulation (Huang *et al* 2007a). In addition, 53BP1 was shown to bind to di-methylated lysine 382 of p53 in response to DNA damage. However, the functional consequence of this

interaction is currently unknown (Kachirskaia *et al* 2008). Taken together these observations indicate that 53BP1 does associate with p53 and may potentially act as a transcriptional regulator of p53.

1.4 p53

Since its discovery in 1979, the role of the p53 protein in cancer has been intensively studied (Levine and Oren 2009). p53 is a sequence specific DNA binding transcription factor that has been described as the 'guardian of the genome' because of its role in coordinating diverse cellular responses to a plethora of cellular stresses including DNA damage, hypoxia and oncogene activation (Lane 1992). Depending on the type and level of cellular stress, p53 can induce cell cycle arrest, DNA repair, cellular senescence, apoptosis, differentiation, cell metabolism and angiogenesis. Transactivation of its vast array of target genes is essential for p53 to impact on so many cellular processes, although it has been suggested that some effects of p53 may be independent of transcription (Bensaad and Vousden 2007, Marchenko and Moll 2007, Vousden and Lane 2007). In light of the complex role of p53, this section of the introduction will mainly focus on the regulation and functions of p53 in response to DNA damage.

1.4.1 p53 has a fundamental role in tumour suppression

p53 is a crucial tumour suppressor gene, as evidenced by the fact that >50% of all human cancers harbour somatic mutations in *TP53* gene and that dysregulation of the p53 pathway commonly occurs in tumours that retain a wild type *TP53* gene. Most of the mutations are missense mutations resulting in the production of a dominant negative protein that is capable of complexing with wild type p53 and overriding normal p53 cellular functions (de Vries *et al* 2002). In addition, germline mutations in *TP53* are the underlying cause of Li-Fraumeni syndrome, which is characterised by a susceptibility to a broad spectrum of cancers including breast cancer, sarcomas, brain tumours and adrenal cortical carcinoma (Malkin *et al* 1990, Varley 2003). Further confirmation of the importance of p53 in tumour suppression came from the *TP53* knockout mouse, which develops cancer rapidly and with 100% penetrance (Donehower *et al* 1992, Jacks *et al* 1994).

1.4.2 Structure of p53

The N-terminus of the p53 protein comprises of two transcriptional activation domains (TADs), TAD1 and TAD2, which span residues 20-40 and 40-60. These domains can enhance transcription of p53 target genes by recruiting histone modifying enzymes, components of the basal transcriptional machinery and co-activator complexes. This region also contains the binding sites for the p53 inhibitory proteins HDM2 (human double minute protein 2) and HDMX (also known as HDM4, human double minute protein 4), which bind to the TAD1 and regulate both its stability and transcriptional activity. C-terminal to the TADs lies the proline rich domain (residues 60-90), which has been proposed to be involved in mediating protein-protein interactions due to the presence of PxxP motifs that mediate binding of Src homology 3 (SH3) domain-containing proteins. The central core region of p53 mediates the sequence specific DNA binding of the protein to response elements in its target genes. Although, bioinformatics studies predict that there may be >4000 human genes that contain putative p53 binding sites (Wang *et al* 2001b), analysis using various chromatin immunoprecipitation based techniques have recently placed this number to be between 500 and 1600 genes (Cawley *et al* 2004, Wei *et al* 2006). The vast majority of cancer associated *TP53* mutations occur in this domain, which includes the six most frequent mutations: R248Q, R273H, R175H, R282W, R249S and G245S. These mutations fall into two categories:1. Those that alter residues required for contacting the DNA (R248, R273) and 2. Those that are important for maintaining the structural conformational of the domain (R175, G245, R249, R282) (Brosh and Rotter 2009, Petitjean *et al* 2007). In addition, to abrogating the ability of p53 to bind to DNA, these mutant p53 proteins can acquire new oncogenic properties, known as 'gain of-function' properties that enable it to promote tumourigenesis (Brosh and Rotter 2009). For p53 to act as an efficient transcription factor, it requires the ability to self-associate with four molecules of the p53 monomer required to bind its response elements. This ability of p53 requires its oligomerisation domain, which is located towards the C-terminal end of the protein. Finally, p53 contains a serine and lysine rich domain at the extreme C-terminus (residues 363-393). This domain undergoes extensive post-translational modifications such as phosphorylation, acetylation, methylation and ubiquitylation that have been demonstrated to be critical in modulating both its stabilisation and transcriptional activity.

1.4.3 p53 functions

Low levels of DNA damage result in the activation of cell cycle checkpoints leading to a transient cell cycle arrest. This allows the cell time to repair the DNA damage and thereby prevents the damaged DNA from undergoing replication or mitosis. p53 plays a role in regulating the checkpoints during the G1 and G2 phases of the cell cycle. In the case of severe levels of DNA damage, p53 promotes apoptosis.

1.4.3.1 G1 growth arrest

The ability of p53 to induce a G1 arrest in response to DNA damage is largely dependent on its ability to induce the expression of p21 (el-Deiry *et al* 1993). p21 mediates p53-dependent G1 arrest by inhibiting the activity of the CDK-cyclin complexes, CDK4/6-cyclin D1 and CDK2-cyclin E, which are associated with regulating the G1 phase of the cell cycle (He *et al* 2005, Lundberg and Weinberg 1998). These kinases are responsible for sequentially phosphorylating pRb resulting in its inactivation. CDK-mediated phosphorylation of pRb promotes the release of the E2F transcription factor from inhibitory effects of pRb, subsequently allowing transactivation of E2F responsive genes required for the G1- to S-phase transition. In its hypophosphorylated state, pRb sequesters the E2F transcription factor, thereby preventing entry into S phase (Harbour and Dean 2000). This model is supported by the fact that over-expression of E2F1 can drive quiescent cells into S phase by relieving p21-mediated inhibition of CDK activity (Johnson *et al* 1993).

p53 is also capable of inducing a G1 arrest by non-transcriptional mechanisms through its ability to bind to cyclin H, which is part of the CDK7/cyclin H/Mat1 CDK activating kinase complex (CAK). CAK plays a crucial role in activating cell cycle progression by phosphorylating and activating CDK2, and, as part of the TFIIH complex by controlling transcriptional activity of RNA polymerase II (Schneider *et al* 1998).

1.4.3.2 G2 growth arrest

In addition to the G1 arrest, p53 also influences the damage-induced G2/M arrest. In response to DNA damage, cells lacking p53 and p21 are capable of initiating a G2 arrest, but are unable to sustain the arrest, resulting in mitotic catastrophe (Bunz *et al* 1998). Progression from G2 phase to mitosis requires the nuclear accumulation and subsequent activation of the mitosis promoting complex CDK1-cyclin B1. Activation of CDK1 involves dephosphorylation of

threonine 14 and tyrosine 15 by the CDC25 phosphatases and phosphorylation by CAK on threonine 161. It is thought that the involvement of p21 in G2 arrest is through its ability to bind to CDK1-cyclin B1 and inhibit CAK-mediated CDK1 activation since DNA damage or high levels of p21 have been demonstrated to reduce the phosphorylation of CDK1 on threonine 161 (Medema *et al* 1998, Smits *et al* 2000).

In addition to the p53 regulated expression of p21, the G2 arrest also appears to require additional transcriptional targets of p53, including GADD45 and 14-3-3σ protein (Hermeking *et al* 1997). After DNA damage, the G2/M cell cycle arrest is initiated by a p53-independent pathway involving the phosphorylation of the CDC25C phosphatase on serine 216 by CHK1, thereby creating a binding site for the 14-3-3 proteins. Phosphorylated CDC25C is sequestered in the cytoplasm by the 14-3-3 proteins, which prevents the dephosphorylation of nuclear CDK1 required to activate it (Lopez-Girona *et al* 1999, Peng *et al* 1997). Even though 14-3-3σ is not essential for the initial G2 arrest, it is required for maintaining the G2 arrest, as demonstrated by the observation that cells deficient in 14-3-3σ undergo premature G2/M checkpoint release and subsequent mitotic catastrophe. This is likely due to the inability of these cells to anchor CDK1-cyclin B1 complex in the cytoplasm (Chan *et al* 1999). GADD45 has been shown to activate G2/M checkpoint by disrupting the CDK1-cyclin B1 complex (Zhan *et al* 1999). Furthermore, p53 is also capable of repressing cyclin B1 expression following DNA damage, which is required by CDK1 to initiate the onset of mitosis (Innocente *et al* 1999).

1.4.3.3 Apoptosis

Apoptosis induced by p53 is a crucial mechanism of tumour suppression and involves the coordination of transcription-dependent and transcription-independent functions of p53. The role of p53 in apoptosis was firmly established by studies in knockout mice, which showed that $p53^{-/-}$ thymocytes exhibit a pronounced resistance to the induction of apoptosis following exposure to IR (Clarke *et al* 1993, Lowe *et al* 1993). A large proportion of p53 responsive genes are pro-apoptotic genes, which belong to the intrinsic and extrinsic apoptotic pathways. Within the intrinsic pathway p53 induces the expression of the pro-apoptotic Bcl-2 family members such as Bax (Bcl-2-associated protein X) and the BH3-only proteins, PUMA (p53 up-regulated modulator of apoptosis), NOXA and BID (Bcl-2 interacting domain death agonist). BH3 only proteins most likely trigger apoptosis by binding and displacing the Bcl-2

pro-survival proteins such as Bcl-2 and Bcl-$_X$l from Bax and Bak leading to their homo-oligomerisation and subsequent activation. Activation of Bax and Bak results in mitochondrial outer membrane permeabilisation allowing release of apoptosis inducing factor (AIF), the inhibitor of IAPs Smac/DIABLO and cytochrome c. Consequently, the caspase cascade is activated, ultimately resulting in cell death (Wei *et al* 2001, Zong *et al* 2001).

Cell surface death receptors transmit rapid apoptotic signals initiated by the binding of their ligands, which results in activation of the extrinsic rather than intrinsic apoptosis pathway (Ashkenazi and Dixit 1998). p53 induces the expression of the TNFR (Tumour necrosis factor receptor) family members FAS/CD95/Apo1, death receptor 4 (DR4) and DR5 (also known as TRAIL-R1 and TRAIL-R2). Transcription of FAS was shown to be induced by p53 through a p53 response element located within the first intron of the CD95 gene (Muller *et al* 1998). Although, the involvement of FAS transactivation in p53-mediated apoptosis appears to be cell type dependent (Bouvard *et al* 2000). Expression of both DR4 and DR5 have been reported to be increased upon DNA damage in a p53-dependent manner. However, the expression of these receptors also involves p53-independent mechanisms (Guan *et al* 2001, Takimoto and El-Deiry 2000, Wu *et al* 1997). Finally, there are numerous other genes not mentioned here that influence the cell type and stress specific apoptotic responses induced by p53 (Riley *et al* 2008).

While the transactivation activities of p53 clearly play an important role during the induction of apoptosis, several studies have shown that under certain conditions p53 can activate apoptosis through transcriptionally independent mechanisms (Caelles *et al* 1994, Haupt *et al* 1995). These functions of p53 involve its ability to promote mitochondrial outer membrane permeabilisation by directly interacting with members of the Bcl-2 family leading to activation of the BH3 only proteins (Moll *et al* 2005). The importance of this role for p53 is supported by the fact that the compound, pitithrin μ which selectively inhibits the mitochondrial function of p53, dramatically reduced p53-mediated apoptosis in irradiated thymocytes without hindering the transcriptional activity of p53 (Strom *et al* 2006). Despite this, it is clear that p53 coordinates both its transcription-dependent and -independent pro-apoptotic activities to cause cell death.

1.4.4 p53 target gene selection

p53 is clearly capable of stimulating the expression of genes involved in cell cycle arrest and apoptosis, which compromises the two major p53-dependent responses to DNA damage. Multiple factors can contribute to influencing p53 target gene selection and therefore the final outcome of p53 activation. These factors are likely to be dependent on the cell type, nature of the stress and the severity of the stress (Murray-Zmijewski *et al* 2008).

It has been proposed that p53 selects which genes to activate based on its abundance and affinity for a particular promoter. In other words, when p53 levels are low, it activates genes with high affinity promoters that tend to be associated with cell cycle arrest, and when p53 levels are high, it activates low-affinity promoters that tend to be involved in the apoptotic response (Chen *et al* 1996, Weinberg *et al* 2005, Zhao *et al* 2000). However, it is evident that promoter selectivity is not as simple as this. Certain p53 cofactors and p53 post-translational modifications have been demonstrated to be important for fine tuning the p53 response to specific stresses by enabling p53 to discriminate among its target genes (discussed below).

1.4.5 Regulation of p53 stability

Due to the crucial role of p53 in maintaining the integrity of the genome, a multitude of mechanisms have evolved to regulate its activity. Having a short half-life, p53 is normally maintained at low levels under 'non-stressed' conditions through its ubiquitin-mediated proteasomal degradation. Following cellular stress, p53 is post-translationally modified on a number of residues, increasing its stability and accumulation in the nucleus, as well as inducing its transcriptional activity (Figure 1.8).

A key player in the regulation of the p53 protein is the HDM2. As a result of its interaction with the transactivation domain within the N-terminal domain of p53, HDM2 can repress the transcriptional activity of p53 by blocking its interaction with components of the transcriptional machinery (Momand *et al* 1992, Oliner *et al* 1993). In addition, HDM2 has also been shown to function as an E3 ubiquitin ligase, which targets p53 for degradation by the proteasome, thereby maintaining low levels of p53 protein in unstressed cells (Haupt *et al* 1997, Honda *et al* 1997, Kubbutat *et al* 1997). Proteasomal degradation of p53 is facilitated by the transcriptional co-activator proteins, CBP and p300 acting as E4 polyubiquitin ligases (Ferreon *et al* 2009, Grossman *et al* 2003, Shi *et al* 2009). CBP and p300-dependent

polyubiquitylation of p53 is dependent on prior monoubiquitylation of p53 by HDM2. Furthermore it is believed that HDM2 can translocate p53 from the nucleus to the cytoplasm where it undergoes degradation (Roth *et al* 1998). HDM2 is also a transcriptional target of p53, which creates an autoregulatory loop in which p53 positively regulates HDM2 expression while HDM2 negatively regulates p53 levels and activity (Wu *et al* 1993) The importance of effective negative regulation of p53 activity is highlighted by the embryonic lethality of *MDM2* knockout mice, which die due to aberrant p53-induced apoptosis. This phenotype is completely rescued following deletion of the p53 gene, demonstrating that it is the uncontrolled activity of p53 that prevents development of the mice (Jones *et al* 1995, Montes de Oca Luna *et al* 1995).

Another prominent negative regulator of p53 is HDMX. Like HDM2, deletion of HDMX in mice causes embryonic lethality, which again is completely rescued by inactivation of p53 (Migliorini *et al* 2002, Parant *et al* 2001). Despite its sequence homology to HDM2 and the presence of a RING domain, HDMX does not have any intrinsic E3 ligase activity. HDMX has been demonstrated to inhibit p53 transcriptional activity through its ability to directly bind to the transactivation domain of p53 (Sabbatini and McCormick 2002, Shvarts *et al* 1996). In addition, HDMX can form a complex with HDM2 and promote its E3 ligase activity (Linares *et al* 2003, Uldrijan *et al* 2007).

To further complicate matters, HDM2-mediated regulation of p53 stability is controlled by the deubiquitylating enzyme, HAUSP (also known as USP7). HDM2 is an unstable protein, primarily due to its autoubiquitylation (Fang *et al* 2000). HAUSP was originally reported to deubiquitylate p53 (Li *et al* 2002a). However, it was subsequently shown to have an additional role in modulating HDM2 ubiquitylation (Cummins *et al* 2004, Li *et al* 2004). Structural studies revealed that HAUSP binds to HDM2 and p53 in a mutually exclusive manner, but that HAUSP has a higher binding affinity for HDM2 than p53 (Hu *et al* 2006). Therefore, it has been proposed that under ‘non-stressed’ conditions HDM2 rather than p53 is the preferred substrate for HAUSP. This results in HDM2 stabilisation and subsequent degradation of p53. Following DNA damage, the HAUSP-HDM2 interaction is abrogated by ATM-mediated phosphorylation of HDM2 enabling HAUSP to interact with p53 (Meulmeester *et al* 2005). Furthermore, the adaptor protein DAXX was shown to regulate HAUSP-mediated deubiquitylation of HDM2 under ‘non-stressed’ conditions by associating

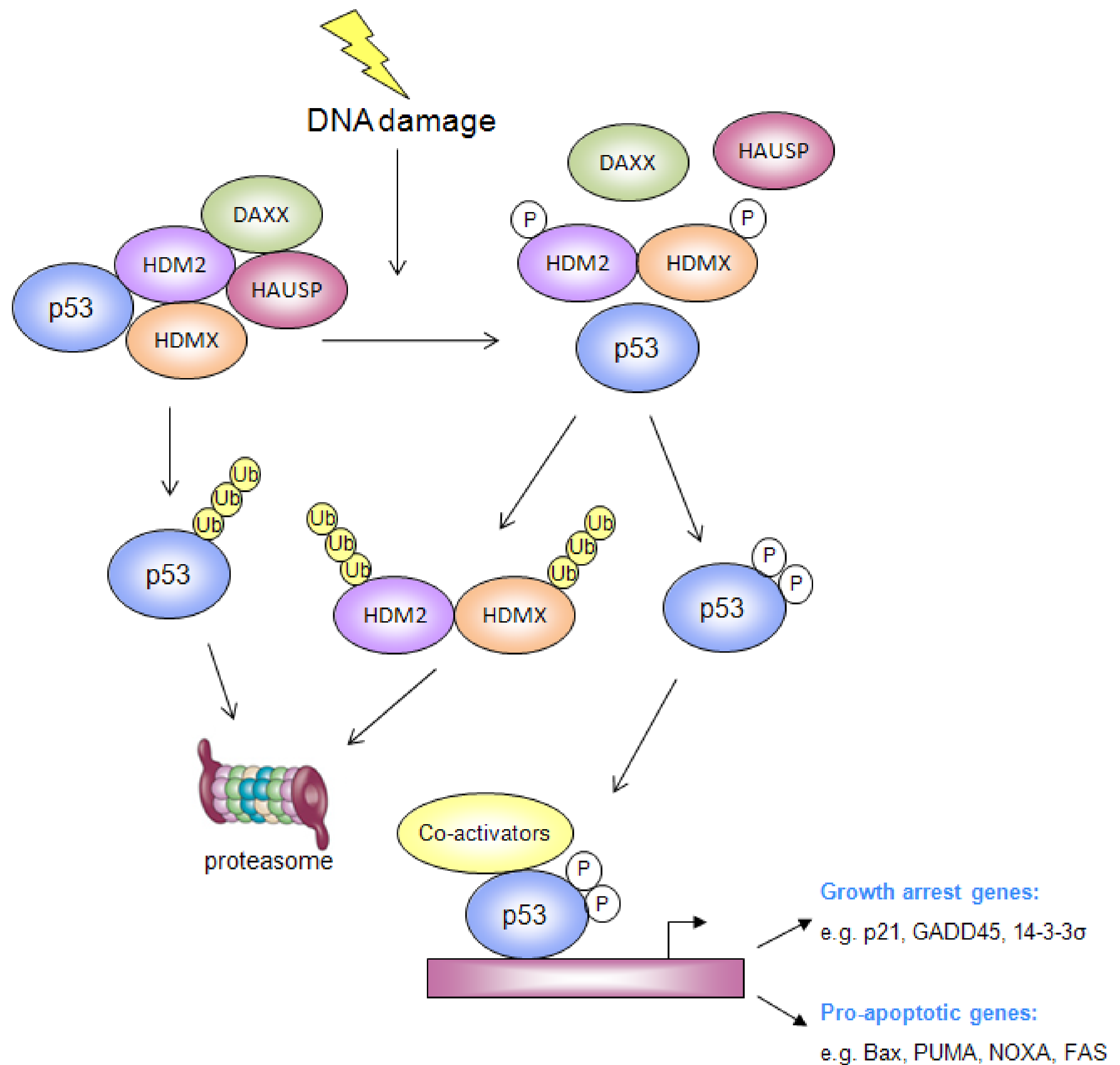


Figure 1.8 ***Activation of p53 in response to DNA damage.*** *Under non-stressed conditions, DAXX and HAUSP interact with HDM2 resulting in the stabilisation of HDM2 and HDMX. HDM2 ubiquitylates p53 and targets it for degradation by the proteasome. In response to DNA damage, HDM2 and HDMX are phosphorylated causing disruption of the HDM2-DAXX-HAUSP-HDMX complex. Subsequently, HDM2 ubiquitylates itself and HDMX. This results in degradation of HDM2 and HDMX by the proteasome allowing stabilisation and activation of p53. Activated p53 then induces the expression of its target genes including growth arrest and pro-apoptotic genes following various post-translational modifications.*

with HDM2 and HAUSP (Tang *et al* 2006a). More recently, the assembly of this multi-protein complex is in part regulated by RASSF1A. RASSF1A disrupts the interaction between HDM2, DAXX and HAUSP, thereby promoting HDM2 ubiquitylation and consequently resulting in p53 stabilisation (Song *et al* 2008). As demonstrated, there is an ever growing number of proteins being identified that affect the levels or activities of HDM2 and therefore influence p53 levels.

1.4.6 Post-translational modifications of p53

p53 is subject to a complex and diverse array of post-translational modifications, which induce its transcriptional activity and influence the expression of its target genes. These modifications include mono- and polyubiquitylation, sumoylation, neddylation, methylation, and acetylation, all of which compete for the same lysine internal residues, and proline prolyl-isomerisation, as well as phosphorylation, which targets serine and threonine residues of p53. In this section, I will mainly focus on phosphorylation, acetylation and methylation of p53.

1.4.6.1 Phosphorylation

Human p53 is phosphorylated by a variety of kinases such as ATM, ATR, DNA-PK, CHK1 and CHK2 on sites that are primarily in the N-terminal TADs and the C-terminal regulatory domain of p53. Most sites that are phosphorylated in response to DNA damage lead to p53 stabilisation and thereby enhance its function and/or affect its ability to bind to promoter sequences (Bode and Dong 2004). Serine 15, threonine 18 and serine 20 have all been implicated in inhibiting the interaction of p53 and HDM2 in response to DNA damage. Initially, it was proposed that phosphorylation of p53 at serine 15 reduced ability of HDM2 to interact with p53 (Shieh *et al* 1997). However, subsequent studies disputed this finding and suggested that phosphorylation of serine 15 does not affect the ability of p53 to be targeted for HDM2-mediated degradation but rather increases the transactivation potential of p53 through increasing its interaction with CBP and p300 (Dumaz and Meek 1999, Lambert *et al* 1998). Instead, damage-induced phosphorylation of threonine 18 and serine 20 has been demonstrated to interfere with the interaction with HDM2, thereby promoting stabilisation of p53 (Chehab *et al* 1999, Sakaguchi *et al* 2000). Phosphorylation of other residues including threonine 18 and serine 20 was shown to be dependent on prior phosphorylation of serine 15 (Dumaz *et al* 1999, Saito *et al* 2003). Further studies using phospho-specific antibodies have

indicated that in response to a variety of DNA damaging agents, p53 can be phosphorylated on a multitude of other serine and threonine residues, most of which are associated with p53 activation (Toledo and Wahl 2006).

Interestingly, p53 phosphorylation at serine 46 has been implicated in mediating selectivity in promoter binding by p53. In response to UV and genotoxic stress, p53 is phosphorylated on serine 46 by the kinases HIPK-2 (homeodomain-interacting protein kinase 2) and the dual specificity tyrosine phosphorylation regulated kinase-2 (DYRK-2) respectively (D'Orazi *et al* 2002, Hofmann *et al* 2002, Taira *et al* 2007). This phosphorylation site specifically promotes the induction of apoptosis inducing genes such as p53-regulated apoptosis-inducing protein 1 (p53AIP) (Oda *et al* 2000). This is accompanied by the down-regulation of p21 expression, ultimately resulting in p53-dependent apoptosis. Some sites on p53, serine 376, serine 378 and threonine 55 are constitutively phosphorylated in unstressed cells, which are thought to be involved in promoting proteasomal-mediated degradation of p53. In contrast to damage-induced phosphorylation, these sites have been shown to be dephosphorylated in response to DNA damage, resulting in activation of p53 (Li *et al* 2007, Waterman *et al* 1998).

Several studies have shown that phosphorylation of p53 represents a ‘priming event’ for subsequent modifications. For example, phosphorylation of serine 15, threonine 18 and serine 20 stimulates the recruitment of the HATs p300, CBP and PCAF that promote C-terminal acetylation (Li *et al* 2002b). Furthermore, phosphorylation of p53 on serine 33, threonine 81 and serine 315 in response to stress leads to efficient interaction with the prolyl isomerise Pin1, which in turn induces a conformational change in p53 that enhances its activity (Zacchi *et al* 2002, Zheng *et al* 2002).

1.4.6.2 Acetylation and methylation

HATs not only acetylate lysine residues in histones, but also acetylate p53 (Gu and Roeder 1997). CBP and p300 acetylate lysines 305, 370, 372, 373, 381, 382 and 386 within the C-terminal domain and lysine 164 in the DNA binding domain (Gu and Roeder 1997, Sakaguchi *et al* 1998, Tang *et al* 2008, Wang *et al* 2003b), whereas lysine 320 is acetylated by the p300/CBP-associated factor, PCAF (Liu *et al* 1999, Sakaguchi *et al* 1998). In addition, within the MYST family of acetyl transferases, hMOF and Tip60 have also been shown to acetylate lysine 120 in the DNA binding domain (Sykes *et al* 2006, Tang *et al* 2006b).

Several roles for p53 acetylation have been suggested. Early studies showed that acetylation of p53 enhanced the DNA binding activity of p53 (Gu and Roeder 1997, Sakaguchi *et al* 1998). However, others have suggested that the main function of p53 acetylation was to promote the interaction of p53 with co-activators (Barlev *et al* 2001). More recently, it has been demonstrated that acetylation of p53 is important for perturbing its interaction with HDM2 at the promoters of p53 responsive genes (Tang *et al* 2008).

As with phosphorylation, acetylation of certain lysine residues has differential effects on which target genes are activated by p53. Using acetylation-mimicking lysine-to-glutamine mutations, functional differences between lysine 320 and lysine 373 were reported. Acetylation of lysine 320 in p53 was shown to favour interaction with high affinity p53 binding sites such as those found in *p21*, thereby promoting cell cycle arrest and cell survival. In contrast, acetylation of lysine 373 increased the ability of p53 to transactivate low-affinity binding sites such as those found in the pro-apoptotic genes *PIG3*, *BAX* and *p53AIP1*, thereby promoting cell death (Knights *et al* 2006). p53-dependent apoptosis can also be specifically enhanced following DNA damage through acetylation of lysine 120. Acetylation of this site leads to an increase in recruitment of p53 specifically to pro-apoptotic target genes such as *PUMA* and *BAX*. This modification appears to be required for p53-dependent apoptosis, as mutating this site to an arginine residue was shown to diminish the ability of p53 to induce apoptosis. However, p53-mediated cell cycle arrest was not affected (Sykes *et al* 2006, Tang *et al* 2006b).

Acetylation levels of p53 can be regulated via deacetylation by HDAC1 containing complexes or SIRT1. Deacetylation of the C-terminal lysines was shown to repress p53-dependent transcriptional activation and modulate p53 effect on apoptosis and cell growth (Luo *et al* 2000, Luo *et al* 2001, Vaziri *et al* 2001). Furthermore, p53 stability is also affected by deacetylation, as several studies have demonstrated that HDM2 inhibits acetylation of p53 by p300 and PCAF and promotes HDAC1-mediated acetylation of p53 (Ito *et al* 2001, Ito *et al* 2002, Jin *et al* 2002). More recently, lysine 120 has been shown to be deacetylated by HDAC1 and this is dependent on KAP1 and other components of the NuRD co-repressor complex. This represses lysine 120-dependent p53-mediated apoptosis (Mellert *et al* 2011).

Finally, lysine residues that can be subjected to acetylation are also targeted by methyltransferases. Methylation of lysine 372 by the SET domain methyltransferase Set9 has

been shown to increase the stability of p53 and enhance p53-dependent transcription (Chuikov *et al* 2004). Interestingly, methylation of lysine 382 by Set8 can suppress the activation of several strong p53 targets such as *p21* and *PUMA*, but not others that are weakly induced (Shi *et al* 2007). Lysine 370 is methylated by the methyltransferase Smyd2 and causes repression of p53 transcriptional activity, although lysine 370 methylation itself is inhibited by Set9 methylation of lysine 372 (Huang *et al* 2006). However, as previously mentioned, di-methylation of lysine 370 increases p53 activity by promoting the interaction with 53BP1. LSD1, a lysine demethylase, can reverse lysine 370 di-methylation, thereby inhibiting this interaction (Huang *et al* 2007a). The functional roles of p53 lysine modifications are further complicated by the crosstalk that exists between methylation and acetylation. Specifically, methylation of lysine 372 by Set7/9 is induced by DNA damage and correlates with increased acetylation of C-terminal lysines including lysine 382 resulting in stabilisation of the p53 protein (Ivanov *et al* 2007). More recently, a new p53 modification was discovered, arginine methylation. The protein arginine methyltransferase 5 (PRMT5) was reported to be involved in methylating arginines 333, 335 and 337 within the oligomerisation domain and this influences p53 promoter selectivity (Jansson *et al* 2008). Depletion of PRMT5 leads to loss of p21, as well as a modest increase in pro-apoptotic proteins PUMA and NOXA, resulting in increased apoptosis.

1.4.7 p53-binding proteins that modulate p53 activity

A final mechanism of modulating p53 transcriptional activity is via binding to other intracellular proteins, which act as cofactors. These cofactors can affect p53 function by either cooperating directly with p53 or indirectly with p53 by regulating proteins that are directly involved in influencing its transcriptional activity such as CBP and p300. Many of these cofactors influence promoter selectivity by p53 and therefore the cellular outcome of the p53 response.

1.4.7.1 Cofactors that directly bind to p53

The best studied p53 interactors are its negative regulators, HDM2 and HDMX, which regulate p53 at multiple levels, affecting localisation, stability and transcriptional activity. In addition, the transcriptional co-activators, CBP and p300 have also been shown to be multifunctional regulators of p53, affecting the stability and transcriptional activity of p53 by

their polyubiquitin (E4) ligase and acetylase activities. In contrast to these cofactors which do not show a preference for either pro- or anti-apoptotic p53 target genes, several cofactors have been identified that specifically promote either apoptosis or cell cycle arrest.

The first example of this group of proteins is the ASPP (apoptosis stimulating protein of p53) family of proteins, which specifically affects the apoptotic response, but not the cell cycle arrest function of p53. ASPP1 and ASPP2 bind to the DNA binding domain (DBD) of p53 and enhance the pro-apoptotic function of p53 by selectively promoting the interaction of p53 to pro-apoptotic gene targets such as *BAX*, *PUMA* and *PIG3* (Samuels-Lev *et al* 2001). Conversely, the anti-apoptotic family member iASPP (inhibitor ASPP) binds to p53 and prevents the transactivation of pro-apoptotic genes, thereby inhibiting p53-mediated apoptosis (Bergamaschi *et al* 2003). Phosphorylation of p53 at serine 46 has been reported to facilitate the dissociation of iASPP from p53 in a Pin1-dependent manner, thereby enabling p53 to promote apoptosis (Mantovani *et al* 2007).

Another example of a protein that binds to p53 and directs cellular outcomes is the p38-regulated and DNA damage-inducible protein 18 (p18/Hamlet). Through associating with p53 p18/Hamlet was shown to increase both p53-mediated apoptosis and activation of some p53 target gene promoters such as *NOXA*, but not others such as *BAX*, *p21* and *PUMA* (Cuadrado *et al* 2007). The Brn3 family of transcription factors also modulates p53 target gene selection. Brn3A promotes cell survival by diminishing the ability of p53 to induce BAX and NOXA expression, while stimulating p21 expression (Hudson *et al* 2005). In contrast, Brn3b functions in the opposite manner as Brn3A by assisting p53 to activate BAX expression, but not p21 (Budhram-Mahadeo *et al* 2006).

An interesting regulator of p53 is the p52 subunit of the transcription factor NF-κB, which inhibits p21 expression, but acts in concert with p53 to increase PUMA, DR5 and GADD45 expression (Schumm *et al* 2006). The transmembrane protein, MUC1 has been found to activate p21 in a p53-dependent manner, while at the same time repressing BAX in a p53-independent manner (Wei *et al* 2005). Similarly, Hzf, a zinc finger protein that is itself a transcriptional target of p53, promotes p53 binding and transactivation of *p21* and *14-3-3*, but not pro-apoptotic genes such as *BAX* and *PUMA* (Das *et al* 2007). The Y-box binding protein YB1 also has a similar impact on p53. YB1 associates with p53 and blocks its activation of *BAX* expression, but does not affect p53 induction of *p21* (Homer *et al* 2005). BRCA1 has

also been shown to interact with the C-terminal region of p53 where it can selectively direct p53 to activate cell cycle arrest and DNA repair instead of apoptotic genes (MacLachlan *et al* 2002, Zhang *et al* 1998). APAK, a KRAB-type zinc finger protein has been shown to bind to p53 unstressed cells and prevents transactivation of pro-apoptotic target genes, thereby negatively regulating p53-dependent apoptosis (Tian *et al* 2009).

1.4.7.2 Cofactors that indirectly influence p53

Several proteins have been identified that affect p53 transcriptional activities and therefore the outcome of the p53 response without directly interacting with p53. The transcriptional repressor Slug, which is a p53 target gene itself, binds to PUMA and represses both gene expression and IR-induced apoptosis in hematopoietic cells (Wu *et al* 2005). Another transcriptional repressor, ZBT4 represses p53-mediated p21 induction and cell cycle arrest by forming a heterotetrameric complex with the Sin3 co-repressor and the transcription factor, MIZ1 (Weber *et al* 2008). The chromatin remodelling complex, human cellular apoptosis susceptibility protein (CAS/CSE1L) can also bind to a subset of p53 target genes independently of p53 including *PIG3* and *AIP1*, but not *p21* or *PUMA* (Tanaka *et al* 2007b). Even though CAS only binds to a subset of pro-apoptotic genes, this is sufficient to enhance apoptosis.

Interestingly, it has been demonstrated that certain p300 cofactors can influence the p53 response. Through interacting with p300, junction-mediating and regulatory protein (JMY) can augment p53-dependent transcription of *BAX*, but not *p21* resulting in apoptosis (Shikama *et al* 1999). Another p300 cofactor, Strap has been shown to increase p53-dependent apoptosis by facilitating the interaction between JMY and p53 and also stabilising p53 by preventing HDM2-mediated down-regulation of p53 levels (Demonacos *et al* 2001). In contrast, Skp2 suppresses p53 transactivation and apoptosis by blocking the interaction between p300 and p53 (Kitagawa *et al* 2008).

1.5 NF-κB

Nuclear factor κB (NF-κB) is a family of transcription factors with a highly diverse spectrum of modulating stimuli and an ever expanding array of responsive genes. The transcriptional regulation of these genes enables NF-κB to control a wide variety of cellular processes including cell proliferation, cell survival, differentiation, inflammation and apoptosis.

In mammalian cells, there are five members, p65 (also known as RELA), RELB, c-REL, p50 and p52 that are capable of forming homodimers, as well as heterodimers with one another. p50 and p52 are derived from larger precursor proteins, p105 and p100 respectively through ubiquitin-dependent proteolytic processing. All these proteins are defined by an N-terminal 300 amino acid highly conserved Rel homology domain (RHD) which mediates DNA binding, dimerisation, nuclear localisation and binding to the inhibitor of NF-κB proteins (IκBs). In addition, c-REL, RELB and p65 proteins contain a C-terminal TAD enabling them to induce gene expression from DNA sequences, known as κB elements within the promoters/enhancers of target genes. In contrast, p50 and p52 lack the TAD, but p50 and p52 homodimers can still bind to the κB elements in the DNA and therefore can function as transcriptional repressors (Hayden and Ghosh 2008).

1.5.1 NF-κB activation

There are several distinct NF-κB activation pathways, with the two most well established pathways being the canonical and the non-canonical pathways (Figure 1.9). p65 or c-Rel containing homo- and heterodimers are predominantly activated by the canonical pathway while the non-canonical pathway mainly activates the p52-RelB heterodimer (Perkins 2007). The canonical pathway is induced in response to a variety of inflammatory stimuli including the pro-inflammatory cytokines tumour necrosis factor-α (TNFα) and interleukin-1 (IL-1), exposure to bacterial products such as lipopolysaccharide (LPS) and engagement of antigen on the TCR. In addition to inflammatory stimuli, the canonical pathway can be activated by DSBs induced by genotoxic agents such as IR and chemotherapeutic drugs including etoposide and camptothecin, as well as by replication stress inducers such as HU and aphidicolin. The non-canonical pathway is activated by a smaller set of inducers including CD40 ligand, lymphotoxin-β, B-cell-activating factor of TNF family (BAFF), LPS and latent membrane protein-1 (LMP1). In this section, I will discuss the function and regulation of the p65-p50 heterodimer, which is the predominant NF-κB complex in many cell types.

1.5.1.1 Canonical pathway

In most unstimulated mammalian cells, NF-κB is found predominantly in the cytoplasm bound to a member of the IκB family, of which the most common are IκBα, IκBβ and IκBε. These function by masking the conserved nuclear localisation signal (NLS) that is found in

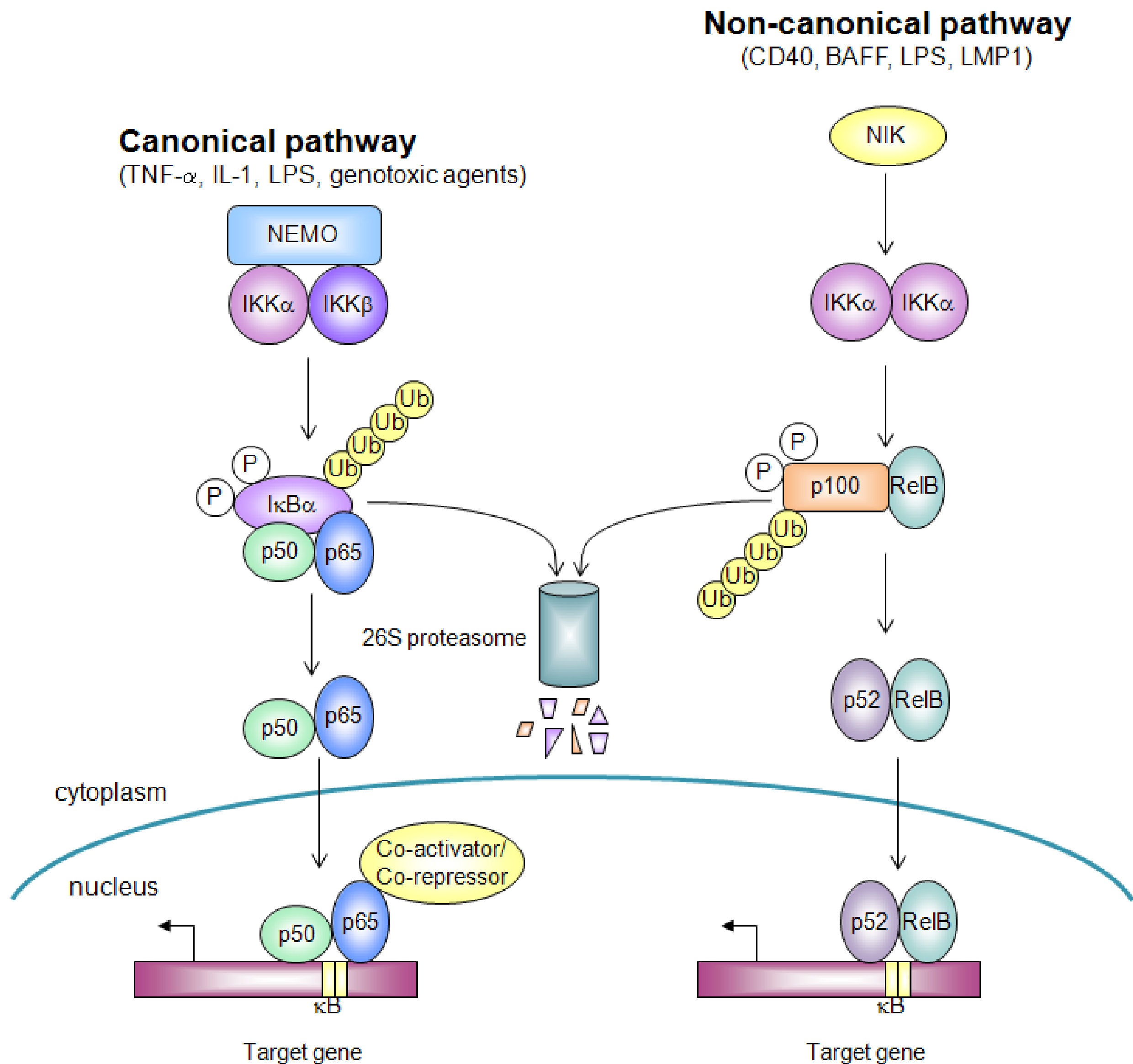


Figure 1.9 ***Pathways leading to NF-κB activation****. Induction of the canonical pathway by a variety of stimuli such as TNF-α results in activation of the IKK complex, which in turn phosphorylates IκBα and targets it for ubiquitylation. Ubiquitylated IκBα is degraded by the 26S proteasome allowing NF-κB to translocate into the nucleus and activate gene transcription. The non-canonical NF-κB pathway results in the activation of IKKα by the NF-κB-inducing kinase (NIK), followed by phosphorylation of p100 by IKKα. Subsequently, p100 is ubiquitylated and targeted for proteasomal processing to p52. p52 forms a complex with RelB, which enters the nucleus and activates gene transcription.*

the RHD of p65 and p50. For IκBα, the major member of the IκB family of proteins, only the NLS of p65 is masked enabling the NF-κB-IκBα complexes to shuttle into the nucleus even in the absence of stimulation (Birbach et al 2002, Huxford et al 1998). However, IκBα also contains a nuclear export signal (NES), which causes rapid export of such complexes back to the cytoplasm (Johnson et al 1999). As the export process is more efficient than the import process, nuclear localisation of NF-κB-IκBα complexes can only be detected when nuclear export is blocked by the inhibitor, Leptomycin B (Huang et al 2000).

Engagement of these activating stimuli with their distinct cell receptors and the formation of DSBs leads to activation of a variety of signalling pathways that all converge on the IκB kinase (IKK) complex, which is composed of the catalytic subunits IKKα and IKKβ and the regulatory subunit called NF-κB essential modifier (NEMO, also known as IKKγ). The activated IKK complex phosphorylates IκBα at serine 32 and serine 36 (DiDonato *et al* 1997, Mercurio *et al* 1997). Analysis of IKKβ and IKKα deficient cells has shown that IKKβ is the predominant kinase in canonical NF-κB activation, although IKKα can induce IκBα phosphorylation in IKKβ deficient cells indicating they can function redundantly (Gerondakis *et al* 1999, Li *et al* 2000, Li *et al* 1999). NEMO appears to be essential for canonical NF-κB activation as NEMO-deficient cells fail to activate NF-κB in response to TNFα, LPS and IL-1β (Makris *et al* 2000, Rudolph *et al* 2000, Schmidt-Supprian *et al* 2000, Yamaoka *et al* 1998). Phosphorylated IκBα is recognised by the $SCF^{\beta TrCP}$ E3 ubiquitin ligase complex, which catalyses the formation of K48-linked polyubiquitin chains at lysine 21 and lysine 22 (DiDonato *et al* 1996, Scherer *et al* 1995, Winston *et al* 1999). Polyubiquitylated IκBα is then targeted for degradation by the 26S proteasome, thereby releasing NF-κB from its inhibitory effects and allowing it to translocate to the nucleus where it can activate its target genes including IκBα (Figure 1.9). Following its resynthesis, IκBα can bind to NF-κB and export it back to the cytoplasm, thereby terminating the NF-κB response.

1.5.1.2 TNFR1 signalling pathways

Stimulation of TNFR1 by TNFα leads to receptor trimerisation and the formation of two sequential protein complexes, namely complex I and complex II. Recruitment of the adaptor protein TNF receptor associated protein with a death domain (TRADD) initiates the formation of complex I. TRADD acts as a scaffold, facilitating the recruitment of the other components

of complex I to the receptor, these include the protein kinase receptor interacting protein (RIP1) and the E3 ubiquitin ligases TNF receptor associated factors (TRAF) 2 and 5 and the cellular inhibitor of apoptosis proteins (cIAP) 1 and 2.

Whilst the kinase activity of RIP1 is not required for signalling to NF-κB, RIP1 itself and the covalent attachment of K63-linked polyubiquitin chains to RIP1 have been shown to be essential (Li *et al* 2006). Initially it was proposed that TRAF2 together with the heterodimeric E2 conjugating enzyme complex UBC13/UBC-like protein (UEV1A) catalysed the polyubiquitylation of RIP1 at lysine 377. However, several studies have disputed this model. Knockdown of UBC13 was shown not impair activation of NF-κB by TNFα (Habelhah *et al* 2004). Consistent with this, cells from conditional *UBC13* knockout mice showed a similar effect (Yamamoto *et al* 2006). Furthermore, although TRAF2 is required for TNFα-mediated NF-κB activation, its RING domain and therefore its E3 ubiquitin ligase activity has recently been shown to be dispensable (Vince *et al* 2009). In addition, structural studies have indicated that it is unlikely that TRAF2 binds to UBC13 or other related E2s (Yin *et al* 2009). These results suggest that TRAF2 and UBC13 are not the E3 or E2 for RIP1 polyubiquitylation. Instead, it seems that TRAF2 acts as an adaptor protein for cIAPs and that these serve as the E3 ubiquitin ligases for TNFα-induced NF-κB activation. TRAF2 mutants that cannot bind cIAPs failed to reconstitute TRAF2/5 double knockout cells, whilst a TRAF2 mutant that can still bind to cIAPs, but has no E3 ligase activity rescued TNFα-mediated activation of NF-κB in these cells (Haas *et al* 2009, Vince *et al* 2009). cIAPs are critical for TNFα signalling, as demonstrated by the fact that loss of cIAPs completely abrogates K63-linked polyubiquitylation of RIP1 and NF-κB activation even though recruitment of TRAF2 to complex I is normal (Bertrand *et al* 2008, Mahoney *et al* 2008, Varfolomeev *et al* 2008). K63-linked polyubiquitylation of RIP1 serves as a scaffold to recruit and activate two downstream complexes, the IKK complex and the TAK1 (transforming growth factor β activated kinase 1) complex. Polyubiquitylated RIP1 recruits the IKK complex through the interaction between the ubiquitin binding motif of NEMO and the K63-linked polyubiquitin chains (Ea *et al* 2006, Wu *et al* 2006a). Similarly, the TAB (TAK1 binding protein) 2 and 3 regulatory proteins of the TAK1 protein kinase complex also contain ubiquitin binding motifs that bind to the K63-linked polyubiquitin chains on RIP1, thereby facilitating the recruitment of the TAK1 complex to the TNFR1 (Kanayama *et al* 2004). Once recruited, TAK1 is

activated by autophosphorylation enabling it to phosphorylate IKKβ on serine 177 and serine 181, resulting in activation of the IKK complex and ultimately NF-κB (Wang *et al* 2001a) (Figure 1.10).

Unlike the formation of complex I, the mechanisms regulating the formation of complex II are poorly understood. Furthermore, rather than activating NF-κB, which predominantly leads to inflammation and cell survival through the expression of pro-inflammatory and anti-apoptotic proteins respectively, complex II promotes apoptosis. Following formation of complex I, the receptor is internalised by endocytosis (Schneider-Brachert *et al* 2004). RIP1, TRADD and TRAF2 then dissociate from the TNFR1 during endocytosis, allowing RIP1 and TRADD to recruit the adaptor protein Fas-associated death domain (FADD), which in turn recruits caspase 8 and 10 (Micheau and Tschopp 2003). The newly formed cytosolic complex II containing TRADD, FADD, RIP1 and procaspase 8/10 promotes activation of these caspases, which then cleave and activate caspase 3, triggering apoptosis. More recently, it has been demonstrated that two distinct caspase 8 activating complexes are formed in response to TNFα (Wang *et al* 2008). Complex IIA comprises of TRADD, FADD and caspase 8 and is controlled by the level of the anti-apoptotic protein, c-FLIP. In contrast, complex IIB consists of non-ubiquitylated RIP1, FADD and caspase 8 and is negatively regulated by cIAPs. Apoptosis can be promoted by the E3 ubiquitin ligase ITCH and the IAP antagonist Smac, which target c-FLIP and cIAPs respectively for ubiquitin-mediated proteasomal degradation (Chang *et al* 2006, Vince *et al* 2007). However, in most cells, TNFα-induced apoptosis is prevented since rapid activation of NF-κB by complex I induces the synthesis of several anti-apoptotic proteins including c-FLIP, cIAP1 and cIAP2, which inhibit caspase 8 activation. Several studies demonstrated that TNFα-induced apoptosis can only occur if either NF-κB activation or new protein synthesis is blocked (Beg and Baltimore 1996, Van Antwerp *et al* 1996, Wang *et al* 1996) (Figure 1.10).

TNFα also promotes apoptosis through activation of the JNK (c-jun N-terminal kinase) signalling pathway (Papa *et al* 2006). Prolonged activation of JNK enhances TNFα-induced apoptosis in part by phosphorylating and activating ITCH, which in turn leads to ubiquitylation and degradation of c-FLIP (Chang *et al* 2006). However, NF-κB activation by

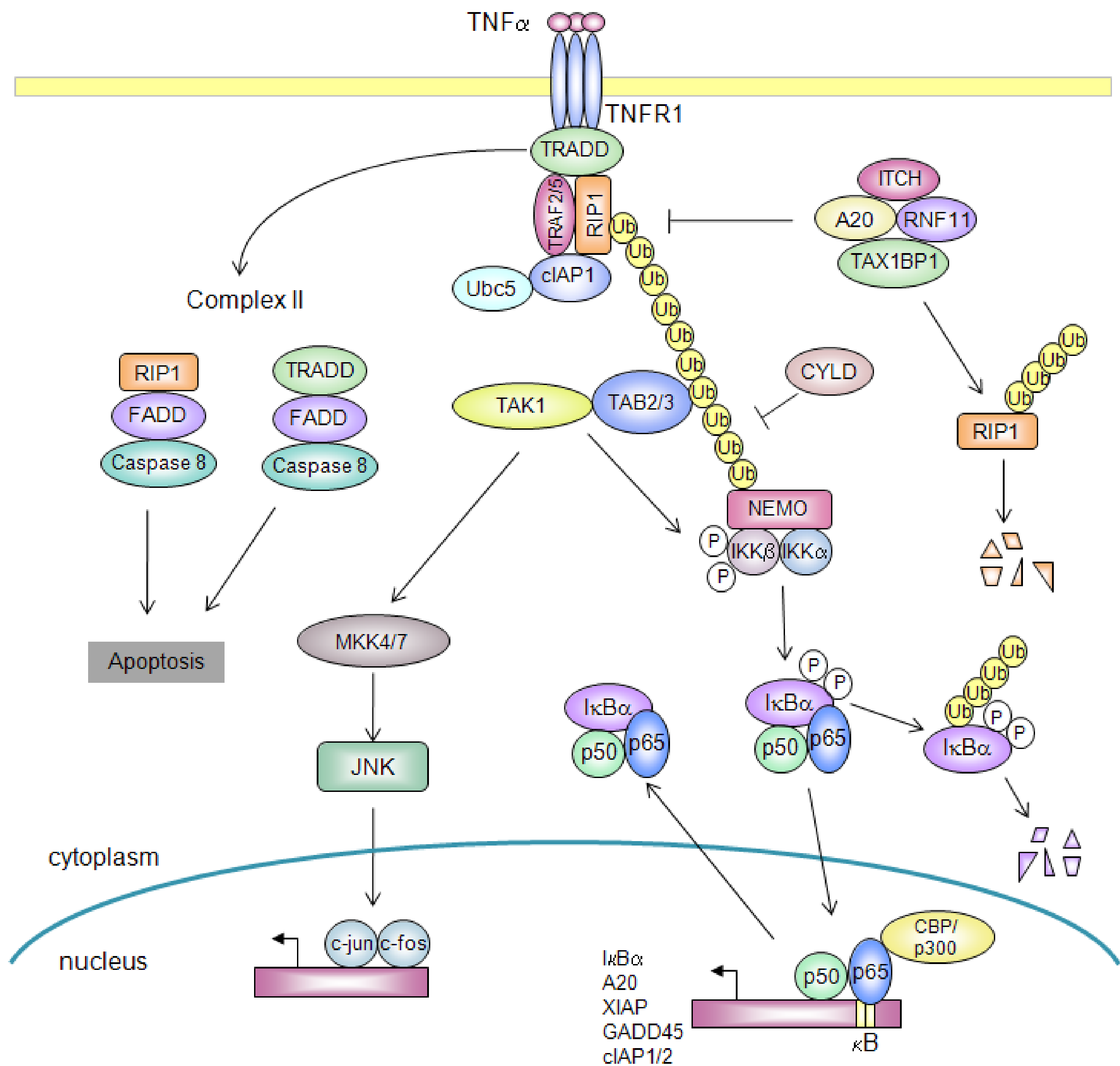


Figure 1.10 ***TNFR1 signalling pathways****. Engagement of TNF with its cognate receptor TNFR1 results in the formation of a proximal signalling complex composed of TRADD, TRAF2, cIAP1 and RIP1. RIP1 recruits the TAK1/TAB2/3 complex thereby promoting activation of the IKK complex. Activated IKK phosphorylates IκBα at serine 32 and 36 leading to ubiquitylation by the SCF$^{\beta TrCP}$ E3 ligase and subsequent degradation by the 26S proteasome. NF-κB is released and enters the nucleus where it can activate its target genes following various post-translational modifications. IκBα is resynthesised, dissociates NF-κB from the DNA and exports NF-κB back to the cytoplasm. TNFR1 activates p38 (not shown) and JNK kinases via recruitment of MKK3/6 and MKK4/7 respectively. These kinases can activate transcription factors such as AP-1 and ATF2. TNFR1 can also initiate events that lead to apoptosis by forming another complex containing FADD and either RIP1or TRADD. This activates caspase 8, which activate effector caspases triggering apoptosis.*

TNFα suppresses prolonged JNK activation through the up-regulation of its target genes, XIAP (X-linked IAP) and GADD45β, the latter of which is an inhibitor of the JNK upstream kinase MKK7 (De Smaele *et al* 2001, Papa *et al* 2004, Tang *et al* 2001). Furthermore, activation of JNK by TNFα requires the generation of ROS, a process that can be counteracted by NF-κB through the induction of genes that encode antioxidant proteins such as manganese-superoxide dismutase (MnSOD) and ferritin heavy chain (FHC) (Kamata *et al* 2005).

1.5.1.3 Toll-like receptor and IL-1 receptor signalling pathways

IL-1 and LPS activate NF-κB in a similar manner because of homology between the cytoplasmic signalling domains in their receptors, known as the Toll-IL-1 receptor (TIR) domain. Binding of IL-1 and LPS to the interleukin 1 receptor (IL-1R) and Toll-like receptor (TLR) respectively leads to the recruitment of the TIR containing adaptor protein, myeloid differentiation primary gene 88 (MYD88). MYD88 in turn recruits two IL-1 receptor-associated kinases, IRAK4 and IRAK1. On recruitment to the receptor complex, IRAK1 is autophosphorylated and associates with E3 ubiquitin ligase TRAF6. These proteins then function together with the E2 conjugating enzyme complex UBC13/UEV1A to catalyse K63-linked polyubiquitylation resulting in recruitment of the TAK1 complex and IKK complex through TAB2/3 and NEMO respectively. TRAF6-mediated K63-linked polyubiquitylation of IRAK1 has been shown to be involved in IKK recruitment and activation (Conze *et al* 2008, Windheim *et al* 2008). Interestingly, a recent study has revealed that free K63-linked polyubiquitin chains, which are not conjugated to any target protein, can directly activate the TAK1 complex (Xia *et al* 2009). These free polyubiquitin chains, which are synthesised by TRAF6 and UBC13/UEV1A associate with TAB2 (and possibly TAB3) promoting activation TAK1, which subsequently activates IKK complex leading to activation of NF-κB.

1.5.1.4 T-cell receptor pathway

Activation of NF-κB in T cells requires engagement of TCRs with antigens presented by host major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells (APC) as well as activation of the co-stimulatory receptor, CD28 (Weil and Israel 2006). In response to stimulation, TCRs initiates a tyrosine phosphorylation cascade that leads to activation of the serine/threonine protein kinase PKCθ and the subsequent recruitment of the

CBM protein complex consisting of the CARD domain proteins CARMA1, BCL10 and a caspase-like protein MALT1. Through MALT1, the CBM complex can recruit TRAF6, which together with UBC13/UEV1A activates TAK1 and IKK complexes (Sun *et al* 2004). In addition, BCL10 and MALT1 have been shown to be polyubiquitylated by TRAF6, which facilitates the recruitment and activation of the IKK complex through the NEMO subunit (Oeckinghaus *et al* 2007, Wu and Ashwell 2008).

1.5.1.5 NF-κB activation by genotoxic stress

Unlike cell surface receptor initiated signalling pathways, the signalling pathways induced by genotoxic agents that result in NF-κB activation are poorly characterised. With the exception of a few genotoxic agents such as UV and hydrogen peroxide (H_2O_2), which can activate NF-κB via IKK-independent pathways, the majority of genotoxic agents activate the IKK-dependent canonical NF-κB pathway (Janssens and Tschopp 2006, Perkins 2007). Several studies have demonstrated that ATM is required for NF-κB activation in response to DSB inducers including IR, the topoisomerase inhibitor camptothecin and the topoisomerase II inhibitors etoposide and doxorubicin (Huang *et al* 2003, Li *et al* 2001, Piret *et al* 1999, Wu *et al* 2006b). In addition to ATM, NEMO which is not bound to IKKα or IKKβ has also been shown to play a key role in transducing the nuclear DNA damage signal to the cytoplasmic IKK complex (Huang *et al* 2003).

In response to DSBs, IKK unbound NEMO translocates to the nucleus where it is conjugated by SUMO-1 at lysine 277 and lysine 309. Nuclear translocation of NEMO was found to be prevented when NEMO was bound to the IKKs (Huang *et al* 2003). Sumoylation of NEMO is mediated by the SUMO E3 ligase PIASγ together with the E2 conjugating enzyme UBC9 (Mabb *et al* 2006). Furthermore, it was found that p53-induced protein with death domain (PIDD) and RIP1 were required to promote NEMO sumoylation (Janssens *et al* 2005). More recently, Stilmann *et al* (2009) revealed that poly (ADP-ribose) polymerase 1 (PARP1) can also promote NEMO sumoylation by acting as a scaffold protein for the assembly of PARP-1, PIASγ, ATM and NEMO into a multi-protein complex (Stilmann *et al* 2009). In response to DNA damage, PARP1 catalyses poly-ADP-ribosylation (PAR) of numerous substrates, as well as itself. NEMO is recruited to this complex by interacting with PARP-1 while PIASγ and ATM are recruited to PAR modified PARP-1 through PAR binding motifs (PARBM).

Subsequently, PIASγ induces sumoylation of NEMO. Notably, neither PIDD or RIP1 were identified in this PARP1 signalling complex, therefore it is currently unknown what the precise roles are for PIDD and RIP1 in DNA damage-induced NF-κB activation. Following DNA damage-induced activation, ATM phosphorylates NEMO at serine 85 (Wu *et al* 2006b). Phosphorylation of NEMO is a prerequisite for its monoubiquitylation at lysine 277 and lysine 309 by cIAP1, which displaces the SUMO-1 attachment at these residues on NEMO. (Huang *et al* 2003, Jin *et al* 2009). Ubiquitylation of NEMO facilitates the nuclear export of the ATM-NEMO complex. This complex stimulates K63-linked polyubiquitylation of the adaptor protein ELKS by the E3 ubiquitin ligase XIAP and UBC13 leading to recruitment of the TAK1 complex through the ubiquitin binding proteins TAB2/3 and subsequent activation of the IKK complex (Wu *et al* 2010). In addition to ATM promoting XIAP-dependent ELKS polyubiquitylation in the cytoplasm, ATM has also been shown to be required for inducing UBC13-mediated K63-linked polyubiquitylation of TRAF6 (Hinz *et al* 2010). Synthesis of K63-linked polyubiquitin chains triggers the recruitment and activation of the TAK1 and IKK complexes as described previously. In this pathway nuclear export of ATM is independent of NEMO, PARP-1 and PIASγ and occurs before NEMO sumoylation indicating that ATM has a dual function in activation of NF-κB. Interestingly, in order for the IKK complex to be activated, NEMO is required to be monoubiquitylated at lysine 85, which is mediated by either cIAP1 or TRAF6 (Hinz *et al* 2010). In the absence of NEMO monoubiquitylation, active TAK1 is unable to phosphorylate IKKβ. Furthermore, monoubiquitylation of NEMO at lysine 285 does not appear to be specific to genotoxic stress, but rather appears to be a common modification since it is also required for TNFα, LPS and IL-1 induced IKK activation (Abbott *et al* 2007, Hinz *et al* 2010, Walsh *et al* 2008) (Figure 1.11).

Recently, it has been demonstrated that replication stress inducers such as HU and aphidicolin can also activate NF-κB in an ATM and NEMO-dependent manner similar to DSB inducers (Wu and Miyamoto 2008). Surprisingly, in contrast to activation of NF-κB by DSB inducers, which generally promote cell survival through the expression of anti-apoptotic proteins, NF-κB activation by replication stress promotes apoptosis through induction of pro-apoptotic genes such as *FAS* and repression of anti-apoptotic genes such as *Bcl-$_{Xl}$* (Wu and Miyamoto 2007, Wu and Miyamoto 2008). This is similar to previous studies that demonstrated that NF-κB can repress anti-apoptotic genes in response to DNA damaging agents including UV and

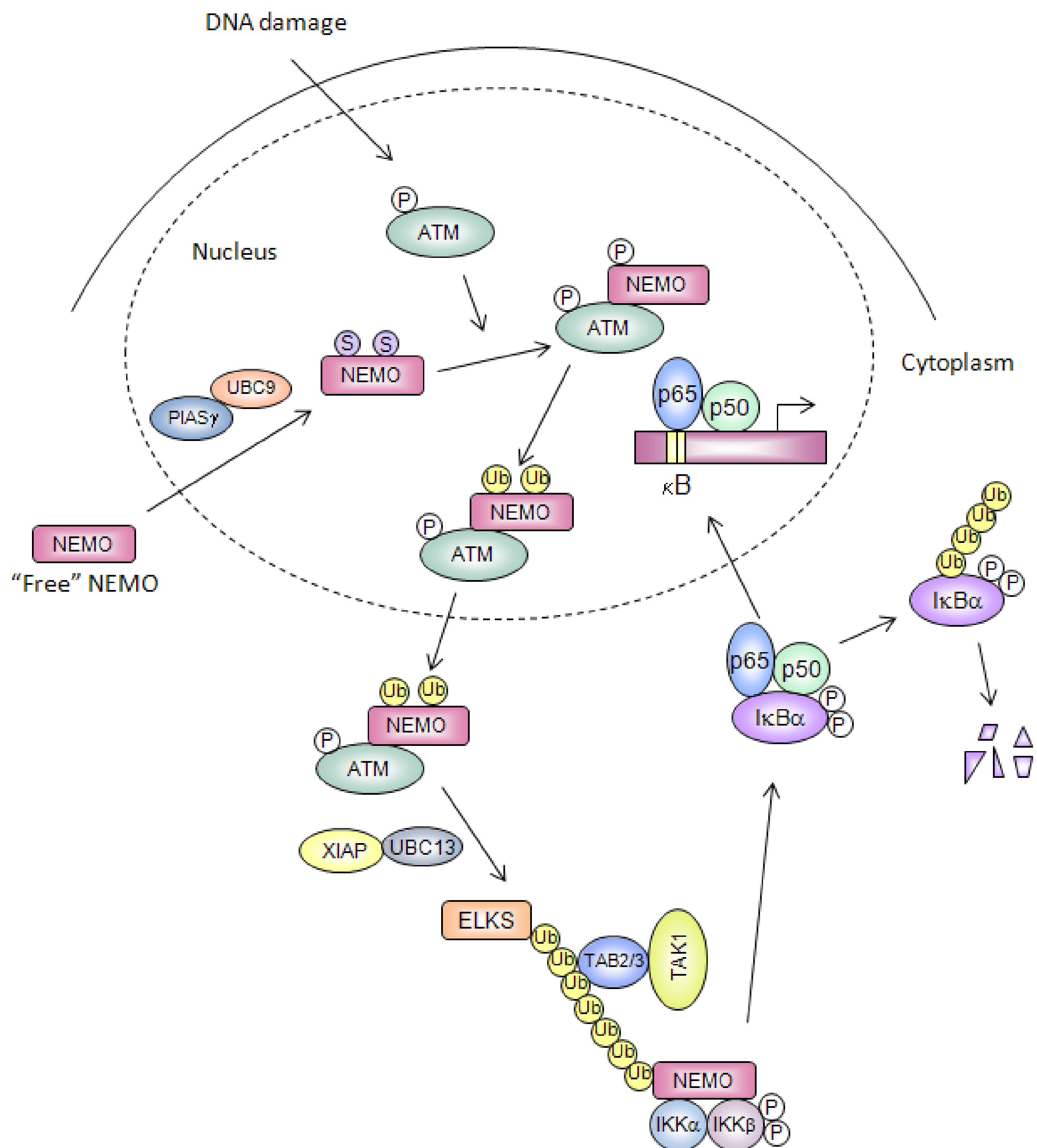


Figure 1.11 ***Activation of NF-κB by DNA damage.*** *DNA damage induces the activation of ATM and sumoylation of NEMO via the SUMO ligase, PIASγ. ATM then phosphorylates NEMO, which in turn promotes monoubiquitylation of NEMO by cIAP1. Subsequently, ATM and NEMO are exported to the cytoplasm where they stimulate K63-linked polyubiquitylation of ELKs by XIAP leading to activation of TAK1 and IKK. Activated IKK phosphorylates 1κBα, which results in the ubiquitylation and proteasomal degradation of 1κBα. NF-κB is released and translocates to the nucleus where it activates its target genes (Adapted from Wu et al 2010).*

cisplatin, thereby promoting its pro-apoptotic function (Campbell *et al* 2004, Campbell *et al* 2006). Together these studies demonstrate that the functional consequences of genotoxic stress-induced NF-κB activation can differ depending on the genotoxic agent.

1.5.1.6 Negative regulation of NF-κB signalling pathways

Activation of IKK is negatively regulated by a variety of mechanisms, one of which is deubiquitylation. CYLD and A20 are two deubiquitylating enzymes that are important for suppressing NF-κB upstream of the IKK complex by cleaving K63-polyubiquitin chains or preventing polyubiquitin chain synthesis.

CYLD was originally identified as a tumour suppressor gene since mutations in the *CYLD* gene predispose individuals to familial cylindromatosis, a rare autosomal recessive disease characterised by numerous benign tumours of the skin appendages (Bignell *et al* 2000). The cancer-associated mutations in CYLD are frequently found in the C-terminal region, which contains a ubiquitin C-terminal hydrolase (UCH) domain. Several studies later showed that through this domain CYLD could negatively regulate NF-κB signalling. Over-expression of CYLD but not mutants defective in DUB activity reduced IKK and NF-κB activation whereas siRNA against CYLD enhanced IKK and NF-κB activation (Brummelkamp *et al* 2003, Kovalenko *et al* 2003, Trompouki *et al* 2003). CYLD inhibits IKK activation by cleaving K63-linked polyubiquitin chains on several proteins including TRAF2, TRAF6, RIP1 and NEMO (Brummelkamp *et al* 2003, Kovalenko *et al* 2003, Trompouki *et al* 2003, Wright *et al* 2007) (Figure 1.10).

A20 is a well known NF-κB target gene that inhibits NF-κB in a negative feedback loop (Jaattela *et al* 1996, Opipari *et al* 1992). A20 deficient mice develop severe inflammatory diseases in multiple organs partly due to enhanced and prolonged activation of IKK by pro-inflammatory stimuli including LPS and TNFα (Lee *et al* 2000). Interestingly, A20 contains an ovarian tumour (OTU) type DUB domain that catalyses deubiquitylation of K63-linked polyubiquitin chains on RIP1, as well as seven zinc finger domains that promote K48-linked polyubiquitylation and proteasomal degradation of RIP1 (Wertz *et al* 2004). Together with the ubiquitin binding protein, Tax1 binding protein 1 (TAX1BP1) and the E3 ubiquitin ligases, immune modulating protein ITCH and RNF11, A20 forms a ubiquitin editing complex that suppresses IKK activation in a two step sequential process. A20 first removes

K63-linked polyubiquitin chains from RIP1 and then catalyses the formation of K48-linked polyubiquitin chains onto RIP1, targeting RIP1 for proteasomal-mediated degradation (Iha *et al* 2008, Shembade *et al* 2007, Shembade *et al* 2008, Shembade *et al* 2009). A20 has also been shown to disassemble K63-linked polyubiquitin chains from TRAF6, thereby suppressing NF-κB responses elicited by LPS stimulation (Boone *et al* 2004). However, the mechanism by which A20 inhibits TRAF6 activation in the TLR/IL-1R pathways has been shown to be different to the mechanism used by A20 to inhibit RIP1 (Shembade *et al* 2010). A20 inhibits the E3 ubiquitin ligase activity of TRAF6 by antagonising the interaction with its E2 ubiquitin conjugating enzyme UBC13. UBC13 is subsequently targeted for ubiquitin-mediated proteasomal degradation by A20 together with its regulatory protein TAX1BP1. Furthermore, this study also showed that this mechanism was not restricted to the TLR/IL-1R pathways, but also functioned in the TNFR1 pathway since A20 can disrupt the interaction between TRAF2 and cIAP1 with their E2 enzymes UBC13 and UBCH5C respectively (Figure 1.10).

1.5.2 Regulation of NF-κB transcriptional activity

Following activation, NF-κB induces the expression of over 150 target genes, which are involved in a wide variety of cellular processes (http://people.bu.edu/gilmore/nf-kb/index.html, (Pahl 1999)). The majority of proteins encoded by these genes play a role in immune and inflammatory responses including cytokines and chemokines such as TNF, IL-1. IL-6, CXCL8, RANTES and CXCL 11, as well as receptors required for immune recognition such as CD80, CCR5 and MHC molecules. Cell survival is another key cellular process that NF-κB promotes through the expression of genes encoding anti-apoptotic proteins including members of the Bcl-2 family such as Bcl-$_{X}$l, Bcl-2, NR13 and BFL1, which function at the mitochondria level and prevent release of cytochrome c, AIF and Smac/DIABLO and cIAP1, cIAP2, XIAP and c-FLIP, which prevent caspase activation. In addition to its anti-apoptotic role, NF-κB also induces cell proliferation and cell cycle progression by regulating the expression of target genes including growth factors such as IL-2 and granulocyte-macrophage colony stimulating factor (GM-CSF) and cell cycle regulators such as c-myc and cyclin D1. In addition, NF-κB regulates genes involved in cell adhesion such as ICAM, cell migration such as MMP9, angiogenesis such as VEGF and the stress response such as COX-2. Interestingly, although NF-κB is generally considered anti-apoptotic, under certain circumstances, NF-κB

can up-regulate its pro-apoptotic target genes such as the death receptors FAS (CD95), DR4 and DR5, the death-inducing ligands Fas ligand (FASL) and TRAIL and also the pro-apoptotic Bcl-2 family member BAX, thereby promoting apoptosis (Dutta *et al* 2006).

Due its role in regulating cell survival, cell proliferation, angiogenesis, cell migration and inflammation, it is not surprising that constitutive activation of NF-κB is often associated with many types of cancer including Hodgkin's lymphoma (HL), chronic myeloid leukemia (CML), multiple myeloma (MM), breast cancer and colorectal cancer, as well as inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease and asthma (Courtois and Gilmore 2006, Karin 2006, Li and Verma 2002). Consequently, NF-κB transcriptional activity is tightly regulated through multiple post-translational modifications as well as by resynthesis of its inhibitor IκBα.

1.5.3 Post-translational modifications

Phosphorylation and acetylation are the two key modifications that control the transcriptional activity and target gene specificity of NF-κB. These modifications are stimulus specific and are also likely to be cell type specific.

1.5.3.1 Phosphorylation

The p65 subunit is the principal target for phosphorylation by various kinases. These p65 phosphorylation events occur in the cytoplasm or in the nucleus and are stimuli specific and probably cell-type specific.

The catalytic subunit of protein kinase PKA (PKAc) is maintained in an inactive form by binding to the IκBα-NF-κB complex. Following stimulus-induced IκBα degradation by LPS, activated PKAc phosphorylates p65 on serine 276, which is located within the RHD of p65 (Zhong *et al* 1997). Phosphorylation of this site promotes the interaction of p65 with CBP and p300 (Zhong *et al* 1998), and displaces transcriptionally repressive HDAC complexes, specifically p50-HDAC1 complexes, that are frequently bound to the κB enhancers of target genes under unstimulated conditions (Zhong *et al* 2002). However, phosphorylation of serine 276 is not exclusively mediated through PKAc. In response to TNFα stimulation, serine 276 is phosphorylated by the mitogen and stress activated kinases (MSK) 1 and 2, which enhances NF-κB transcriptional activity (Vermeulen *et al* 2003). Interestingly, PKAc phosphorylates

p65 in the cytoplasm whereas MSK1 and MSK2 function in the nucleus. In addition, serine 276 phosphorylation is also important for determining whether p65 forms homodimers or heterodimers. Serine 311 is another residue within the N-terminal RHD that is targeted for phosphorylation by protein kinase C (PKC)ζ in TNFα stimulated cells (Duran *et al* 2003). Similar to the mechanism described for PKAc, PKCζ-mediated phosphorylation of p65 enhances its interaction with CBP.

Within the C-terminal TAD, serine 529 is phosphorylated by CK2 in the cytoplasm in response to TNFα and IL-1 treatment and phosphorylation at this site has been shown to potentiate NF-κB activity (Wang *et al* 2000). Stimulation with TNFα and IL-1β results in phosphorylation of serine 468 by IKKβ (Schwabe and Sakurai 2005), while IKKε phosphorylates serine 468 in response to the genotoxic agent etoposide and following T-cell co-stimulation (Mattioli *et al* 2006, Renner *et al* 2010). Interestingly, phosphorylation of serine 468 by these kinases stimulates p65 transactivation whereas phosphorylation of this site in unstimulated cells by glycogen synthase kinase 3β (GSK3β) inhibits the transcriptional activity of p65 (Buss *et al* 2004a). p65 is phosphorylated at serine 536, a site within the TAD, by a variety of kinases via various signalling pathways and in most cases phosphorylation at this site enhance the transactivation potential of p65. Similar to serine 276 phosphorylation, phosphorylation of serine 536 has also been shown to displace HDAC co-repressor complexes, in particular HDAC3-SMRT complexes enabling p65 to interact with CBP and p300 (Chen *et al* 2005, Hoberg *et al* 2006). Serine 536 phosphorylation induced by TNFα, IL-1 and T-cell co-stimulation is mediated by IKKα, IKKβ, IKKε and NF-κB activating kinase (NAK) (Buss *et al* 2004b, Mattioli *et al* 2004, Sakurai *et al* 1999, Sizemore *et al* 1999). Interestingly, it has been shown that IKKβ-dependent phosphorylation of p65 at serine 536 requires the activity of AKT, a component of the PI3K/AKT signalling pathway (Madrid *et al* 2001, Sizemore *et al* 1999). Furthermore, serine 536 can be phosphorylated by an IKK-independent mechanism following treatment with etoposide and doxorubicin that involves activation of the ribosomal S6 kinase 1 (RSK1) by p53 and promotes nuclear translocation of NF-κB (Bohuslav *et al* 2004).

Serine 205, serine 281, threonine 435 and threonine 254 have all been shown to be targets for phosphorylation in response to TNFα and LPS treatment, although the kinases responsible have yet to be identified (Anrather *et al* 2005, O'Shea and Perkins 2010, Ryo *et al* 2003).

Phosphorylation at serine 205, serine 281 and threonine 435 influences NF-κB promoter selectivity and phosphorylation at threonine 254 creates a binding site for the prolyl isomerise Pin1. The action of Pin1 disrupts the interaction between p65 and IκBα and induces p65 translocation to the nucleus (Ryo *et al* 2003). Furthermore, it also protects p65 from ubiquitin-mediated proteasomal degradation, thereby increasing p65 stability.

Phosphorylation of threonine 505 requires CHK1 and is induced by the ARF tumour suppressor or treatment with cisplatin. This phosphorylation event inhibits p65 transactivation by enhancing the association of p65 with HDAC1 (Campbell *et al* 2006, Rocha *et al* 2005). This results in repression of *Bcl-χl* expression, thereby sensitising cells to apoptosis. p50 has also been shown to be phosphorylated by PKAc at serine 337 and this increases the DNA binding ability of p50 (Hou *et al* 2003).

Phosphorylation of the NF-κB subunits is a reversible process and consequently several phosphatases have been identified that dephosphorylate p65 thereby inhibiting NF-κB transcriptional activity. These include protein phosphatase 2A (PP2A) and the type 2C protein phosphatise (PP2C) WIP1, which has recently been shown to specifically dephosphorylate serine 536 in response to TNFα (Chew *et al* 2009, Yang *et al* 2001a)

1.5.3.2 Acetylation

Numerous lysines in p65 are acetylated and these have been shown to modulate its activity. The HATs CBP and p300 can acetylate p65 at lysines 218, 221 and 310. Acetylation of lysine 221 enhances the DNA binding activity of p65 and in conjunction with acetylation of lysine 218 impairs the NF-κB-IκBα interaction, thereby preventing IκBα-dependent nuclear export of NF-κB and prolonging the NF-κB response. Deacetylation of these sites by HDAC3 enhances the binding of NF-κB to IκBα promoting its nuclear export, which results in termination of the NF-κB response. Lysine 310 acetylation has been shown to be important for stimulating NF-κB transcriptional activity and is enhanced by serine 276 and serine 536 phosphorylation of p65, which promotes the assembly of p65 with CBP and p300 (Chen *et al* 2005). Lysine 310 deacetylation by the histone deacetylase SIRT1 or alternatively by the HDAC3-SMRT co-repressor complex inhibits p65 transcriptional activity (Hoberg *et al* 2006, Yeung *et al* 2004). Furthermore, it has been demonstrated that HDAC1-mediated deacetylation of p65 also reduces the transactivation potential of p65 (Ashburner *et al* 2001).

CBP, p300 and PCAF-mediated acetylation of lysine 122 and lysine 123 has also been described, although modifications at these sites have inhibitory effects on NF-κB activity by reducing the ability of p65 bind to the κB sites in its target genes and consequently suppressing the transactivation potential of NF-κB (Kiernan *et al* 2003).

The p50 subunit is also subject to stimulus-induced acetylation of lysine 431, lysine 440 and lysine 441 by CBP and p300. These modifications appear to enhance the DNA binding activity of p50 and increase the transcriptional activity of the NF-κB complex (Deng *et al* 2003, Furia *et al* 2002).

1.5.4 Crosstalk between NF-κB and p53

p53 has long been implicated in regulating NF-κB at multiple levels and vice versa. In response to inflammatory stimuli, NF-κB induces anti-apoptotic genes that antagonise the pro-apoptotic function of p53. Moreover, the IκB family member Bcl-3 or NF-κB activation by IKK complex can induce the expression of HDM2, thereby enhancing ubiquitylation and degradation of p53 (Kashatus *et al* 2006, Tergaonkar *et al* 2002). Additional, antagonism comes from the competition between p53 and p65 for binding to CBP and p300 (Webster and Perkins 1999).

In contrast to these observations, there is evidence that p53 and NF-κB can cooperate with each other. For example, as previously mentioned, p53 can induce RSK1 activity resulting in serine 536 phosphorylation of p65 and nuclear localisation of NF-κB (Bohuslav *et al* 2004). Furthermore, in some circumstances, p65 and p53 can cooperatively induce apoptosis, which can occur through induction of apoptotic target genes containing promoters with both p53 and NF-κB response elements such as DR5 (Aleyasin *et al* 2004, Fujioka *et al* 2004, Ryan *et al* 2000, Shetty *et al* 2005).

Alternatively, modifications of NF-κB can promote p53-induced apoptosis. Activation of ARF tumour suppressor by oncogenes, results in activation of p53 through binding to and inactivating HDM2 (Sherr 2006). Concomitantly, ARF expression induces ATR leading to activation of CHK1 and subsequent phosphorylation of p65 at threonine 505. This results in repression of Bcl-$_{X}$l and sensitises cells to TNF-induced apoptosis (Rocha *et al* 2005). Furthermore, the effect of this will also reduce the ability of p65 to oppose the pro-apoptotic

function of p53. Interestingly, ARF-induced ATR activity can also regulate p53, thereby ARF can coordinantly regulate and integrate NF-κB and p53 function (Rocha *et al* 2003). In addition, NF-κB transcriptional activity can also increase p53 expression, partly by directly inducing the *TP53* gene (Fujioka *et al* 2004, Wu and Lozano 1994).

1.5.5 Termination of NF-κB activity

Due to the detrimental effects caused by constitutive NF-κB activation, it is critical that NF-κB activity is terminated following cellular stimulation. However, the mechanisms by which the activity of NF-κB is terminated remain poorly understood. It is generally accepted that termination of NF-κB activity mainly relies on the resynthesis of IκBα by NF-κB. Newly synthesised IκBα associates with NF-κB, displaces it from the DNA and exports its out of the nucleus, thereby creating a negative feedback loop (Arenzana-Seisdedos *et al* 1997). This model is supported by studies in IκBα$^{-/-}$ deficient cells (Cheng *et al* 1998, Hoffmann *et al* 2002). Furthermore, IκBβ and IκBε have also been shown to regulate NF-κB activity through a negative feedback loop, albeit with different kinetics (Hoffmann *et al* 2002, Kearns *et al* 2006). However, in order for IκBα to bind to p65 efficiently, p65 needs to be de-modified (Chen and Greene 2003). Therefore, dephosphorylation and deacetylation of p65 by phosphatases and HDACs respectively plays a key role in suppressing NF-κB-dependent transcription.

Another mechanism by which NF-κB transcriptional activity is terminated involves removal of NF-κB from the promoters of some of its target genes by ubiquitin-mediated proteasomal degradation. In the absence of IκBα, this was shown to be the major termination mechanism. However, in cells containing IκBα, proteasomal degradation and resynthesised IκBα were shown to act synergistically to efficiently terminate transcription of NF-κB dependent genes. If proteasome activity is blocked, NF-κB is not removed from some of its target genes despite IκBα resynthesis and consequently NF-κB transcriptional activity is sustained (Saccani *et al* 2004). Currently, two ubiquitin ligase complexes have been reported to be responsible for the degradation of nuclear NF-κB. PDLIM2 is a nuclear ubiquitin ligase that has recently been shown to function in the negative regulation of NF-κB. PDLIM2 polyubiquitylates p65 and transports it to proteasome enriched promyelocytic leukemia (PML) nuclear bodies where it

is rapidly degraded, thereby terminating NF-κB activity (Tanaka *et al* 2007a). The other is the suppressor of cytokine signalling-1 (SOCS1)-containing ECS ubiquitin ligase complex, which also contains Elongin B and Cullin 2 (Willems *et al* 2004). SOCS1 was demonstrated to interact with p65 and decrease its stability by ubiquitin-mediated proteasomal degradation (Ryo *et al* 2003, Strebovsky *et al* 2010). Recently, the COMMD1 (copper metabolism MURR1 domain containing protein 1) protein was shown to interact with the ECS complex and promote the associations between p65 and SOCS1 and between SOCS1 and Cullin 2, thereby facilitating the ubiquitylation of p65 by the ECS complex and its subsequent proteasomal degradation (Maine *et al* 2007). More recently, Mao *et al* (2009) have reported that the HAT, GCN5 associates with the COMMD1 containing ECS ubiquitin ligase promoting p65 ubiquitylation and repressing NF-κB activity (Mao *et al* 2009). Under certain circumstances ECS-induced ubiquitylation and degradation of p65 can be inhibited by Pin1 (Ryo *et al* 2003). Over-expression of Pin1 was shown to reverse the decrease in p65 stability that is observed following SOCS1 over-expression.

1.6 CBP AND p300

CBP and p300 are highly homologous non-DNA binding transcriptional co-activators that were originally identified by their interactions with cAMP-response element binding protein (CREB) and the adenoviral E1A protein respectively (Chrivia *et al* 1993, Eckner *et al* 1994). Subsequently, CBP and p300 have been found to interact with a wide variety of other intracellular proteins including numerous transcription factors such as nuclear hormone receptors (Chakravarti *et al* 1996), p53 (Lill *et al* 1997), p65 (Gerritsen *et al* 1997), c-Jun (Lee *et al* 1996a), c-fos (Janknecht and Nordheim 1996b) and the CCAAT box/enhancer-binding protein (C/EBP)-β (Mink *et al* 1997), as well as components of the basal transcription machinery such as RNA polymerase II, TBP and TFIIB (Imhof *et al* 1997, Kee *et al* 1996, Nakajima *et al* 1997). Since so many proteins interact with CBP and p300, it is not surprising that many physiological processes including cell proliferation, differentiation, DNA repair, development and apoptosis are dependent on CBP and p300 (Goodman and Smolik 2000). Several lines of evidence highlight the importance of CBP and p300. Firstly, CBP and p300 are required for embryonic development and viability, as demonstrated by the fact that *CBP* and *p300* knockout mice are embryonic lethal (Yao *et al* 1998). Secondly, mutations in both *CBP* and *p300* are associated with Rubinstein-Taybi syndrome (RTS), a developmental

disorder characterised by mental retardation, skeletal abnormalities and an increased predisposition to cancer (Miller and Rubinstein 1995, Petrij *et al* 1995, Roelfsema *et al* 2005). Finally, bi-allelic somatic mutations in *p300* have been detected in a range of epithelial cancers such as gastric, colon, pancreatic and breast cancers (Gayther *et al* 2000) and frequent chromosomal translocations of CBP and p300 have been observed in haematological malignancies, particularly acute myeloid leukemia (AML) (Janknecht 2002).

1.6.1 Structure

CBP and p300 are large, modular proteins that contain several conserved functional domains including three zinc binding cysteine-histidine (CH) rich domains, a KIX domain, a HAT domain and a bromodomain. These regions of CBP and p300 share a high degree of sequence similarity (approximately 95%) and as a result the functions of these proteins, albeit with a few exceptions, are generally interchangeable (Goodman and Smolik 2000, Kasper *et al* 2006, Kawasaki *et al* 1998) (Figure 1.12). Therefore, CBP and p300 will subsequently be referred to as CBP/p300. The CH1 and CH3 domains (sometimes referred to as TAZ1 and TAZ2 domains respectively) mediate interactions with the majority of CBP/p300 interacting proteins including p53, HDM2 and p65 (Goodman and Smolik 2000). Additional sites for protein-protein interactions include the KIX domain and the glutamine rich C-terminus. The KIX domain is the region that binds to CREB, the archetypical CBP-binding transcription factor as well as several other transcription factors and transcriptional regulatory proteins including c-jun and BRCA1 (Goodman and Smolik 2000). The glutamine rich region forms contacts with other transcriptional co-activators, most notably those involved in nuclear hormone receptor signalling including SRC-1 and p/CIP, which are members of the steroid receptor co-activator (SRC)/p160 family (Kamei *et al* 1996, Xu *et al* 1999a). CBP/p300 are HATs and the respective HAT domain resides in the central region of these proteins. This domain encompasses the CH2 domain and catalyses acetylation of all four core histones as well as several non-histone nuclear proteins (discussed below). The bromodomain that resides N-terminal to the HAT domain is found in many chromatin associated proteins (Dhalluin *et al* 1999) and is capable of binding to acetylated lysines (Winston and Allis 1999). Via the bromodomain, CBP/p300 can not only bind to acetylated lysines, but also to acetylated transcription factors such as MyoD (Polesskaya *et al* 2001). Acetylation of MyoD was demonstrated to strengthen the interaction between MyoD and CBP/p300. In addition to

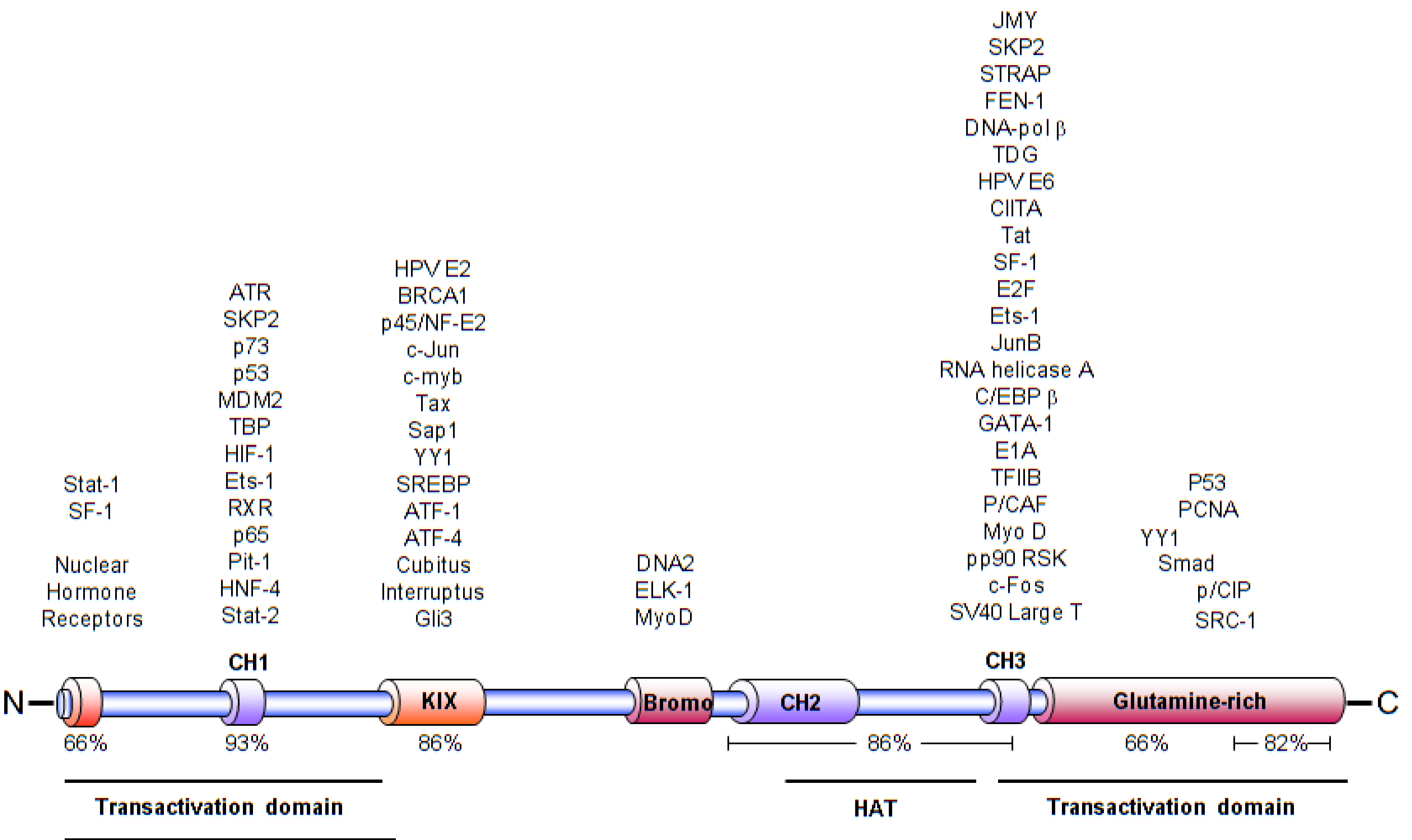


Figure 1.12 ***Schematic diagram of CBP/p300****. A selection of interacting proteins are shown at the top of the figure and the functional domains are depicted below with the percentage of amino acid identity between the two proteins indicated.*

acetyltransferase activity, CBP/p300 possess transcriptional activity since the N- and C-terminus of CBP/p300 can act as transactivation domains (Bisotto *et al* 1996, Lee *et al* 1996b, Swope *et al* 1996, Yuan *et al* 1996). When fused to the DNA binding domain of the yeast transcription factor Gal4, these domains can potentially activate the transcription from Gal4 DNA binding sites. This is likely to be because these regions are capable of interacting with the basal transcription machinery. More recently, it has been found that the N-terminal region of CBP/p300 harbours both E3 and E4 ubiquitin ligase activities (Grossman *et al* 2003, Shi *et al* 2009). Through their E3 activities, CBP/p300 are autoubiquitylated, whereas they promote polyubiquitylation of p53 via their E4 activities (discussed below). Interestingly, unlike their other intrinsic activities, the ubiquitin ligase activities of CBP/p300 were shown to be exclusively cytoplasmic.

1.6.2 Transcriptional regulation by CBP/p300

By acting as transcriptional co-activators, CBP/p300 play a central role in coordinating and integrating multiple signal transduction pathways with the transcriptional machinery, thereby regulating gene expression (Bedford *et al* 2010). Current evidence suggests that CBP/p300 mediate transcriptional co-activation in several ways. Since CBP/p300 can interact with transcription factors and components of the basal transcription machinery, it has been proposed that CBP/p300 act as a molecular bridge to connect the DNA binding transcription factors with the basal transcriptional machinery, thereby stabilising the transcription complex (Chan and La Thangue 2001).

Secondly, CBP/p300 may act as a scaffold for the formation of multi-subunit complexes containing transcription factors and cofactors. The size of CBP/p300 provides them with many different interaction surfaces, thereby enabling them to bind concurrently to various proteins. By serving as a scaffold for the assembly of transcription factors and transcriptional regulatory proteins, CBP/p300 can concentrate these factors into a local transcription environment (Chan and La Thangue 2001). This scaffolding function for CBP/p300 has been demonstrated to be important for the transcriptional regulation of the human interferon β gene. CBP/p300 assembles the IFNβ enhancesome by mediating the simultaneous recruitment of several transcription factors such as c-jun, NF-κB and interferon regulatory factor 1 (IRF1) and architectural proteins including high mobility group (HMG) to the IFNβ promoter (Kim *et al* 1998, Munshi *et al* 1998). By bringing together nuclear proteins and the basal

transcription machinery, CBP/p300 plays a crucial role in the transcriptional activation of the interferon β gene (Yie *et al* 1999b). Depletion of CBP/p300 from this complex decreases the rate of transcription (Yie *et al* 1999a).

Finally, CBP/p300 are able to acetylate all four core histones through their histone acetyltransferase activity (Bannister and Kouzarides 1996, Ogryzko *et al* 1996). Two models regarding how acetylation disrupts the structure of the chromatin have been described. Acetylation of lysine residues within the histone tails can result in neutralisation of the positively charge associated with lysines, thereby decreasing the interaction between histones and the DNA molecule. As a consequence, the condensed chromatin is transformed into a more relaxed conformation, which enables the promoters to become more accessible to transcription factors and the basal transcription machinery. Conversely, histone deacetylation by HDACs is thought to prevent access by restoring the positive charge and strengthening the interaction between histones and DNA (Wolffe and Hayes 1999). In addition to this charge-neutralisation model, it has been demonstrated that acetylation may de-stabilise higher order chromatin structure, thereby facilitating the binding of transcription factors to their target gene promoters (Tse *et al* 1998a, Tse *et al* 1998b).

Studies by Gu and Roeder demonstrated that p53 could be acetylated by CBP/p300 (Gu and Roeder 1997). This provided the first example that CBP/p300 could acetylate non-histones proteins and since then a number of other proteins have been identified that are acetylated by CBP/p300 including several transcription factors such as NF-ATc2 (Garcia-Rodriguez and Rao 1998), p65 (Chen *et al* 2001), E2F-1, E2F-2 and E2F-3 (Martinez-Balbas *et al* 2000, Marzio *et al* 2000), c-Myb (Tomita *et al* 2000) and Sp3 (Braun *et al* 2001), as well as other co-activator proteins such as MAML-1 (Hansson *et al* 2009) and PC4 (Kumar *et al* 2001), components of basal transcription machinery including TFIIEβ and TFIIF (Imhof *et al* 1997) and DNA repair proteins (see below).

1.6.3 Non-transcriptional functions of CBP/p300

Like transcription, proteins involved in DNA repair and DNA replication also require an open chromatin structure in order to access the DNA. Several studies have shown CBP/p300 are involved in these processes through their ability to modify histones and interact with proteins involved in DNA repair and DNA replication. PCNA, a vital component of DNA replication

and repair machinery, recruits p300 to the DNA where it acetylates histones in preparation for DNA replication or repair (Hasan *et al* 2001a). The ring shaped heterotrimeric PCNA encircles and slides along double stranded DNA tethering DNA polymerases to the DNA, thereby facilitating DNA synthesis. PCNA is acetylated by p300 and this modification increases PCNA binding efficiency for the DNA polymerases, Polβ and Polδ (Naryzhny and Lee 2004). In addition, flap endonuclease 1 (FEN1), which is involved in processing DNA ends during DNA replication and repair, is acetylated by p300 (Hasan *et al* 2001b). Acetylation reduces its DNA binding activity, as well as its exo- and endonuclease activities (Friedrich-Heineken *et al* 2003, Hasan *et al* 2001b). Recently, p300 has been shown to acetylate another DNA processing nuclease involved in DNA replication and repair, DNA2 (Balakrishnan *et al* 2010). However, in contrast to FEN1, this modification stimulates the enzymatic activities of DNA2. p300 also binds and acetylates several other DNA repair proteins including DNA polymerase β and the DNA glycosylases, thymine DNA glycosylase (TDG) and 8-oxoguanine-DNA glycosylase (OGG1) (Bhakat *et al* 2006, Hasan *et al* 2002). Acetylation modifies the enzymatic functions of these proteins. Furthermore, CBP/p300 can interact with ATR and this is required for the DNA replication checkpoint since loss of CBP/p300 results in a defective DNA replication checkpoint (Stauffer *et al* 2007). More recently, p300 has been shown to interact and acetylate NBS1 which facilitates the stabilisation and recruitment of NBS1 to sites of DNA damage. A prerequisite for NBS1 acetylation is ATM-dependent phosphorylation of p300 on serine 106 (Jang *et al* 2010, Jang *et al* 2011).

CBP/p300 play a key role in regulation of p53 stability through their cytoplasmic E4 ubiquitin ligase activities and depletion of CBP/p300 in unstressed cells results in the stabilisation of p53 in the cytoplasm (Grossman *et al* 2003, Shi *et al* 2009). In the absence of stress, p53 can be both monoubiquitylated and polyubiquitylated by HDM2. Monoubiquitylation has been shown to enhance the nuclear export of p53, whereas polyubiquitylation results in its proteasomal degradation in the cytoplasm. In conjunction with HDM2, CBP/p300 have been shown to catalyse polyubiquitylation of p53, thereby regulating the turnover of p53 in unstressed cells. However, HDM2-mediated monoubiquitylation of p53 is required for CBP/p300-dependent polyubiquitylation of p53. In support of this, an HDM2 mutant that is defective in binding to p300, but can still bind to p53, can promote ubiquitylation, but not degradation of p53 (Zhu *et al* 2001). This demonstrates that CBP/p300 can regulate p53

differently depending on the cellular compartment. In the cytoplasm CBP/p300 can inhibit p53 whereas in the nucleus, CBP/p300 activate p53, presumably by acetylation.

1.6.4 Regulation of CBP/p300 activity

The intrinsic activities of CBP/p300 are regulated by multiple signalling pathways through post-translational modifications. Although, it is well known that phosphorylation of CBP/p300 is cell cycle dependent, relatively little is known about how phosphorylation regulates CBP/p300 function (Yaciuk and Moran 1991). CDK2-cyclin E complex was reported to phosphorylate CBP/p300 in the C-terminal. In the case of p300, this phosphorylation event negatively regulates its co-activator function. Since *p21* gene requires p300 for its transcription, it was proposed that p21 relieves repression of p300 by CDK2-cyclin E complex creating a positive feedback loop (Perkins *et al* 1997). In support of this, p21 was shown to increase p300-dependent transcription of NF-κB. However, this is inconsistent with another report, which showed that CDK2-cyclin E stimulated the intrinsic HAT activity of CBP, promoting entry into S phase (Ait-Si-Ali *et al* 1998). An explanation for these distinct effects was found upon discovery of a domain within CBP and p300 termed cell cycle regulatory domain (CRD1). This domain is a strong transcriptional repression domain that functions independently of CBP/p300 HAT activity (Snowden *et al* 2000). This can be derepressed by expression of p21 in a promoter dependent manner demonstrating that p21 can have multiple effects on CBP/p300 function. Inhibition of the interaction between CDK2-cyclin E complex and CBP/p300 would inhibit HAT activity and therefore contribute to cell cycle arrest while derepression of CRD1 would selectively activate CBP/p300-dependent transcription at specific promoters.

The transactivation potential of CBP/p300 can be increased through phosphorylation PKA (Xu *et al* 1998), C/EBPβ (Schwartz *et al* 2003), Ca2+/calmodulin-dependent protein kinase IV (CAMKIV) (Chawla *et al* 1998) and members of the MAP kinase (MAPK) signalling pathway including MEKK1 and ERK1/2 (Gusterson *et al* 2002, Janknecht and Nordheim 1996a, Sang *et al* 2003, See *et al* 2001), although the phosphorylation sites on CBP/p300 remain to be elucidated. In addition, AKT-mediated phosphorylation of p300 at serine 1834 has been shown to enhance its HAT and transcriptional activities (Huang and Chen 2005). Furthermore, the transactivation activity of CBP is augmented through phosphorylation by IKKα on serine 1382 and serine 1386 (Huang *et al* 2007b). Interestingly, IKKα-induced CBP

phosphorylation switches the binding preference of CBP from p53 to NF-κB. Recently, ATM has been demonstrated to phosphorylate p300 on serine 106 resulting in activation of p300 (Jang *et al* 2011). Conversely, phosphorylation of p300 at a conserved residue, serine 89 by PKCδ and AMP-activated protein kinase reduces p300-dependent transactivation (Yang *et al* 2001b, Yuan *et al* 2002).

Methylation of the CBP/p300 by the CARM1 methyltransferase has been shown to both activate and repress the transcriptional activity of CBP/p300. Xu *et al* (2001) demonstrated that methylation of CBP/p300 at several arginine residues within the KIX domain blocked the interaction between CBP/p300 and CREB, resulting in inhibition of CREB-dependent transcription (Xu *et al* 2001b). In contrast, methylation of different arginine residues on CBP/p300 has been demonstrated to potentiate their transactivation activity, thereby facilitating activation of nuclear hormone receptors (Chevillard-Briet *et al* 2002). Furthermore, a recent report has shown that CARM1-mediated methylation of arginine 754 of p300 stimulated its co-activator activity leading to activation of BRCA1 and subsequently induction of p21 and GADD45 (Lee *et al* 2011). In addition, p300 has been shown to be sumoylated and modification of p300 by SUMO-1 provides a binding site for HDAC6 resulting in repression of p300 transcriptional activity (Girdwood *et al* 2003). As well as post-translational modifications, CBP/p300 intrinsic activities are modulated via interactions with other proteins. The transcriptional activities of CBP/p300 are enhanced through interacting with the p68 RNA helicase (Rossow and Janknecht 2003), as well as p21 through binding to the CRD1 domain (Snowden *et al* 2000). In contrast, Cyclin D1 was shown to repress p300 transactivation in a CDK-independent manner through the recruitment of HDACs (Fu *et al* 2005). As previously mentioned, several p300 cofactors have been identified that influence both the transcriptional activity of p53 and the expression of p53 target genes. In response to DNA damage, JMY and Strap form a complex with p300 and stimulate the transcriptional activity of p300 (Demonacos *et al* 2001, Shikama *et al* 1999), whereas Skp2 binds to p300 and inhibits its transactivation activity (Kitagawa *et al* 2008).

1.7 AIMS AND OBJECTIVES

Although 53BP1 is best known for its involvement in the DNA damage response where its functions as a mediator protein that facilitates checkpoint activation and DNA repair, it was

initially proposed to function as a transcriptional regulator of p53 (Iwabuchi *et al* 1998). However, due to conflicting studies regarding the mechanism by which 53BP1 regulates the transactivation potential of p53, the function of 53BP1 in transcriptional regulation remains unclear. Despite this, other proteins involved in the cellular response to DNA damage such as BRCA1, MCPH1 and TOPBP1 have been shown to play a role in transcription indicating that DSB repair proteins can potentially also function as regulators of transcription. Furthermore, 53BP1 is a chromatin-associated protein that via its Tudor domain interacts with H4K20me2 and H3K79me2, which are found in regions of chromatin that are transcriptionally active (Botuyan *et al* 2006, Huyen *et al* 2004, Ng *et al* 2003). In addition, mass spectrometric analysis identified 53BP1 as a CBP/p300 interacting protein (GS Stewart, unpublished). Taken together these observations indicate that 53BP1, as well as being DNA damage responsive protein may also function as a transcriptional regulator. Therefore to investigate the role of 53BP1 as a transcriptional regulator, the main objectives were:

- To perform gene expression profiling using a microarray approach to identify genes that are regulated by 53BP1, both in the presence and absence of overt DNA damage.
- To characterise the interaction between 53BP1 and CBP/p300 and to assess if 53BP1 functions as a transcriptional co-activator of p53 and CBP/p300.

CHAPTER 2

CHAPTER 2 MATERIALS AND METHODS

2.1 CHEMICALS

TNFα was reconstituted according to the manufacturer's instructions (Peprotech). Aliquots were stored at -20°C. Caffeine was dissolved in deionised water to make a 80mM stock solution, which was filter sterilised (Merck). Cells were treated with 8mM caffeine for 12-18 hours at 37°C.

2.2 CELL BIOLOGY TECHNIQUES

2.2.1 Maintenance of cell lines

Cell lines were maintained in McCoys 5A medium or Dulbecco's modified Eagle's medium (DMEM) supplemented with 2mM glutamine and 10% fetal calf serum (FCS, Invitrogen). 100units/ml penicillin (Invitrogen) was also added to the medium to prevent infection. All media was stored at 4°C and solutions were pre-warmed to 37°C before cell culture use. Cells were incubated in a humidified atmosphere at 37°C with 5% CO_2. To subculture cells, the medium was removed from the cell monolayer and the cells were washed with 1X PBS. 1X Trypsin-EDTA (Invitrogen) was added (2mls per 75cm^2 flask) and incubated at 37°C for ~2 minutes. Fresh medium was added to inactivate the trypsin and cells were seeded into a new 75cm^2 flask at the appropriate density.

2.2.2 Cell lines

Human osteosarcoma U2OS cells, human non-small cell lung carcinoma H1299 cells, and human small cell lung adenocarcinoma A549 cells were obtained from the American Tissue Culture Collection (ATCC). U2OS cells were routinely maintained in McCoys 5A medium whereas, H1299, HeLa and A549 cells were maintained in DMEM.

2.2.3 Cryopreservation of cell lines

Cells from a 70% confluent flask were trypsinised and centrifuged at 1200rpm for 5 minutes at room temperature. The supernatant was gently removed and discarded. Cells were resuspended in 1ml of medium containing 50% FCS, 40% medium and 10%

dimethylsulphoxide (DMSO) (Sigma). Cells were transferred into a 1.5ml cryovial and were placed at -80°C for 24 hours before being stored in liquid nitrogen.

2.2.4 Recovery of cells from liquid nitrogen

To recover cells from liquid nitrogen, the cells were thawed quickly in a 37°C waterbath and the contents were added drop wise to 10mls of pre-warmed medium. The cells were pelleted at 1200rpm for 5 minutes at room temperature, the supernatant gently removed and the cells resuspended in fresh medium before being placed in a 75cm^2 flask.

2.2.5 Testing cells for mycoplasma

Before cells were used for experiments, they were tested for mycoplasma using the MycoAlert mycoplasma detection kit (Lonza) according to the manufacturer's instructions. Briefly, 2mls of cells were centrifuged at 1500rpm for 5 minutes and 100µl of the supernatant was transferred to a white 96 well flat-bottomed plate (Nunc). 100µl of MycoAlert® reagent was added to the sample and after 5 minutes the luminescence was recorded on a Victor 1420 multi-label plate reader (reading A). The plate was then removed and 100µl of MycoAlert® substrate was added to the sample. Following a 10 minute incubation, the luminescence was recorded (reading B) and the ratio of reading B to reading A was calculated. Ratios of greater than 1 indicate that the cells are infected with mycoplasma.

2.2.6 RNA interference

The siRNA duplexes were 21 bases with a 2 base deoxynucleotide overhang (Table 2.1, Dharmacon Research). Oligofectamine reagent (Invitrogen) was used to deliver the siRNA duplexes into eukaryotic cells. 24 hours prior to transfection, cells were seeded into 6cm dishes, so they were 30-50% confluent the following day. 20µl of 20µM siRNA was diluted in 350µl Opti-MEM (Invitrogen) and 8µl oligofectamine was diluted in 22µl Opti-MEM. After 5-10 minutes at room temperature, the oligofectamine mix was added to the siRNA mix and incubated for 20 minutes at room temperature to allow the siRNA complexes to form. Cells were washed once with Opti-MEM before the addition of 1.6ml of Opti-MEM per 6cm dish. 400µl of siRNA complexes were added to the appropriate dish, left for 4 hours at 37°C, before 1ml of medium supplemented with 30% FCS was added. Cells were harvested either 72 or 96 hours post transfection.

Target Protein	Sense sequence
Control	CGUACGCGGAAUACUUCGAdTdT
53BP1	GAACGAGGAGACGGUAAUAdTdT

Table 2.1 siRNA sequences used in this study.

2.2.7 Transient transfections

Transient expression of plasmids was achieved using Lipofectamine LTX with Plus reagent (Invitrogen). Plus reagent was used to enhance transfection efficiency. Cells were seeded into 24 well plates, so they would be 50-80% confluent after 24 hours. 500ng DNA was diluted in 100µl Opti-MEM and after the addition of 0.5µl Plus reagent, the DNA was left for 5 minutes at room temperature. Lipofectamine LTX was added at 1:2.5 or 1:4 DNA: Lipofectamine LTX ratio for H1299 and U2OS cells respectively. After 30 minutes at room temperature, 100µl of the DNA-Lipofectamine LTX complexes were overlaid on to the cells. Cells were incubated for 4-6 hours at 37°C before the medium was replaced. Cells were harvested 24 or 48 hours post-transfection.

2.2.8 Luciferase reporter assay

Luciferase expression was assayed using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. 500ng of the relevant constructs were transfected into cells in a 24 well plate in triplicate using Lipofectamine LTX and Plus reagent (Table 2.4). In each luciferase assay, a Renilla plasmid was co-transfected in as a control for transfection efficiency and the DNA amount was equalised using an empty vector, pcDNA 3.1. 24 hours post transfection, medium was removed and cells washed in PBS. Cells were lysed in 100µl of 1X passive lysis buffer (PLB) per well for 15 minutes on a rocker at room temperature. Lysates were then transferred to autoclaved microfuge tubes and placed at -20°C until the luciferase assay was conducted.

The assays for firefly luciferase activity and Renilla luciferase activity were performed sequentially in a white 96 well flat-bottomed plate (Nunc). 20µl of cell lysate was added per well, followed by the addition of 50µl of Luciferase Assay Reagent II (LAR II).

Luminescence was recorded on a Victor 1420 multi-label plate reader. After quantifying the firefly luminescence, the plate was removed and 50µl of 1X Stop and Glo Reagent was added to quench the firefly luciferase reaction whilst simultaneously initiating the Renilla luciferase reaction. Subsequently, the *Renilla* luminescence was measured.

Data was analysed and presented as relative luciferase units, which were calculated by dividing the firefly luciferase values with the Renilla luciferase values. Experiments were repeated 3-5 times to ensure reproducibility.

2.3 PROTEIN CHEMISTRY TECHNIQUES

2.3.1 Preparation of whole cell lysates

Cells were centrifuged at 1200rpm for 5 minutes at 4°C in a universal tube, the cell pellet was resuspended in 10mls of ice cold PBS and then centrifuged at 1200rpm for 5 minutes at 4°C. The cell pellet was resuspended in a volume of UTB buffer (8M urea, 150mM β-mercaptoethanol, 50mM Tris pH7.5) and transferred to a microfuge tube. The samples were sonicated for 2 x 10 seconds using a Microson XL ultrasonic cell disruptor (Misonix) on setting 4 and then centrifuged at 16,000rpm for 20 minutes at 4°C. The supernatant was aliquoted, snap frozen in liquid nitrogen and stored at -80°C.

2.3.2 Protein determination

Protein concentrations were determined according to the Bradford method. Protein samples were diluted 1:10 with sterile distilled water and 10ul aliquoted in quadruplet into a 96 well flat bottom plate. A standard curve was generated using a range of bovine serum albumin (BSA, Sigma) concentrations made from 1mg/ml stock (0-500µg/ml). 10µl of each standard was added in triplicate to the same 96 well plate. The Bradford reagent (Bio-Rad) was diluted 1:5 with water and 200µl was transferred to each well. The plate was incubated at room temperature for 5 minutes and then the absorbance was measured at 595nm using a plate reader. The protein concentrations were determined by comparing the sample absorbance with the BSA standard curve.

2.3.3 *SDS-polyacrylamide gel electrophoresis*

Proteins were analysed by SDS-polyacrylamide electrophoresis and were made with varying amounts of acrylamide depending on the relative molecular weight of the protein of interest. Routinely a 6% acrylamide gel was used to detect proteins with a molecular weight of >80kDa, a 10% acrylamide gel was used to detect proteins with a molecular weight of >40kDa and a 12% acrylamide gel was used to detect proteins with a molecular weights of <40kDa. Polyacrylamide gels were made from 30% Acrylamide, 0.8% NN'methylenebisacrylamide solution (Biorad) diluted in sterile distilled water to give the desired final percentage. 0.1M Tris, 0.1M Bicine (N, N-bis[2-hydroxy-ethyl-glycine]) (pH8.3), 0.1% (w/v) SDS and 0.2% (v/v) TEMED were then added to give a final volume of 39.88mls. After the addition of 200µl of freshly prepared 10% (w/v) ammonium persulphate, which initiates polymerisation, the gel was cast in the gel apparatus (Hoefer Scientific Instruments) and a well forming comb inserted.

Following the gel setting, the wells were rinsed with running buffer (0.1M Tris, 0.1M Bicine, 0.1% SDS (w/v) pH8.3). An equal volume of 2X sample buffer (0.125M Tris/HCl pH6.8, 4% (w/v) SDS, 20% Glycerol, 0.2M DTT, 0.02% Bromophenol blue) was added to each protein sample before it was boiled at 100°C for 5 minutes to denature the protein. Samples were loaded onto the gel alongside a pre-stained molecular weight marker (Amersham). 30-50µg of whole cell lysate was routinely used per lane. Electrophoresis was carried out at 25mA for 5 hours or overnight at 8mA.

2.3.4 *Western blotting*

Following SDS-PAGE gel electrophoresis, the separated proteins were transferred onto a nitrocellulose membrane (GE Healthcare). A blotting cassette was set up containing the gel and nitrocellulose membrane sandwiched between two pieces of Whatmann 3MM filter paper and this by two sponge sheets. All components of the cassette were pre-soaked in transfer buffer (48mM Tris, 390mM glycine, 20% (v/v) methanol) before the cassette was constructed. The cassette was placed into the blotting tank (Hoefer transblot electroblotter) that was filled with transfer buffer with the nitrocellulose facing the positive electrode. Blotting was carried out overnight at 200mA for 18 hours.

After blotting the proteins were visualised on the nitrocellulose membrane by staining with Ponceau S stain (Sigma) for 1 minute. Excess stain was removed by rinsing with distilled water. The membrane was then washed in either phosphate-buffered saline with 0.1% Tween-20 (PBS-T) or Tris-buffered saline (200mM Tris, 1.36M NaCl pH7.6) with 0.1% Tween-20 (TBS-T) until the stain was completely removed. TBS-T was used if phosphorylated proteins were being analysed. The membrane was blocked at room temperature for 2 hours in milk (5% (w/v) skimmed milk powder in either PBS-T or TBS-T) to block any unoccupied binding sites on the membrane and to prevent non-specific binding of antibodies to the membrane. Following this, the nitrocellulose was incubated with the primary antibody diluted to the appropriate concentration in milk block with gentle rocking (Table 2.2). Excess primary antibody was removed by washing the membrane three times in PBS-T or TBS-T for 10 minutes. The relevant horse-radish peroxidise-labelled, anti-species secondary antibody conjugate diluted in milk block was incubated at room temperature with the membrane for 1 hour with gentle rocking (Table 2.3). The membrane was then washed three times in PBS-T or TBS-T for 10 minutes to remove any excess secondary antibody. The membrane was treated for 1 minute in equal volumes of the two reagents supplied with the enhanced chemiluminescence system (ECL) (GE Healthcare) and exposed to Hyperfilm^{TM} (GE Healthcare).

2.3.5 Immunofluorescence

Cells were grown to 70% confluency on either 12 well poly-L-lysine coated glass slides (Hendley) in 10cm dishes or on poly-L-lysine coverslips (BD Biosciences). Alternatively, 50µl of cell suspension was added to one well on the slides or 100µl cell suspension was added to coverslip and the cells were left to adhere for at least 1 hour. Slides were fixed in ice cold paraformaldehyde (3.6% paraformaldehyde in PBS, pH 7.2) for 10 minutes and then placed into ice-cold extraction buffer (10mM PIPES, 300mM sucrose, 20mM NaCl, 3mM $MgCl_2$, 0.5% triton X-100, pH6.8) for 7 minutes. Cells grown on coverslips were fixed and extracted in ice-cold methanol for 15 minutes at -20°C. Cells were washed in PBS (3 x 5 minutes) before being blocked in filter sterilised 10% FCS in PBS for 1 hour at room temperature or overnight at 4°C. Cells were then washed for 3 x 5 minutes in PBS and incubated for 1 hour in primary antibody diluted to the appropriate concentration in 1% FCS in PBS (Table 2.2). Two ‘dip’ washes in PBS were performed to remove excess antibody,

followed by 3 x 5 minute washes in PBS. Cells were then incubated with secondary antibodies diluted to the appropriate concentration in 1% FCS in PBS for 1 hour in a dark moist box (Table 2.3). To remove the excess antibody two 'dip' washes in PBS were performed proceeded by 3 x 5 minute washes in PBS. Slides and coverslips were mounted with a drop of Vectashield Mounting Medium (Vector Laboratories) containing 4′, 6-diamidino-2-phenylindole (DAPI). Slides were protected with glass coverslips (Surgipath) and coverslips were mounted on glass slides (Surgipath). The edges were sealed with nail varnish and slides were stored at 4^oC in the dark. Cells were visualised using Nikon Eclipse E600 microscope and images were recorded and analysed using the Hamamatsu C4742-95 digital camera and image analysis software Velocity version 4.

2.3.6 Immunoprecipitation

Cell pellets were lysed for 30 minutes with rotation in lysis buffer containing 50mM Tris pH7.5, 150mM NaCl, 1% Nonidet P-40, supplemented with 2mM $MgCl_2$, 90U/ml Benzonase (Novagen) and a EDTA-free protease inhibitor tablets (Roche). Cell lysates were centrifuged at 44000rpm for 30 minutes at 4^oC to remove cell debris. To prevent any non-specific proteins that may bind to the beads, the cell lysates were pre-cleared by the addition of either Protein G-Sepharose (Sigma) or Protein A-Sepharose (GE Healthcare) beads depending on the antibody species to be used for 1 hour at 4^oC with rotation. Protein A/G was removed by centrifugation at 16,000rpm for 5 minutes at 4^oC. 10μg of antibody was added to cleared lysate and antigen-antibody complexes were left to form for 3 hours at 4^oC on a vertical rotating wheel (Table 2.2). Non-specific species-matched IgG was used as an antibody control. Lysates were centrifuged at 44000rpm for 15 minutes at 4^oC. Immune complexes were precipitated by rotating at 4^oC for 2 hours with 25μl of protein A or protein G beads. Bead bound immunoprecipitates were washed three times in wash buffer (50mM Tris, pH 7.5, 150mM NaCl, 0.5% Nonidet P-40) supplemented with a complete protease inhibitor tablet. Samples were boiled in 2X sample buffer for 5 minutes at 100^oC in preparation for SDS-PAGE and Western blotting.

Antigen	Species	Company	Dilution	Application	Incubation Conditions for WB
53BP1	Rabbit	Novus Biologicals	1:1000 10μg per IP	WB, IF IP	2hr RT
SMC1	Rabbit	Bethyl Laboratories	1:1000	WB	2hr RT
Phospho-Ser966 SMC1	Rabbit	Bethyl Laboratories	1:1000	WB	2hr RT
NBS1	Mouse	Novus Biologicals	1:10,000	WB	2hr RT
Phospho-Ser343 NBS1	Rabbit	SAB	1:500	WB	O/N 4^oC
H2A	Rabbit	Millipore	1:5000	WB	O/N 4^oC
Phospho-Ser139 H2AX	Mouse	Millipore	1:1000	WB	O/N 4^oC
p53 (DO-1)	Mouse	Donated by R.Grand	1:1000	WB	2hr RT
Phospho-Ser15 p53	Rabbit	Cell Signalling	1:1000	WB	O/N 4^oC
MDM2 (2A10)	Mouse	Donated by R.Grand	1:10	WB	O/N 4^oC
p21	Mouse	Cell Signalling	1:1000	WB	O/N 4^oC
PUMA	Rabbit	Abcam	1:500	WB	O/N 4^oC
CBP	Rabbit	Bethyl Laboratories	1:1000 10μg per IP	WB IP	O/N 4^oC
p300	Rabbit	Bethyl Laboratories	1:5000 10μg per IP	WB IP	O/N 4^oC
p300	Mouse	Millipore	1:1000	WB	O/N 4^oC
HA	Mouse	Sigma	1:1000 1:200	WB IF	2hr RT
p65	Rabbit	Santa Cruz	1:1000 1:50	WB IF	O/N 4^oC
p65	Mouse	Santa Cruz	1:1000 1:50 10μg per IP	WB IF IP	O/N 4^oC
Phospho-Ser536 p65	Rabbit	Cell Signalling	1:1000	WB	O/N 4^oC
Phospho-Ser468 p65	Rabbit	Cell Signalling	1:1000	WB	O/N 4^oC
IκBα	Rabbit	Santa Cruz	1:1000	WB	O/N 4^oC
IKKα	Rabbit	Santa Cruz	1:50	WB	O/N 4^oC
IKKβ	Mouse	Santa Cruz	1:200	WB	O/N 4^oC

NEMO	Rabbit	Santa Cruz	1:500	WB	O/N 4°C
p50	Rabbit	Enzo Life Sciences	1:1000	WB	O/N 4°C
G3BP2	Rabbit	Bethyl Laboratories	1:5000	WB	O/N 4°C
FADD	Mouse	BD Transduction	1:1000	WB	O/N 4°C
BIRC4/XIAP	Rabbit	Cell Signalling	1:1000	WB	O/N 4°C
BAG4/SODD	Rabbit	Abcam	1:500	WB	O/N 4°C
IKIP	Goat	Abcam	1:500	WB	O/N 4°C
IgG	Rabbit	DAKO	10µg per IP	IP	
β-actin	Mouse	Sigma	1:50,000	WB	1hr RT

Table 2.2 ***Primary antibodies used for Western blotting (WB), immunofluorescence (IF) and immunoprecipitation (IP).*** *O/N denotes overnight and RT denotes room temperature.*

Antibody	Company	Dilution	Application
Polyclonal swine anti-rabbit IgG HRP	DAKO	1:1000	WB
Polyclonal goat anti-mouse IgG HRP	DAKO	1:3000	WB
Alexa flour® 594 goat anti-mouse IgG	Invitrogen	1:1000	IF
Alexa flour® 488 goat anti-rabbit IgG	Invitrogen	1:1000	IF

Table 2.3 ***Secondary antibodies used for Western blotting (WB) and immunofluorescence (IF).***

2.3.7 *GST pull-down assay*

Glutathione S-transferase (GST) pull-down assays were carried out using GST-fusion proteins and [^{35}S]-labelled proteins (Table 2.4). Typically, 20-30µg of GST-fusion protein was mixed with 10µl of [^{35}S]-labelled protein for 1 hour at 4°C with rotation. Protein complexes were isolated by incubation with 35µl glutathione-agarose beads for 1 hour with rotation at 4°C. Beads were washed three times in GST lysis buffer (1% Triton-X100, 1mM EDTA in PBS) and twice in GST wash buffer (1mM EDTA in PBS). GST-protein complexes were eluted with 50µl of GST elution buffer (25mM glutathione, 50mM Tris, pH8.0) for 1 hour at 4°C with rotation. Samples were mixed with 4X sample buffer (250mM Tris pH6.8, 8% SDS, 10% glycerol, 0.04% Bromophenol blue, 400mM DTT), boiled for 5 minutes at 100°C and loaded on to an SDS-PAGE gel.

After gel electrophoresis, gels were stained in a solution containing 0.1% (w/v) Coomassie Brilliant Blue R-250 (Sigma), 10% glacial acetic acid and, 40% methanol on a shaker for 10 minutes at room temperature. Subsequently, gels were placed in destaining solution containing 10% glacial acetic acid and 30% methanol until proteins were clearly visible. Destained gels were incubated in Amplify Fluorographic Reagent (Amersham Bioscience) for 30 minutes on a shaker. Gels were dried under vacuum at 80°C for 2 hours and exposed for autoradiography at -20°C using Hyperfilm (GE Healthcare).

2.3.8 *Nuclear extract fractionation*

HeLa S3 nuclear extract was purchased from Cilbiotech (http://www.cilbiotech.be/index.htm) and was first subject to ion exchange chromatography to separate proteins based on their molecular charge. The extract was loaded on to a positively charged anion exchange column called a diethylaminoethyl (DEAE) cellulose column, which had been equilibrated with equilibration buffer (20mM Tris-HCl pH8, 100mM KCl, 0.5mM DTT). The protein fractions were eluted step wise in elution buffer containing 20mM Tris-HCl pH8.0, 0.5mM DTT and increasing salt concentrations 0.2M, 0.3M and 0.4M KCl. Fractions were collected and subject to SDS-PAGE on a 4-12% gradient gel (Biorad) and Western blotting.

Peak fractions were then used for gel filtration chromatography to separate proteins by molecular weight. The Superose 6 column was equilibrated with 20mM HEPES (pH 7.5),

Gene	Vector	Tag	Application	Source
53BP1 (full length)	pcDNA 3.1	Flag	IVT, *in vivo* overexpression and luciferase assays	Dr G Stewart
53BP1 (aa 1-356)	PGEX 4T-1	GST	GST pull down assays	Dr G Stewart
53BP1 (aa 333-759)	PGEX 4T-1	GST	GST pull down assays	Dr G Stewart
53BP1 (aa 722-1039)	PGEX 4T-1	GST	GST pull down assays	Dr G Stewart
53BP1 (aa 992-1331)	PGEX 4T-1	GST	GST pull down assays	Dr G Stewart
53BP1 (aa 1309-1620)	PGEX 4T-1	GST	GST pull down assays	Dr G Stewart
53BP1 (aa 1584-1972)	PGEX 4T-1	GST	GST pull down assays	Dr G Stewart
CBP (full length)	pcDNA3.1		IVT	Dr A Turnell
CBP (aa 1-451)	PGEX 4T-1	GST	GST pull down assays	Dr G Stewart
CBP (aa 451-721)	PGEX 4T-1	GST	GST pull down assays	Dr G Stewart
CBP (aa 721-1100)	PGEX 4T-1	GST	GST pull down assays	Dr G Stewart
CBP (aa 1099-1460)	PGEX 4T-1	GST	GST pull down assays	Dr G Stewart
CBP (aa1460-1891)	PGEX 4T-1	GST	GST pull down assays	Dr G Stewart
CBP (aa 1892-2441)	PGEX 4T-1	GST	GST pull down assays	Dr G Stewart
p300	pBS		IVT	Dr A Turnell
p300 (aa 19-596)	PGEX 4T-1	GST	GST pull down assays	Dr G Stewart
p300 (aa 596-957)	PGEX 4T-1	GST	GST pull down assays	Dr G Stewart
p300 (aa 863-1382)	PGEX 4T-1	GST	GST pull down assays	Dr G Stewart
p300 (aa 1302-1756)	PGEX 4T-1	GST	GST pull down assays	Dr G Stewart
p300 (aa 1716-1961)	PGEX 4T-1	GST	GST pull down assays	Dr G Stewart
p300 (aa 1916-2414)	PGEX 4T-1	GST	GST pull down assays	Dr G Stewart
GST	PGEX 4T-1		GST pull down assays	Dr G Stewart
Adenovirus E1a 13S	PGEX 4T-1	GST	GST pull down assays	Dr R Grand
	pcDNA3.1		DNA transfections	Dr Philip Byrd
Renilla	pRL-TK		luciferase reporter assays	Dr John O'Neil

p53 luciferase reporter	PG13-Luc		p53 luciferase reporter assay	Dr Ester Hammond
p53 wild type	pBK-CMV		p53 luciferase reporter assay	Dr Ester Hammond
p53 mutant (R175H)	pBK-CMV		p53 luciferase reporter assay	Dr Ester Hammond
p21 luciferase reporter	p21-Luc/ WWP-Luc		p21 luciferase reporter assay	Dr A Turnell
Gal4 luciferase reporter	pGL3-E1B		p300 luciferase reporter assay	Dr Neil Perkins
Gal4 DBD (aa 1-147)	pcDNA3	Gal4 DBD	p300 luciferase reporter assays	Dr A Turnell
Gal4 p300 (full length)	pVR1012	Gal4 DBD	p300 luciferase reporter assays	Dr Neil Perkins
	pGL2		NF-κB luciferase reporter assays	Dr John O'Neil
	3enh-κb-ConA-Luc		NF-κB luciferase reporter assays	Dr John O'Neil
53BP1 (full length)	pCMH6K	HA	luciferase reporter assays, DNA transfections	Dr Michal Goldberg
53BP1 (aa 1-1710)	pCMH6K	HA	luciferase reporter assays, DNA transfections	Dr Michal Goldberg
53BP1 (aa 1-1052)	pCMH6K	HA	DNA transfections	Dr Michal Goldberg
53BP1 (aa 1052-1972)	pCMH6K	HA	luciferase reporter assays, DNA transfections	Dr Michal Goldberg
53BP1 (aa 1483-1972)	pCMH6K	HA	luciferase reporter assays, DNA transfections	Dr Michal Goldberg
53BP1 (aa 1052-1710)	pCMH6K	HA	luciferase reporter assays, DNA transfections	Dr Michal Goldberg
53BP1 (Δ1235-1616)	pCMH6K	HA	luciferase reporter assays, DNA transfections	Dr Sagar Sengupta

Table 2.4 ***Gene expression constructs used in this study.***

0.2M KCl and 0.5mM DTT and the fraction applied to the column. The fractions were collected and an equal volume was loaded on to a 4-12% SDS polyacrylamide gel (Invitrogen) and Western blotting carried out.

2.4 MOLECULAR GENETICS TECHNIQUES

2.4.1 Preparation of media and agar plates

All bacterial cultures were grown in Luria broth (LB) (Sigma), which was prepared by dissolving 25g of LB in 1L of distilled water and sterilised by autoclaving at 121°C, 15psi for 30 minutes.

LB-agar was made by supplementing LB with 1.2% agar and sterilised by autoclaving. Following boiling, the LB-agar was allowed to cool to approximately 50°C before supplemented with antibiotics. 25mls of liquid LB-agar was dispensed into a sterile 9cm diameter Petri dish. Plates were then allowed to set at room temperature and stored at 4°C until used for bacterial transformations. Before use, the plates were dried in a fume hood for 1 hour.

2.4.2 Antibiotics

Ampicillin and kanamycin were made up as stock solution of 100mg/ml in sterile distilled water stored at -20°C. Ampicillin and kanamycin were and used at a final concentration of 50μg/ml and 25μg/ml respectively.

2.4.3 Transformation of bacteria

Bacterial transformations were carried out in competent *Escherichia coli* cells. MAX efficiency DH5α competent cells and MAX efficiency Stbl2 competent cells (Invitrogen) were used for plasmid production, whereas BL21-Gold competent cells were used to generate recombinant proteins. For each transformation, 50μl aliquot of competent cells was thawed on ice before addition of up to 100ng DNA. Cells were incubated on ice for 30 minutes, before being heat shocked at 42°C for either 30 seconds for DH5α cells, 25 seconds for Stbl2 cells or 20 seconds for BL21 cells. Cells were placed back on ice for 2 minutes before the addition of 450μl preheated (42°C) super optimal catabolite (SOC) medium (Invitrogen). Transformation

reactions were placed in an incubator at either 30°C for 90 minutes (Stbl2 cells) or 37°C for 1 hour for DH5α and BL21 cells with shaking at 225rpm. The transformation reaction was spread on to the LB-agar plates and incubated overnight at 37°C.

2.4.4 Growth of overnight cultures from colonies

Single colonies of competent cells were picked using a sterile tip and grown in 150μl of LB medium in a 96 well flat-bottomed plate at 37°C for 2 hours. Plates were sealed and placed at 4°C until required.

2.4.5 Mini-preparation of DNA

Mini-preparations of plasmid DNA were prepared using the GenElute™ plasmid miniprep kit (Sigma) according to the manufacturer's instructions. Initially, 5mls of LB broth supplemented with the appropriate antibiotic was inoculated with 10μl of culture grown in the 96 well plate, and grown overnight at 37°C with shaking. The overnight culture (3-5mls) was pelleted by centrifugation at 12,000 x g for 1 minute. Bacterial cell pellets were resuspended in 200μl of resuspension solution; following this the cells were lysed in 200μl alkaline lysis buffer by gentle inversion (6-8 times). The alkaline lysis of the bacterial cells was halted by the addition of 350μl of neutralisation solution. The lysed samples were then centrifuged at 12,000 x g for 10 minutes in order to pellet the cell debris. The column was equilibrated with column preparation solution before the supernatant was applied. The column was washed in a solution containing ethanol and the plasmid DNA was then eluted in 100μl of elution solution. The quality of the DNA was analysed by agarose gel electrophoresis and the DNA was stored at -20°C.

2.4.6 Polymerase chain reaction (PCR)

PCR amplification was performed using Taq Expand Long Template (Roche) and primers from Sigma (Table 2.5). Reaction mixtures were made up in thin-walled PCR tubes in a 50μl volume containing 5μl of 10X PCR buffer, 0.5μl-1μl DNA, 2μl (250ng) forward primer, 2μl (250ng) reverse primer, 1.5μl dNTP mix (10mM each dNTP) (Roche) and sterile distilled water. 0.75μl of Taq polymerase (2.5U/μl) was added last. The tubes were placed in the PCR machine (Veriti 96 well thermal cycler, Applied Biosystems) and the following PCR

Primer Name	Sequence
Human 53BP1 1	Forward: 5'-TCTGGAAGGCTGCGCAGTATTGG-3' Reverse: 5'-GGTGCTGAAGATACTGCCTCATC-3'
Human 53BP1 2	Forward: 5'-ATCAGTCAGGTCATTGAGCAG-3' Reverse: 5'-CTGCAGATTCAGAAGTCACCAGA-3'
Human 53BP1 3	Forward: 5'-CTGCAAGGTCAGAGGACATGC-3' Reverse: 5'-GTTGATGCTTTCTACAAGTGA-3'
Human 53BP1 4	Forward: 5'-GTCTGGAAGAACAATCAAATG-3' Reverse: 5'-CAGGGTCTGAGGTGGAAGAAAT-3'
Human 53BP1 5	Forward: 5'-GTCACCAGCTACTCGATCTGAG-3' Reverse: 5'-GTGAGAGCAGATGATCCTTTAAG-3'
Human 53BP1 6	Forward: 5'-TAGAACCATGTGCTGAGAATAG-3' Reverse: 5'-CTACTGCTGTTGCTGAGTCTGT-3'
Human 53BP1 7	Forward: 5'-GTCATGTCTGAAAGCATGGTGGAG-3' Reverse: 5'-GTGGTGAGAAACCAGTCAGTGCT-3'
Human 53BP1 8	Forward: 5'-CATAGGAATCCAAACCATGGAGTG-3' Reverse: 5'-CAGTGTGTGAGGAGGATGGTGAT-3'
Human 53BP1 9	Forward: 5'-ACAAGTCTCTCAGCTATGCACAG-3' Reverse: 5'-GATGATGGGTACGAATGTGATGT-3'
Human 53BP1 10	Forward: 5'-AATAGCTTTGTAGGGCTCCGTG-3' Reverse: 5'-GAGGTCGCAAGTCTGCCACAGTA-3'
Human 53BP1 11	Forward: 5'-TCCAGTAGCAGCAGCACAACC-3' Reverse: 5'-GTATCAGACCAACAGCAGACAT-3'
Human 53BP1 12	Forward: 5'-TGGGTCCATGATAGTTGCCATGC-3' Reverse: 5'-GTGTCTTGTGTGTAACTGGATTCCTT-3'
Human 53BP1 13	Forward: 5'-ATGGACCTCACTGGAAGTCAGTTG-3' Reverse: 5'-GAGAATGAGGCTCGAAGTGAGGAT-3'
pcDNA3.1	Forward: 5'-ACTCACTATAGGGAGACCCAAGC-3' Reverse: 5'-GCAACTAGAAGGCACAGTCGAGG-3'

Table 2.5 ***PCR and sequencing primers used in this study.***

conditions were applied: 1 cycle of 95°C for 2 minutes; 30 cycles of 95°C for 20 seconds, 60°C for 30 seconds, 72°C for 1 minute/Kb of plasmid DNA length followed by a final 10 minute extension at 72°C. The resulting DNA products were resolved by agarose gel electrophoresis and purified using an Invitrogen purification kit.

2.4.7 Purification of PCR products

Before the DNA was used for other applications such as sequencing and restriction digestion, any impurities such as primers, dNTPs, enzymes and salts were removed from the PCR product using the PureLink PCR Purification kit (Invitrogen). Briefly, 4 volumes of Binding buffer were added to 1 volume of PCR reaction and mixed well to enable the double stranded DNA (dsDNA) to bind to the column. The sample was transferred to a PureLink Spin Column, centrifuged for 1 minute at 10,000 x g and the flow through was discarded. Impurities were removed by washing the column with 650µl of wash buffer supplemented with ethanol and centrifuging at 10,000 x g for 1 minute. Again the flow through was discarded and the column transferred to fresh tubes. The DNA was eluted in 50µl of low salt elution buffer (10mM Tris-HCl, pH8.5) and the purity of the DNA was analysed by agarose gel electrophoresis. DNA was stored at -20°C until further use.

2.4.8 Agarose gel electrophoresis

Analysis of DNA was performed on agarose gels, which were prepared by dissolving agarose (Sigma) in 1X TBE buffer (89mM Tris, 89mM Boric acid, 2mM EDTA) to a final concentration of 0.8-4% (w/v). The agarose was dissolved by heating the mixture to boiling point. It was then allowed to cool before the addition of 1µl of Syber Safe DNA gel stain (Invitrogen) per 100ml. 5µl of miniprep or 1µl of PCR product was diluted with 6X loading buffer (0.25% Bromophenol blue, 30% glycerol in a 10mM Tris, 1mM EDTA, pH8.0 solution) and then loaded. To ensure the DNA was the correct size, a DNA Molecular Weight Marker (Roche) was run alongside the samples. Gel electrophoresis was performed in 1X TBE at 120V for up to 1 hour and the DNA was visualised with a Safe Imager blue-light transilluminator.

2.4.9 DNA sequencing

Sequencing was performed to validate the DNA sequence of coding regions generated by PCR and to ensure any mutant plasmid DNA contained the correct mutations. Each reaction was carried out using the Big Dye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and consisted of 7μl 2.5x sequencing buffer provided in the kit, 1μl purified PCR product, 1μl (125ng) of primer, 1μl of BigDye® Terminator v3.1 Ready Reaction mix provided in the kit and sterile distilled water up to total volume of 20μl. Reactions were placed in a thermal cycler for the sequencing reaction and run for 30 cycles using the following conditions: 95°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes.

The PCR products were then precipitated with 50μl absolute ethanol and 2μl 3M sodium acetate (pH4.6) for 15 minutes at room temperature. Samples were centrifuged at 13,000rpm for 20 minutes, the supernatant removed and the pellet washed with 250μl of 70% (v/v) ethanol before being centrifuged for a further 5 minutes. The supernatant was removed and any residual ethanol was removed by air drying for at least 1 hour. Following addition of 10μl of 'Hi-Dye' (Applied Biosystems) to dissolve the DNA pellet, the samples were denatured at 95°C for 5 minutes and then put on ice immediately to prevent reduplexing.

Samples were loaded into the appropriate 96 well plates and sequencing was performed using the Applied Biosystems 3100 ABI prism capillary sequencer. Following the run, the data files were extracted from the 3100 data collection software version 1.0 and analysed using the software programme FinchTV version 1.4.0 (Geospiza Inc).

2.4.10 Maxi-preparation of plasmid DNA

Large amounts of DNA were purified from bacterial cultures using the PureLink^{TM} HiPure Plasmid Filter Purification kit (Invitrogen) according to the manufacturer's instructions. Initially, 10mls of LB containing either 50μg/ml of ampicillin or 25μg/ml kanamycin was inoculated with 10μl of bacterial culture from the 96 well plate and grown overnight at 37°C at 225rpm in a shaking incubator. 250μl of this culture was then used to inoculate 200mls of LB containing the appropriate antibiotic, which was grown overnight. The following day, a HiPure Filter Maxi Column containing a filtration cartridge was equilibrated with 30mls of equilibration buffer and the solution allowed to drain by gravity flow. Meanwhile, the cells were harvested by centrifugation at 4,000 x g for 10 minutes at 4°C and resuspended in 10mls

of resuspension solution. Cells were lysed in 10mls alkaline lysis buffer and incubated at room temperature for 5 minutes. The reaction was halted by addition of 10mls of precipitation buffer. The precipitated lysate was transferred into the equilibrated column and allowed to run through the filter by gravity flow. Immediately after the column stopped dripping, the filtration cartridge was removed and the lysate washed with 50mls wash buffer containing ethanol. DNA was collected in a 50ml falcon tube by the addition of 15mls of elution buffer to the column. To precipitate and desalt the DNA, isopropanol was added to the eluted DNA, mixed well and centrifuged at 5,000 x g for 1 hour at 4°C. The supernatant was removed and the pellet washed with 5mls of 70% (v/v) ethanol before being centrifuged again at 5,000 x g for 1 hour at 4°C. The supernatant was discarded, the pellet air dried, resuspended in 100-500µl sterile distilled water and stored at -20°C.

2.4.11 Determination of DNA purity and concentration

The nanodrop spectrophotometer was used to determine DNA concentration and DNA purity. The ratio of absorbance at 260nm and 280nm is used to assess DNA purity and a ratio of ≥1.8 indicates the DNA is pure and free from protein contamination. The DNA concentration was quantified by measuring the absorbance at OD_{260nm} and calculated against 1 OD_{260nm} unit being an equivalent of 50µg of DNA in 1ml.

2.4.12 In vitro transcription/translation (IVT)

[^{35}S] methionine-labelled proteins were produced *in vitro* using a TNT® Coupled T7 Reticulocyte Lysate System (Promega) according to the manufacturer's instructions. Briefly, the reaction mixture consisted of 25µl TNT® rabbit reticulocyte lysate, 2µl TNT® reaction buffer, 1µl TNT® T7 RNA polymerase, 1µl amino acid mixture minus methionine, 2µl [^{35}S]-labelled methionine (1000Ci/mmol at 10mCi/ml; MP Biomedicals), 1µl RNasin® ribonuclease inhibitor (40U/µl) (Promega), 2µl DNA template (0.5µg/µl) and nuclease free water to a final volume of 50µl. The reaction was incubated at 30°C for 90 minutes, centrifuged and stored at -80°C. To verify the presence of *in vitro* translated protein, 5µl of the sample was loaded on to an SDS-PAGE gel. Following electrophoresis, the gel was stained with Coomassie blue, destained overnight, incubated in Amplify and dried under vacuum. [^{35}S] methionine-labelled proteins were visualised by exposure of the dried gel to Hyperfilm at -20 °C.

2.4.13 Production and purification of recombinant proteins

Plasmids encoding GST upstream of the coding sequence of interest were transformed into BL21 competent cells. Colonies from successful transformations were picked and grown up as described in section 2.4.4. 10ml of LB containing 50μg/ml ampicillin was inoculated with 10μl of culture and incubated overnight at 37°C with shaking.

The following day, a mini induction was conducted to test the expression of the protein. The 10ml culture was diluted 1:10 and two cultures were grown for each protein at 37°C in a shaking incubator. After 1 hour, one of the cultures was induced with 0.4mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma) and both the non-induced and induced cultures were incubated for a further 3-4 hours at 30°C with shaking. Cultures were pelleted at 13,000rpm for 5 minutes, the supernatant removed and samples boiled at 100°C for 7 minutes in 50μl 2X loading buffer. 15μl of the samples were loaded on to an SDS-PAGE gel and electrophoresis carried out. The gel was stained with Coomassie blue, destained overnight and dried on to 3MM Whatman filter paper. Positive colonies that induced the GST-fusion protein of interest were selected and then 10mls inoculated into 1L of LB containing 50μg/ml ampicillin. Bacterial cultures were grown in a shaking incubator at 37°C for 4 hours or until the optical density of the cultures had reached A_{600} = ~0.6. Fusion proteins were induced by the addition of 1mM IPTG and cultures were incubated for a further 5 hours at 30°C. The bacterial cultures were then pelleted at 8,000 x g for 15 minutes at 4°C, snap frozen in liquid nitrogen and stored at -80°C until the next stage of the purification.

To purify the GST-fusion proteins, the bacterial cell pellet was thawed and lysed in 25mls ice-cold GST lysis buffer (1% Triton X-100, 1mM EDTA pH8 in 1X PBS) supplemented with a complete protease inhibitor tablet (Roche). Lysates were sonicated five times on ice for 30 seconds at 2 minute intervals and pelleted at 20,000rpm for 10 minutes at 4°C to remove any insoluble material. The supernatant was transferred to a fresh tube and centrifuged again. 2mls of washed glutathione agarose beads was mixed with the resulting supernatant and rotated at 4°C for 2 hours. Beads were centrifuged at 2000rpm for 30 seconds at 4°C, washed three times in lysis buffer and twice in GST wash buffer (1mM EDTA pH8.0 in 1X PBS). GST-fusion proteins were eluted by incubating the glutathione agarose beads in 4mls of elution buffer (25mM glutathione, 50mM Tris, pH8) for 1 hour at 4°C with rotation. Following centrifugation at 2000rpm for 30 seconds at 4°C, the supernatant was removed and set aside.

To ensure all the GST-fusion proteins had been released, the elution process was repeated. The supernatants from the two elutions, which contain the GST-fusion proteins, were transferred to dialysis tubing. Prior to this, the tubing was hydrated by heating the tubing up in a solution of 2mM EDTA pH8.0 and 3% $NaHCO_3$, left to cool for 5 minutes and then rinsed in distilled water. The GST-fusion proteins were dialysed overnight at 4°C in 5L of 50mM Tris pH7.5, 10% (v/v) glycerol and 1mM dithiothreitol (DTT) (Sigma). The following day, GST-fusion proteins were collected, aliquoted and the protein concentration determined by Bradford assay. The purified proteins were visualised by SDS- PAGE gel electrophoresis and Coomassie blue staining. Purified proteins were stored at -80°C.

2.4.14 RNA extraction

Cells were harvested, centrifuged at 1200rpm for 5 minutes at 4°C and the pellet was disrupted by flicking the tube. The sample was homogenised in 5mls Trizol (Invitrogen) by pipetting up and down several times. The homogenised sample was incubated at room temperature for 5 minutes to allow for the complete dissociation of nucleoprotein complexes. 1ml of chloroform was added and the sample tubes were vigorously shaken by hand for 1 minute, followed by incubation at room temperature for 3 minutes. Samples were centrifuged at 12,000 x g for 15 minutes at 4°C, which resulted in the mixture being separated into a lower red, phenol-chloroform phase containing DNA and protein, and a colourless upper aqueous phase, which contains the RNA. The aqueous phase was transferred to a fresh tube and the RNA precipitated by incubating the sample with isopropanol (2.5mls) at -20°C overnight. The next day the sample was centrifuged at 12,000 x g at 4°C for 10 minutes, the supernatant removed and the pellet washed in 5mls of 70% ethanol (v/v). Samples were then mixed by vortexing before further centrifugation at 7,500 x g for 5 minutes at 4°C. The pellet was resuspended in 100μl RNase free water and stored at -80°C.

2.4.15 RNA purification

A Qiagen RNeasy Mini Kit was used to purify the RNA. To create conditions that promote selective binding of RNA to membrane in the RNeasy mini column, 350μl of buffer RLT (without β-mercaptoethanol) was added to the RNA and thoroughly mixed. 250μl of absolute ethanol was then added, mixed, and the sample transferred to an RNeasy mini column placed in a 2ml collection tube. Columns were centrifuged for 15 seconds at 8,000 x g, the flow

through discarded and 500μl of Buffer RPE was added to the RNeasy column to wash the spin column membrane. Columns were centrifuged again at 8,000 x g for 15 seconds, the flow through discarded and the columns washed again in 500μl of 80% (v/v) ethanol, followed by centrifugation at 8,000 x g for 2 minutes. The flow through was discarded and the tubes spun again for an additional 5 minutes at 12,000 x g to remove any residual ethanol. The RNeasy column was transferred to a new 1.5ml collection tube and the RNA was eluted by the addition of 25μl of RNase-free water pipetted directly onto the spin column membrane followed by centrifuging at 8,000 x g for 1 minute.

The RNA was then treated with TURBO DNase free kit (Ambion) to remove any contaminating DNA. 2.5μl of 10X TURBO DNase buffer and 1μl TURBO DNase was added to the RNA, mixed gently and incubated at 37°C for 30 minutes. The DNase was inactivated by adding 2.5μl of DNase Inactivation Reagent and incubated for 2 minutes at room temperature with occasional mixing to redisperse the DNase Inactivation Reagent. Treated RNA was spun at 10,000 x g for 2 minutes and the supernatant, which contains the RNA was transferred to a fresh tube and stored at -80°C.

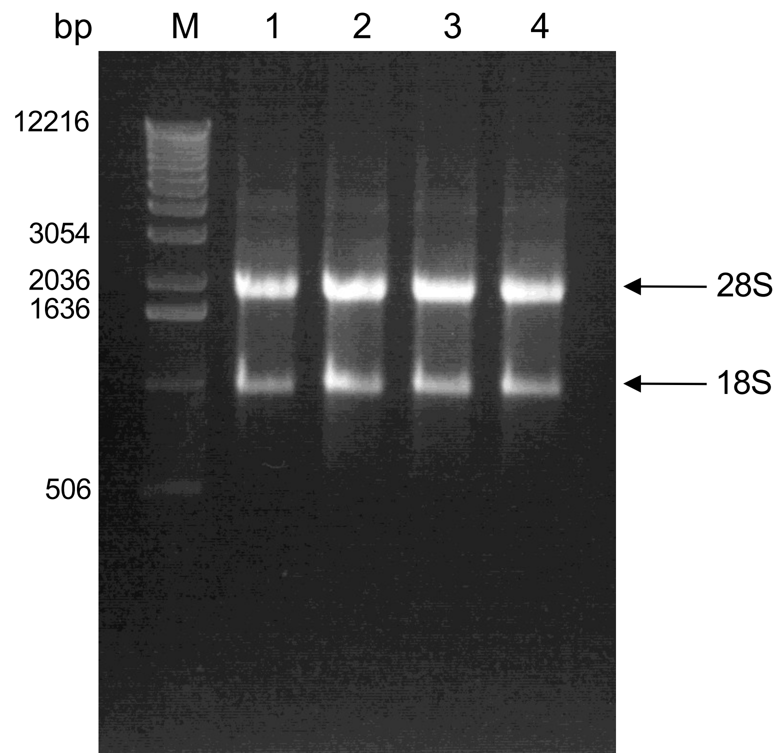
2.4.16 Determination of RNA integrity and concentration

The concentration of the RNA extracted was determined by measuring the optical density (OD) at A_{260nm} using a nanodrop spectrophotometer, with 1 A_{260nm} unit being an equivalent of 40μg of RNA in 1ml distilled water. The integrity of the total RNA extract was determined by agarose gel electrophoresis on a 0.8% agarose gel, visualised using the Safe Imager blue-light transillumінator. Intact RNA exhibits clear 28S and 18S rRNA bands, with the intensity of the 28S band being approximately twice that of the 18S (Figure 2.1). RNA purity was determined by measuring the A_{260}/A_{280nm} ratio. Readings of 1.9-2.1 demonstrated that the RNA was pure and protein free (lower than 1.6 indicates protein contamination).

2.5 QUANTITATIVE RT-PCR (qRT-PCR)

2.5.1 Reverse transcription

To synthesise cDNA, the Reverse Transcription System (Promega) was used. The reaction mixture was made up as follows: 4μl $MgCl_2$, 2μl 10X Reverse Transcription Buffer, 2μl



Sample 1: Control siRNA 0hrs post IR

Sample 2: Control siRNA 4hrs post IR

Sample 3: 53BP1 siRNA 0hrs post IR

Sample 4: 53BP1 siRNA 4hrs post IR

Figure 2.1 ***Assessment of RNA samples on a 0.8% agarose gel.*** *2μl of RNA was run on a 0.8% agarose gel to confirm the integrity of the RNA. M denotes DNA molecular weight marker.*

dNTP mixture (10mM each dNTP), 0.5µl Recombinant RNasin® Ribonuclease Inhibitor, 0.7µl (15U) AMV Reverse Transcriptase (High Concentration), 1µl (0.5µg) Random primers, 1µl of RNA, which had previously been incubated at 70°C for 10minutes, spun briefly and placed on ice and RNase-free water to a total volume of 20µl. The reaction was incubated at room temperature for 10 minutes to allow extension of the primers, then incubated for 15 minutes at 42°C, followed by heating at 95°C for 5 minutes and cooling at 0-5°C for 5 minutes to inactivate the AMV Reverse Transcriptase and prevent it from binding to the cDNA. cDNA was stored at -20°C.

2.5.2 Primer design

Primers were designed using the guidelines outlined by Applied Biosystems, which are:

- Primers can be designed as close as possible to each other provided that they do not overlap (amplicons of 50-200bp are strongly recommended).
- Keep the GC (guanine-cytosine) content in the 20 to 80% range.
- Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided.
- The melting temperature should be 58-60°C
- The five nucleotides at the 3′ end should have no more than two G and/or C bases.

A list of primers used is contained in Table 2.6. All primers were ordered from Thermo Electron (Thermo Fisher Scientific), and resuspended at a concentration of 200µM and stored at -20°C. Stocks were diluted to 20µM for use.

2.5.3 Primer optimisation

RT-PCR was carried out using the dsDNA binding dye SYBR Green 1, which is incorporated into the amplicon during the PCR. Primers were optimised to determine the minimum primer concentrations giving maximum specific product and minimum non-specific product. This is achieved by independently varying the primer concentrations. 0.1µM, 0.05µM and 0.025µM were tested over a temperature range of 50-66°C. cDNA from U2OS cells was used for primer optimisation. Following the PCR reaction, 14µl of the PCR product was run on a 4% agarose gel and analysed to determine the optimal primer conditions (Figure 2.2, Table 2.6).

2.5.4 qRT-PCR reactions

Reactions were performed using SYBR Green 1 Master Mix (Applied Biosystems), which is supplied in 2X concentration and contains SYBR Green 1 dye, AmpliTaq Gold® DNA polymerase, dNTPs with dUTP, passive reference dye and optimised buffer components. Gene specific primers (at the optimal concentration) were added to the SYBR Green 1 master mix and water added to bring the volume of each reaction to 25μl (Table 2.6). This was added to 2.5μl cDNA, which had been dispensed into a 96 well optical plates (Applied Biosystems) (Figure 2.3). Reactions were made up as shown in Table 2.7. These were run on the ABI7500 Real Time PCR machine under the following conditions: 1 cycle of 94°C for 10 minutes, 40 cycles of 94°C for 1 minute, 50-66°C for 1 minute (optimal temperature determined was used), 72°C for 1 minute and 1 cycle of 72°C for 5 minutes.

After completion of the PCR reaction, dissociation curve analysis was carried out, which is independent of the PCR. Dissociation curves allow the purity of the product to be assessed and are useful for determining the presence of multiple species in the samples, which are normally primer-dimer artefacts or co-amplified alleles (Figure 2.2). The results were analysed using the Applied Biosystems Sequence Detection Software version 1.3.1. Relative quantitation was performed using the relative standard curve method, in which quantity is expressed relative to a control sample, which was untreated U2OS cells. The standard curve was generated by using serial dilutions of RNA from U2OS cells (control sample). To generate relative gene expression levels, the threshold cycle (cycle number at which logarithmic PCR plots cross a calculated threshold line, Ct, with Ct values decreasing as template concentration increases) values between the gene of interest and the 18S ribosomal RNA subunit were normalised. 18S ribosomal RNA subunit was used as an endogenous control and each experiment was carried out in triplicate.

Primer Name	Sequence	Concentration (μM)	Annealing Temperature (^{o}C)
BAG4 Forward BAG4 Reverse	5'-GCTTCTCCTGGTGCTTATGG-3' 5'-CTGATGGACACTGCAAGGAA-3'	0.1	58
TNFRSF9 Forward TNFRSF9 Reverse	5'-AAACGGGGCAGAAAGAAACT-3' 5'-GGGGGAATCCTGGGTATTAT-3'	0.1	58
FADD Forward FADD Reverse	5'-CCGATGTCATGGAACTCAGA-3' 5'-GCGGGAGAGGCATTAATAAAC-3'	0.05	60
IKIP 1 Forward IKIP 1 reverse	5'-TGCAAAGGTGGAAAACCAAT-3' 5'-CTGCTCAAACTGGGTCATCA-3'	0.05	60
IKIP 2 Forward IKIP 2 Reverse	5'-TGCAAAGGTGGAAAACCAAT-3' 5'-AAAGCGTCGTCAGACTGTTG-3'	0.1	60
IKIP 3.1 Forward IKIP 3.1 Reverse	5'GCAGAAATCTGAAGCTATCATGG -3' 5'-TCTTGAGAAAGCGTCGTCAG-3'	0.05	62
G3BP2 Forward G3BP2 Reverse	5'-GGAAACGTTGTGGAACTTCG-3' 5'-GCCTAATATCCCTGCGATCA-3'	0.025	60
LMNB1 Forward LMNB1 Reverse	5'-TGGGCGTCAAATTGAGTATG-3' 5'-CGGCTTTCCATCAGTTCTTC-3'	0.05	60
UBE2W Forward UBE2W Reverse	5'-AAGAGACGACCACCGGATAA-3' 5'-CCCAGAATGCACACGAGTAA-3'	0.05	60
RAD23B Forward RAD23B Reverse	5'-AACACCTCAGGAAAAAGAAGC-3' 5'-CCCAAGTCATCCCAGACAAT-3'	0.1	62
SH3MD2 Forward SH3MD2 Reverse	5'-CCTGGGTCCTGTCTTGAATG-3' 5'-TTTGAACCAGCCATCCTCTC-3'	0.025	60
ESCO2 Forward ESCO2 Reverse	5'-TCCAGAATCCCCAAGCTCTA-3' 5'-GGGGTGTTGCAGTACTTGGT-3'	0.05	64
SUMO3 Forward SUMO3 Reverse	5'-GGCAGCCAATCAATGAAACT-3' 5'-TCATCGTGGTGAATGTCCTC-3'	0.05	60
BIRC4 Forward BIRC4 Reverse	5'-GGGGTTCAGTTTCAAGGACA-3' 5'-CGCCTTAGCTGCTCTTCAGT-3'	0.05	60
XRCC4 Forward XRCC4 Reverse	5'-GAGCAGGACCAGCTGATGTA-3' 5'- TTCTGCAATGGTGTCCAAG-3'	0.05	60
TP53INP1 Forward TP53INP1 Reverse	5'-CCTCCAACCAAGAACCAGAA-3' 5'-TCAGCCAAGCACTCAAGAGA-3'	0.025	62

GAS6 Forward GAS6 Reverse	5’-TTGACTTCCGGACCTTTGAC-3’ 5’-GCATCCCTGTTGACCTTGAT-3’	0.025	62
CDKN1B Forward CDKN1B Reverse	5’-AAGAAGCCTGGCCTCAGAAG-3’ 5’-ACAGGATGTCCATTCCATGA-3’	0.05	60
STAT3 Forward STAT3 Reverse	5’-GCTGGTCAAATTCCCTGAGT-3’ 5’-CCGTTGTTGGATTCTTCCAT-3’	0.05	60
REV3L Forward REV3L Reverse	5’-ACAGGCCATATGGGAAGATG-3’ 5’-CTGGGATCCATCGCTGTAGT-3’	0.025	60
CHES1 Forward CHES1 Reverse	5’-CTGGGTGGAAAAACTCAGTG-3’ 5’-ATTGAACACGTGTGGGTGTG-3’	0.05	60
XPA Forward XPA Reverse	5’-ATGCGAAGAATGTGGGAAAG-3’ 5’-CCCATTGTGAATGATGTGGA-3’	0.05	60
18S Forward 18S Reverse	5’-AGGAATTCCCAGTAAGTGCG-3’ 5’-GCCTCACTAAACCATCCAA-3’	0.1	58-64

Table 2.6 ***Sequences of the RT-PCR primers used in this study****. All primers were designed following the guidelines provided from Applied Biosystems.*

Component	Volume per reaction	Final Concentration
2X SYBR Green PCR Master Mix	12.5μl	1X
Forward primer	Variable	0.025-0.1μM
Reverse primer	Variable	0.025-0.1μM
cDNA	2.5μl	2.5μg
Water	7.5-11.25μl	-
Total	25μl	-

Table 2.7 ***Components used to make up the reaction mix for RT-PCR.***

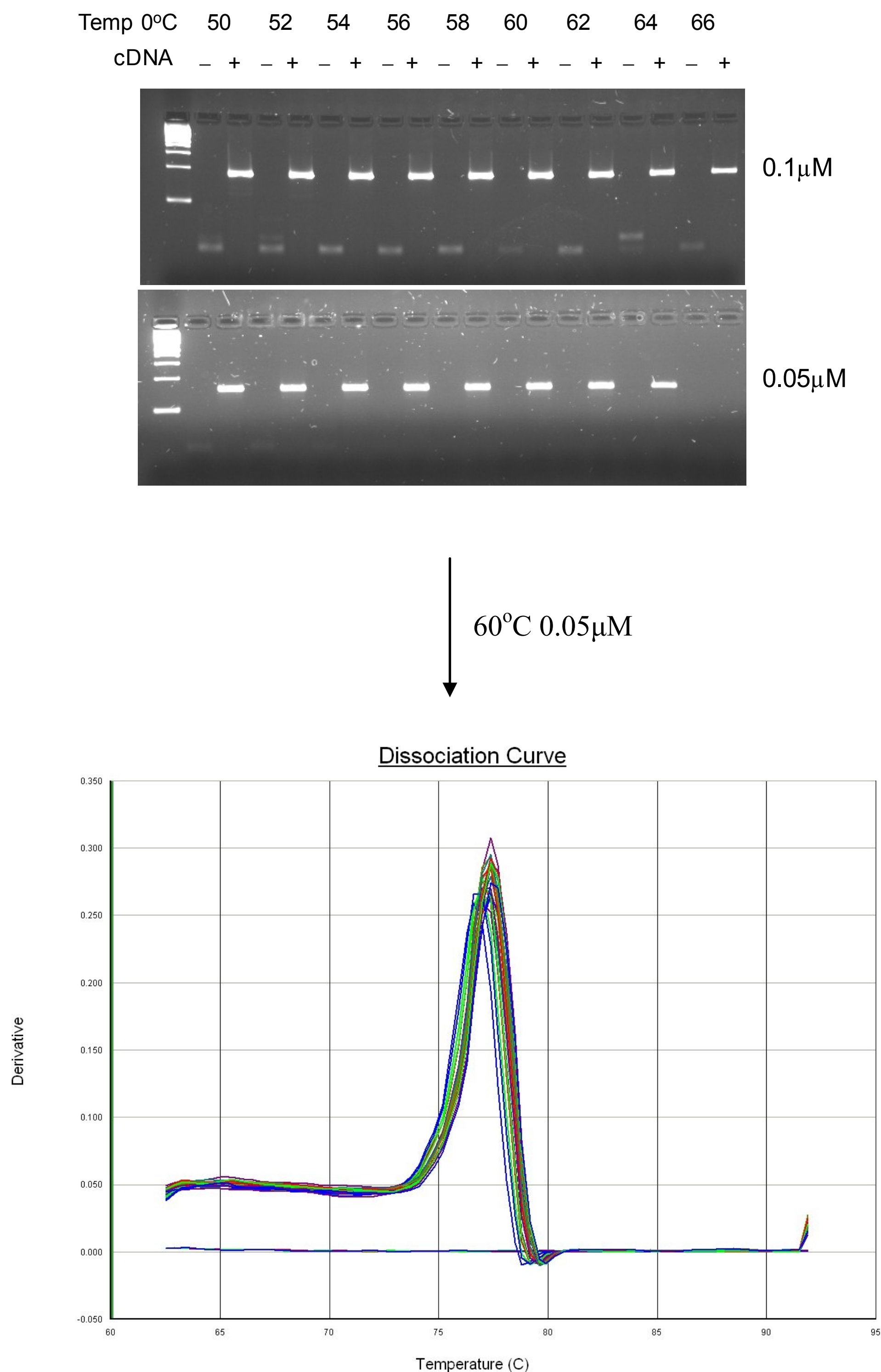


Figure 2.2 ***RT-PCR optimisation.*** *Each set of primers was optimised to see which temperature and concentration resulted in the greatest amount of product in complete absence of any primer dimers. Dissociation curves were then checked on the RT-PCR machine to verify specificity.*

		1	2	3	4	5	6	7	8	9	10	11	12
Gene of interest	A	NTC	NTC	NTC	Con	Con	Con	Con 1:5	Con 1:5	Con 1:5	Con 1:10	Con 1:10	Con 1:10
	B	Con 1:20	Con 1:20	Con 1:20	Con 0hr	Con 0hr	Con 0hr	Con 4hr	Con 4hr	Con 4hr	53BP1 0hr	53BP1 0hr	53BP1 0hr
	C	53BP1 4hr	53BP1 4hr	53BP1 4hr									
18S	D	NTC	NTC	NTC	Con	Con	Con	Con 1:5	Con 1:5	Con 1:5	Con 1:10	Con 1:10	Con 1:10
	E	Con 1:20	Con 1:20	Con 1:20	Con 0hr	Con 0hr	Con 0hr	Con 4hr	Con 4hr	Con 4hr	53BP1 0hr	53BP1 0hr	53BP1 0hr
	F	53BP1 4hr	53BP1 4hr	53BP1 4hr									
	G												
	H												

Figure 2.3 ***RT-PCR plate layout.*** *Each experiment was carried out in triplicate and serial dilutions of RNA from untreated U2OS cells were used for relative quantification.*

2.6 MICROARRAY TECHNIQUES

The microarray study was conducted in collaboration with Dr Mike Hubank at the Institute of Child Health Microarray Facility, University College London.

All the microarray techniques were conducted according to the Gene Chip Expression Analysis Technical Manual from Affymetrix (http://www.affymetrix.com/support/index.affx). An overview of the microarray procedure is given in Figure 2.4

2.6.1 First and second cDNA synthesis

5µg of RNA was reverse transcribed using the T7-Oligo (dT) promoter primer in the first strand synthesis reaction to produce single stranded cDNA. This cDNA then underwent second strand synthesis using *E. coli* RNase H and DNA pol I. The RNase H activity is used to create short RNA fragments in the RNA:DNA heteroduplex that function as primers for second strand cDNA synthesis by *E. coli* DNA pol I. The addition of bacteriophage T4 DNA polymerase to the reaction creates blunt double-stranded DNA. The cDNA was then purified and the integrity was checked by running a sample on an agarose gel before proceeding to the generation of cRNA.

2.6.2 In vitro transcription-cRNA labelling reaction

Biotin-labelled cRNA was synthesised by transcribing *in vitro* the double-stranded cDNA using biotin-labelled ribonucleotides and the T7 RNA polymerase, which binds to its promoter in the double stranded cDNA. The cRNA was purified to remove any enzymes or unincorporated ribonucleotides. The optical density of biotinylated cRNA was measured to determine concentration and confirm purity (260/280 ratio = 1.9-2.1) before it was fragmented for hybridisation.

2.6.3 cRNA fragmentation and hybridisation

Typically, 15µg of cRNA was fragmented into 50-200 base fragments and then hybridised to the probe array for 16 hours. The Affymetrix U133 Plus 2.0 GeneChip oligonucleotide microarray was used, which contains 54,120 probe sets, corresponding to around 38,500 genes. The probe arrays were washed with a series of stringent and non-stringent buffers and then stained using Streptavidin Phycoeruthrin (SAPE), which binds tightly to biotin.

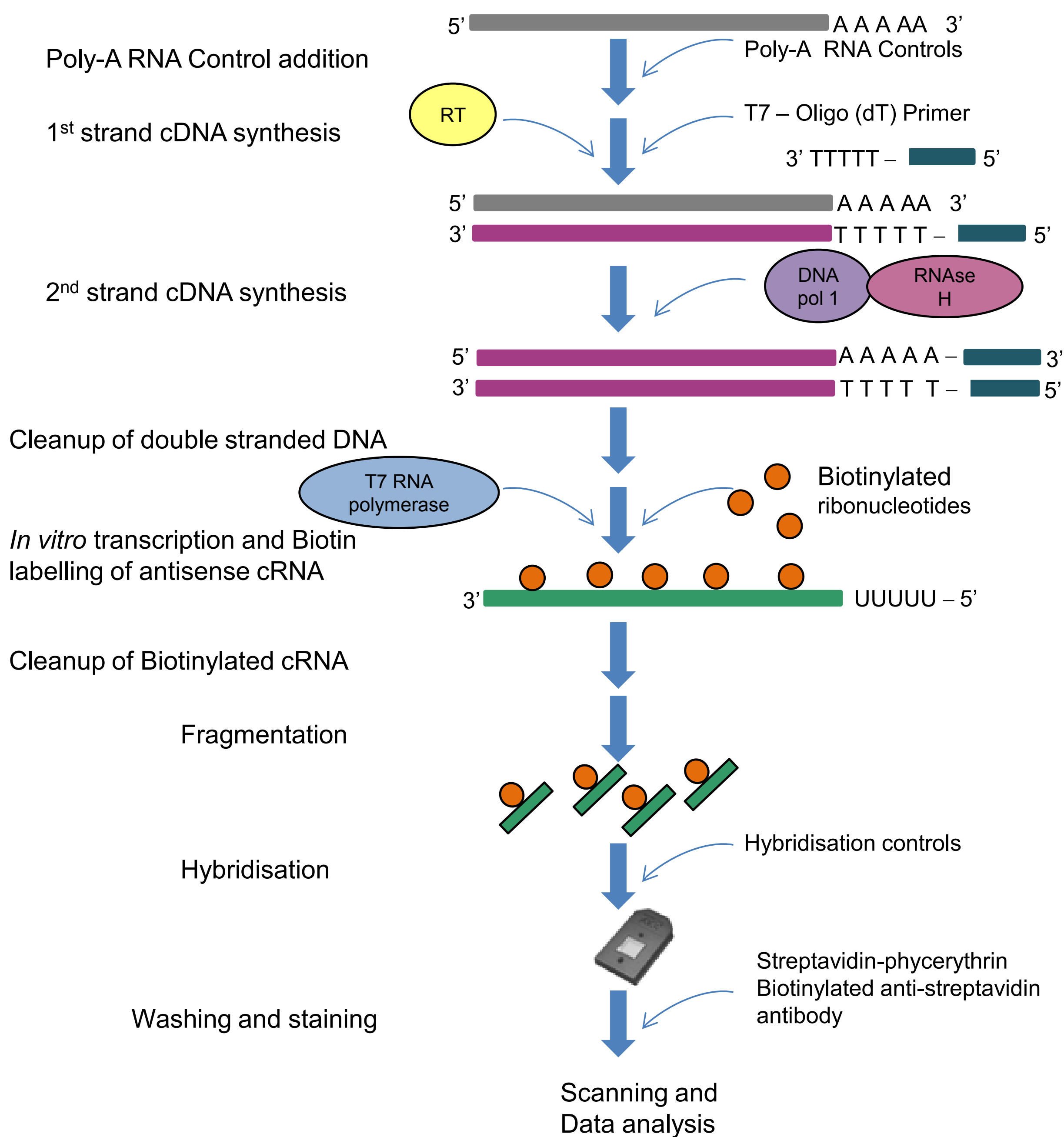


Figure 2.4 ***Schematic representation of microarray procedure.*** *Following extraction, RNA was subjected to first and second strand cDNA synthesis using T7 (dT) primer and dNTPs. The double stranded cDNA was cleaned up, which involved ETOH precipitation. Biotin labelled cRNA was synthesised from the double stranded cDNA by in vitro transcription (IVT) using biotinylated ribonucleotides and subsequently underwent a further ETOH cleanup step. After determination of the purity and concentration, the biotinylated cRNA was fragmented at 94°C followed by hybridisation on to U133 plus 2.0 Affymetrix chip. The hybridised probe array was stained with streptavidin phycoerythrin conjugate and scanned by the GeneChip® Scanner 3000. The amount of fluorescent light emitted is proportional to the abundance of RNA at each location on the probe array. Data analysis was then carried out, which comprised of normalisation, filtration and statistical tests.*

2.6.4 *Probe array scan*

After the wash protocol, the probe arrays were scanned using an Affymetrix GeneChip Scanner 3000, which is controlled by the Affymetrix Microarray Suite (MAS) 5.0 program. The laser scanner determines the amount of bound biotinylated cRNA indirectly through the streptavidin-conjugated phycoerythrin fluorescence at each feature within a probe set. The Affymetrix MAS 5.0 programme analyses the data and computes a single intensity value for each probe set. The software then applies a statistical expression algorithm to determine the expression levels of each gene. Further details of the algorithm applied by the Affymetrix MAS 5.0 software can be found in the GeneChip® Operating Software Manual that can be obtained from www.affymetrix.com. This software also provides indicators of sample integrity, assay execution and hybridisation performance through the assessment of control hybridisations.

2.6.5 *Data analysis using GeneSpring-GX software*

With the help of Dr Eliot Marston, detailed analysis of microarray data was performed using the GeneSpring-GX software, version 7.3.1 (Agilent Technologies). An overview of the microarray data analysis is given in Figure 2.5.

2.6.5.1 Normalisation

The raw data from MAS 5.0 was exported to GeneSpring and normalisation carried out to allow microarray experiments from different samples and different arrays to be compared. (Brazma *et al* 2001, Quackenbush 2002, Bolstad *et al* 2003, Leung and Cavalieri 2003). First, the data was duplicated to allow normalisation to be performed in two separate ways. The results for one of the datasets were normalised to the 50th percentile of total expression value data for each chip, so that the genes at the median level of each array had a value of 1. Per-chip normalisations control for chip-wide variations caused by experimental artefacts. The data was also normalised to the median for each genes across the arrays to enable relative expression values to be assigned per gene per array. This allowed the data to be examined in terms of relative expression levels before and after IR between the control and 53BP1 siRNA treated samples. In contrast, the other dataset was similarly normalised to the 50th percentile of total expression value for each chip, but each gene post-IR was normalised to its pre-IR equivalent. This gave expression values of 1 for each gene pre-IR and fold change values for

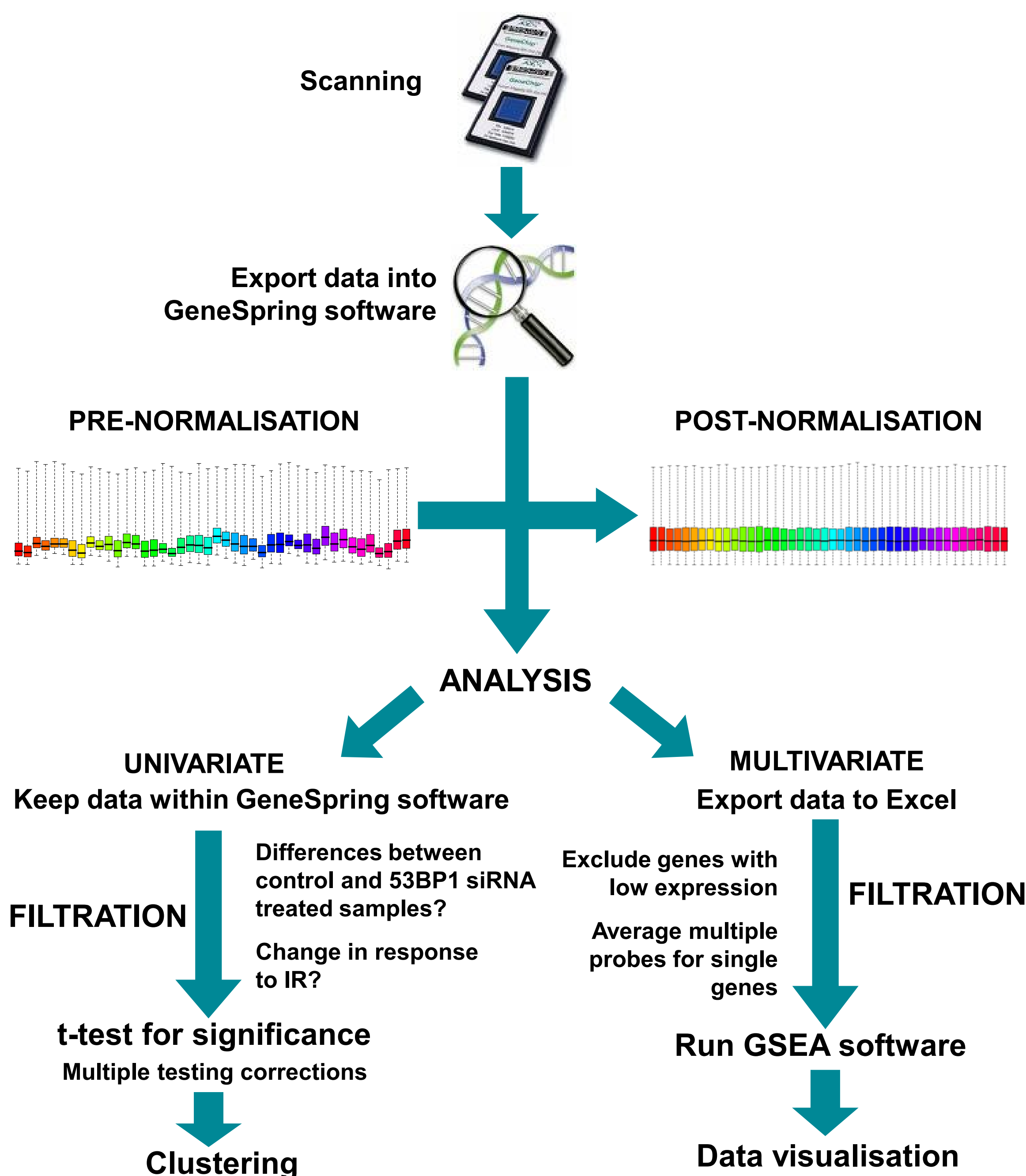


Figure 2.5 ***Schematic diagram illustrating how the microarray data was analysed****. Following scanning of the GeneChips, the data from the MAS 5.0 program was exported to GeneSpring for normalisation and analysed by either univariate (single genes) or multivariate (biological pathways). GeneSpring was used to perform univariate analysis, in which the normalised data was filtered based on p-value and fold change before it was subjected to statistical tests to determine significance. The genes that passed the strict criteria were clustered and visualised on a heat map generated using TreeView. To analyse data by multivariate analysis, the data was first exported and filtered in Excel before being analysed and visualised using GSEA.*

each gene post-IR. This enabled identification of those changes in gene expression levels that occurred solely in response to IR.

2.6.5.2 Filtration

Following normalisation, the results were filtered in two main ways to reduce the number of genes. Firstly, using the detection p-value assigned to each transcript by the MAS 5.0 software. MAS 5.0 evaluates the abundance of each transcript represented and labels it as either present, marginal or absent. The p-value indicates the statistical significance of the presence of each transcript. The p-value cutoff assigned by MAS 5.0 programme is 0.05, however, on recommendation by Dr Mike Hubank only those genes with a p-value of less than 0.1 were retained for analysis. This is because the MAS algorithm is overly-stringent for calculating p-values. Genes that were either not expressed at all or were expressed at very low levels were excluded. Secondly, the data was filtered using fold changes in gene expression levels, so that any gene with less than a 2 fold change before IR or a 1.5 fold change in response to IR between control and 53BP1 siRNA treated samples were omitted from the analysis.

2.6.5.3 Statistical tests

To identify which of the genes selected from the filtering process were significantly differentially expressed, the data was analysed using a paired t-test ('Welch' t-test) and multiple testing corrections (MTC). MTC is very stringent and adjusts the p-values from multiple statistical tests to correct for the occurrence of false positives (i.e. genes that were falsely called differentially expressed when they were not) (Noble 2009). For analysis of pre-IR transcriptional differences, a paired t-test along with MTC was conducted between the unirradiated control and 53BP1 siRNA treated samples. Genes which were over- or under-expressed in the control siRNA treated samples in comparison to the 53BP1 siRNA treated samples were identified. In contrast, to analyse the response to IR, a paired t-test with MTC was conducted between the pre-IR and post-IR expression levels to determine which genes were significant. Following this, the genes were separated into those that were up-regulated or down-regulated in response to IR in the control siRNA treated samples compared to the 53BP1 siRNA treated samples.

2.6.5.4 Clustering

Again with the assistance of Dr Eliot Marston, clustering was performed on the microarray data using the Cluster 3.0 program and the results were visualised using the TreeView software (http://rana.lbl.gov/EisenSoftware.htm, (Eisen *et al* 1998)). Data was exported from GeneSpring and loaded into the Cluster 3.0 program where complete linkage clustering was performed. Clustered data was then displayed as heat maps generated using TreeView. This allowed visualisation of gene expression patterns between control and 53BP1 siRNA treated samples before IR and in response to IR.

2.6.5.5 Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) (Subramanian *et al* 2005) was performed on the microarray data with the help of Dr Carmel McConville. GSEA is designed to test whether differentially expressed genes belong to specific functionally related gene sets (e.g. genes belonging to common pathways or responding to specific stimuli) and is freely available at http://www.broadinstitute.org/gsea/. To decrease the complexity of the data, normalised expression values for 54,614 probe sets were reduced by approximately 35% to 35,462 probe sets by excluding genes with expression values less than 1.5 fold. In addition, multiple probe sets for a given gene were replaced by the probe set with the maximum expression value to prevent inflating the enrichment score for any particular gene. This further reduced the dataset to 15,803 genes. GSEA analysis was then conducted to ascertain the enrichment score for all the functional gene sets in the Molecular Signatures Database (MsigDB) 1.0 geneset database, which had a minimum of 15 genes and maximum of 500 genes (http://www.broadinstitute.org/gsea/msigdb/index.jsp). This resulted in a total of 247 out of a possible 522 gene sets being used for the analysis. Significance values (p-values) were calculated and a false discovery rate (FDR) score produced using adjustment multiple hypothesis testing as described (Subramanian *et al* 2005). FDR score was used as an indicator of the probability that an observed result represents a false positive finding. Significance was determined using a cutoff FDR value of less than 0.25, which means that there is less than a 25% chance of a particular gene set being a false positive result.

CHAPTER 3

CHAPTER 3 53BP1 STIMULATES THE TRANSCRIPTIONAL ACTIVITY OF p53 AND p300

3.1 INTRODUCTION

The tumour suppressor protein p53 plays a crucial role in preventing genomic instability. In the absence of cellular stress, p53 forms a ternary complex with HDM2 and HDMX, which together with CBP/p300 promotes p53 degradation via the ubiquitin-dependent proteasomal system (Espinosa *et al* 2003, Ferreon *et al* 2009, Grossman *et al* 2003). Upon activation by DNA damage, p53 coordinates a complex cellular response, which can lead to cell-cycle arrest at the G1/S or G2/M transition stages, DNA repair, senescence or apoptosis (Sengupta and Harris 2005, Vousden and Prives 2009). Cell cycle arrest is achieved primarily through transactivation of p53 target genes such as p21, GADD45 and 14-3-3σ (Bartek and Lukas 2001, Taylor and Stark 2001), whereas induction of pro-apoptotic genes including PUMA, NOXA, FAS and BAX results in apoptosis (Pietsch *et al* 2008).

Due to the involvement of p53 in coordinating a plethora of cellular processes, it is not surprising that its activity is regulated by a complex series of interconnected mechanisms. Of these, the most well studied is modifying the function of p53 via altering patterns of post-translational modifications such as phosphorylation, acetylation, ubiquitylation and sumoylation (Kruse and Gu 2009). Whilst post-translational modifications play an important role in p53 regulation, an increasing array of cofactors are being identified that influence p53 activity and confer specificity to the p53 response by selectively controlling the expression of certain p53 responsive genes. Under conditions of repairable DNA damage, p53 has been shown to interact with cofactors that strongly promote cell cycle arrest such as iASPP, which antagonises the expression of apoptotic genes (Bergamaschi *et al* 2003). p53 also interacts with MUC1, which facilitates p21 expression but represses BAX expression (Wei *et al* 2005), Hzf, which has been shown to preferentially transactivate cell cycle arrest rather than apoptotic genes (Das *et al* 2007) and YB1, which blocks BAX expression (Homer *et al* 2005). In the presence of irreparable DNA damage, there are also cofactors that associate with p53 and promote transactivation of pro-apoptotic genes and the repression of cell cycle genes. These include ASPP1 and 2 (Samuels-Lev *et al* 2001) and Pin1, which has been shown to

dissociate p53 from iASPP, thereby promoting apoptosis (Mantovani *et al* 2007). Furthermore, both p18/Hamlet and the p52 subunit of NF-κB have been reported to be recruited to the promoters of a subset of pro-apoptotic target genes resulting in induction of their transcription (Cuadrado *et al* 2007, Schumm *et al* 2006).

Two well known p53 transcriptional co-activators are CBP /p300 (Grossman 2001), which are highly homologous proteins that possess acetyltransferase activity (Arany *et al* 1994, Ogryzko *et al* 1996). However, CBP/p300 have also been shown to interact with a wide array of other transcription factors such as the signal transducers and activators of transcription (STAT) family (Horvath 2000), NF-κB (Gerritsen *et al* 1997), the E2F family (Marzio *et al* 2000) and the AP-1 family (Bannister and Kouzarides 1995, Lee *et al* 1996b), as well as components of the basal transcription machinery including TFIIB and TAT-binding protein (Imhof *et al* 1997, Kee *et al* 1996, Nakajima *et al* 1997). CBP/p300 are important regulators of many cellular processes such as proliferation, apoptosis, differentiation and angiogenesis and are involved in preventing the development of cancer (Goodman and Smolik 2000, Iyer *et al* 2004). The ability of CBP/p300 to interact with multiple transcription factors and the basal transcription machinery has led to the proposal that these co-activators function as signal integrators by coordinating complex signal transduction events at the transcriptional level (Kamei *et al* 1996).

CBP/p300 are large modular proteins that share several conserved regions, which constitute most of the known functional domains in these proteins (Arany *et al* 1994, Dyson and Wright 2005). Along with the KIX domain, the CH1 domain and CH3 regions serve as binding sites for the majority of the transcription factors that interact with CBP/p300 (Goodman and Smolik 2000). However, due to the intracellular levels of CBP and p300 being limited and several transcription factors binding to the same domains, it has been shown that there is competition between transcription factors for CBP/p300 and this impacts on the transcriptional response (Horvai *et al* 1997, Kamei *et al* 1996, Webster and Perkins 1999). CBP/p300 also contain two transactivation domains at the N- and C-terminus, which function to potentiate the activity of transcription factors by interacting simultaneously with the basal transcription machinery and the transcription factor (Bisotto *et al* 1996, Lee *et al* 1996b, Swope *et al* 1996, Yuan *et al* 1996). The ability of CBP/p300 to modulate the activity of transcription factors is controlled post-translationally by multiple signalling pathways that

regulate their sumoylation (Girdwood *et al* 2003), methylation (Xu *et al* 2001b), acetylation (Hansson *et al* 2009, Thompson *et al* 2004) and phosphorylation (Vo and Goodman 2001).

The intrinsic activity of CBP/p300 is also regulated via interactions with other proteins. The p68 RNA helicase and the CDK inhibitor, p21 have been shown to enhance CBP/p300 transcriptional activity (Rossow and Janknecht 2003, Snowden *et al* 2000), whereas in contrast, cyclin D1 has been demonstrated to repress p300 transactivation through recruitment of HDACs (Fu *et al* 2005). The p300 cofactors, JMY and Strap form a complex with p300 and increase its transcriptional activity specifically in response to DNA damage. This results in an increase in p53 acetylation and p53-dependent apoptosis (Demonacos *et al* 2001, Shikama *et al* 1999). More recently, Skp2 was identified as a p300 cofactor and was shown to antagonise the interaction between p300 and p53 by binding to p300, thereby preventing the acetylation of p53 and suppressing its ability to induce apoptosis (Kitagawa *et al* 2008). This mode of regulation of CBP/p300 activity adds additional complexity and specificity to the transcriptional response, as it demonstrates that modulating the interaction of CBP/p300 with transcription factors can also influence promoter selectivity.

53BP1 was initially proposed to function as a transcriptional co-activator for p53, based on its ability to bind to p53 and enhance its transcriptional activity (Iwabuchi *et al* 1998). The 53BP1-p53 interaction was found to be mediated by the DBD of p53 and the C-terminal region of 53BP1, which contains tandem BRCT domains (Iwabuchi *et al* 1994, Thukral *et al* 1994). Subsequently, when the crystal structure of the 53BP1-p53 interaction was resolved, it showed that the central DNA binding region of p53 bound to the N-terminal BRCT domain and the linker region of 53BP1, which is the region between the two BRCT domains (Derbyshire *et al* 2002, Joo *et al* 2002). Importantly, structural analysis also revealed that the same p53 residues were involved in binding to both 53BP1 and DNA. This caused controversy within the field because it provided evidence that it was sterically impossible for p53 to bind to 53BP1 and DNA simultaneously. More recently, data from two reports showed that the Tudor domain of 53BP1 can recognise di-methylated lysines on in the C-terminus of p53, in particular lysine 370 and lysine 382 (Huang *et al* 2007a, Kachirskaia *et al* 2008). Di-methylation of both these lysines has been shown to increase the association of p53 with 53BP1. Interestingly, in the case of di-methylated lysine 370, 53BP1 was shown to be required for p53 transactivation of its target genes, whereas no effect was seen on p53 transactivation when 53BP1 was bound to di-methylated lysine 382. This identifies an

alternative way in which 53BP1 can interact and regulate p53, although it remains to be determined how the recognition of different methylation sites on p53 by 53BP1 can result in alternative physiological outcomes. Despite these recent findings, the role of 53BP1 as a transcriptional co-activator of p53 has yet to be firmly established.

Interestingly, 53BP1 was identified as a CBP and p300 interacting protein by mass spectrometric analysis (GS Stewart, unpublished) along with several other novel CBP and p300 interacting proteins. Furthermore, for p300 these included proteins involved in the DNA damage response and DNA repair such as all three components of the MRN complex (Lamarche *et al* 2010), Msh6 (Jiricny 2006) and XAB2 (Hanawalt and Spivak 2008). Only the DNA damage response mediator proteins, 53BP1 and MDC1 (Stewart *et al* 2003, Wang *et al* 2002) were identified as associating with both proteins, although the interaction with CBP was much weaker than with p300 (Figure 3.1). These data suggest that 53BP1 is interacts with CBP and p300 *in vivo*.

The aims of this study were to:

(i) Determine if 53BP1 exists in a complex with CBP and p300 and map the sites of interaction.

(ii) Ascertain if 53BP1 could act as a transcriptional co-activator of p300 and p53.

3.2 RESULTS

3.2.1 53BP1 interacts with CBP and p300 in vivo

To confirm the mass spectrometric analysis, reciprocal co-immunoprecipitation assays were performed. Endogenous CBP and p300 was immunoprecipitated from whole cell extracts prepared from HeLa cells using anti-p300 and anti-CBP antibodies that were different to those originally used in the immunoprecipitation/mass spectrometric analysis and Western blot analysis was used to determine whether CBP and p300 could associate with 53BP1 *in vivo*. Indeed, the co-immunoprecipitation data revealed that 53BP1 interacts with both CBP and p300 (Figure 3.2). To strengthen this observation, the reciprocal experiment was performed in which endogenous 53BP1 was immunoprecipitated using an anti-53BP1 antibody and co-precipitation of CBP and p300 was assessed by Western blot analysis using anti-CBP and

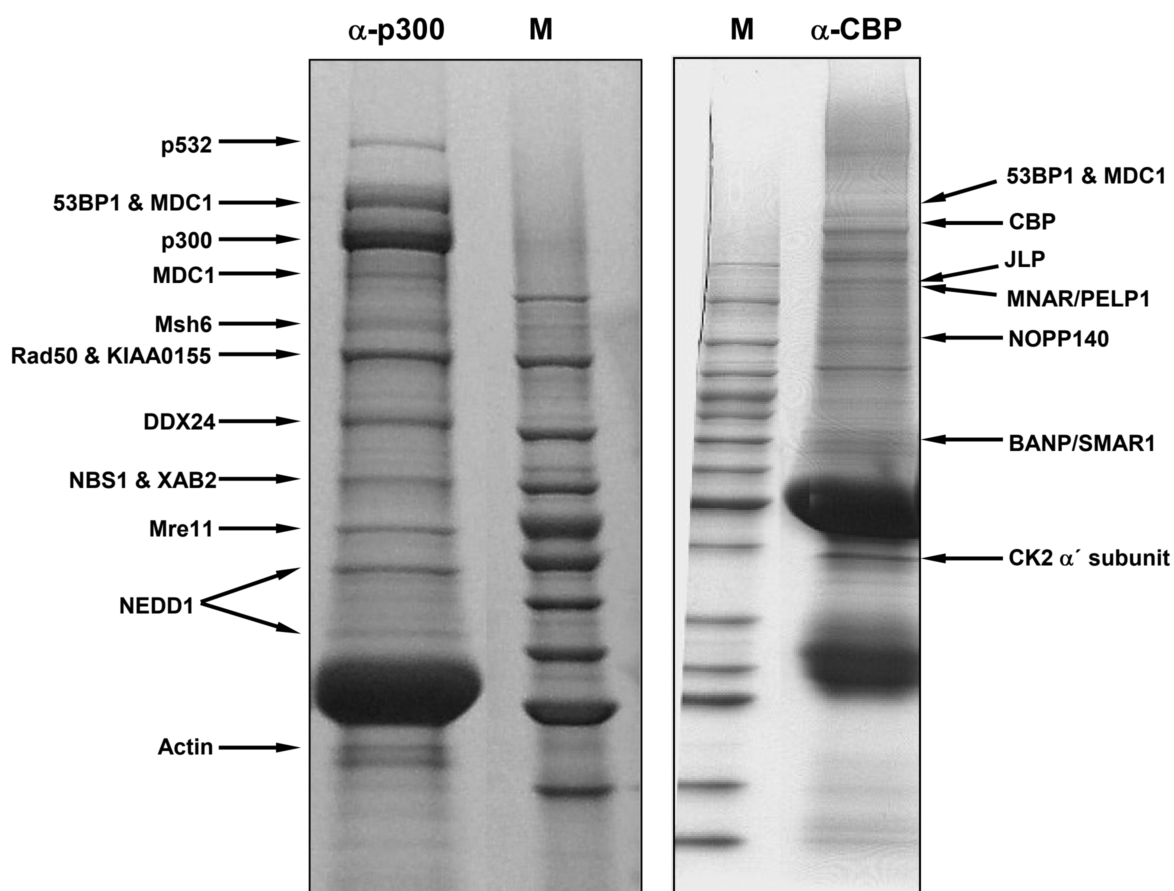


Figure 3.1 ***Identification of CBP and p300 immunocomplexes.*** *CBP and p300 immunocomplexes were isolated from HeLa total cell extract using anti-p300 and anti-CBP antibodies, resolved on a 4-20% SDS-PAGE gel and stained with Coomassie blue. Protein bands were excised and digested with modified trypsin prior to analysis by mass spectrometry. The proteins identified are labelled. M denotes molecular weight marker. (Figure provided by Dr GS Stewart).*

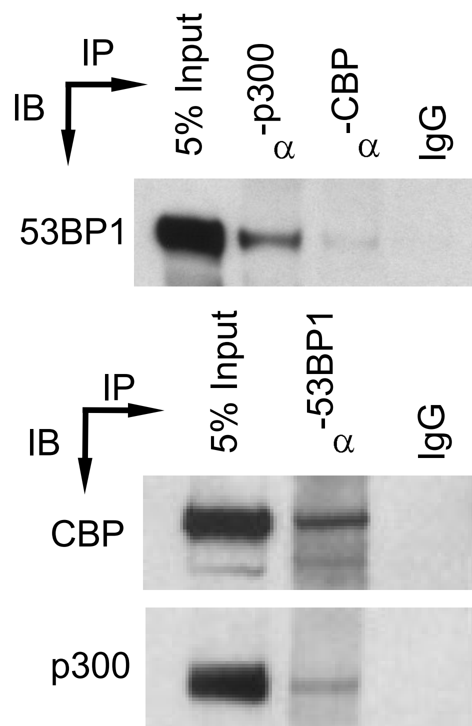


Figure 3.2 ***Transcriptional co-activators CBP/p300 interact with 53BP1 in vivo.*** *53BP1 and CBP/p300 were immunoprecipitated from HeLa whole cell extract and Western blotting was used to assess the binding of CBP/p300 and 53BP1 respectively. IgG denotes immunoprecipitates performed using a non-specific IgG antibody as a control. IP and IB are abbreviations for immunoprecipitation and immunoblot respectively.*

anti-p300 antibodies. As shown in Figure 3.2, CBP and p300 were found in 53BP1 immunocomplexes demonstrating that endogenous 53BP1 physically associates with CBP and p300 *in vivo*, thus confirming the mass spectrometric analysis.

The co-immunprecipitation data suggests that 53BP1, CBP and p300 may reside in the same protein complex. Therefore, to confirm this and to estimate the size of the complex, nuclear extract was subjected to a two step fractionation process. Initially, the nuclear extract was fractionated by ion exchange chromatography and then, the bound proteins were eluted in fractions from the DEAE column by gradually increasing the ionic strength of the elution buffer by increasing the concentration of NaCl. The levels of 53BP1, p300 and CBP in each fraction were detected with SDS-PAGE and Western blot analysis. Initial analysis indicated that all three proteins co-eluted in the same fractions (Figure 3.3). The peak fraction (fraction 7) containing all three proteins was further fractionated by size using a Superose 6 gel filtration column. Again the fractions were analysed by Western blot analysis and 53BP1, CBP and p300 could be shown to co-elute in the same fractions. The majority of 53BP1, CBP and p300 co-fractionated in the void volume (>2 MDa) indicating that these proteins exist in very high molecular weight complexes. However, it is unclear from these data if 53BP1, CBP and p300 exist in the same complex since the complex could not be resolved by the gel filtration column (Figure 3.3).

3.2.2 Mapping interaction sites on 53BP1, CBP and p300

In order to investigate the biological interaction between 53BP1 and CBP /p300, the domains required for the interaction were mapped on each respective protein using GST pull-down assays. GST-fused overlapping fragments covering the entire open reading frames of 53BP1, CBP and p300 were mixed with the corresponding binding protein, which had been *in vitro* translated and labelled with [^{35}S] L-α-methionine. CBP and p300 were found to bind to fragments 5 and 6 of 53BP1, which encompasses the C-terminal end of 53BP1 (Figure 3.4). This region contains the tandem BRCT domains, which have been shown to act as protein-protein interaction moieties (Manke *et al* 2003) and the Tudor domain, which has been demonstrated to bind to proteins containing methylated lysines and arginine residues (Cote and Richard 2005, Kim *et al* 2006). In the reciprocal experiment, 53BP1 bound to fragments 1 and 2 of both CBP and p300, which corresponds to the N-terminal region. In addition, 53BP1 also bound to fragment 5 and 6 of CBP, as well as fragments 4 and 5 of p300 (Figure 3.5,

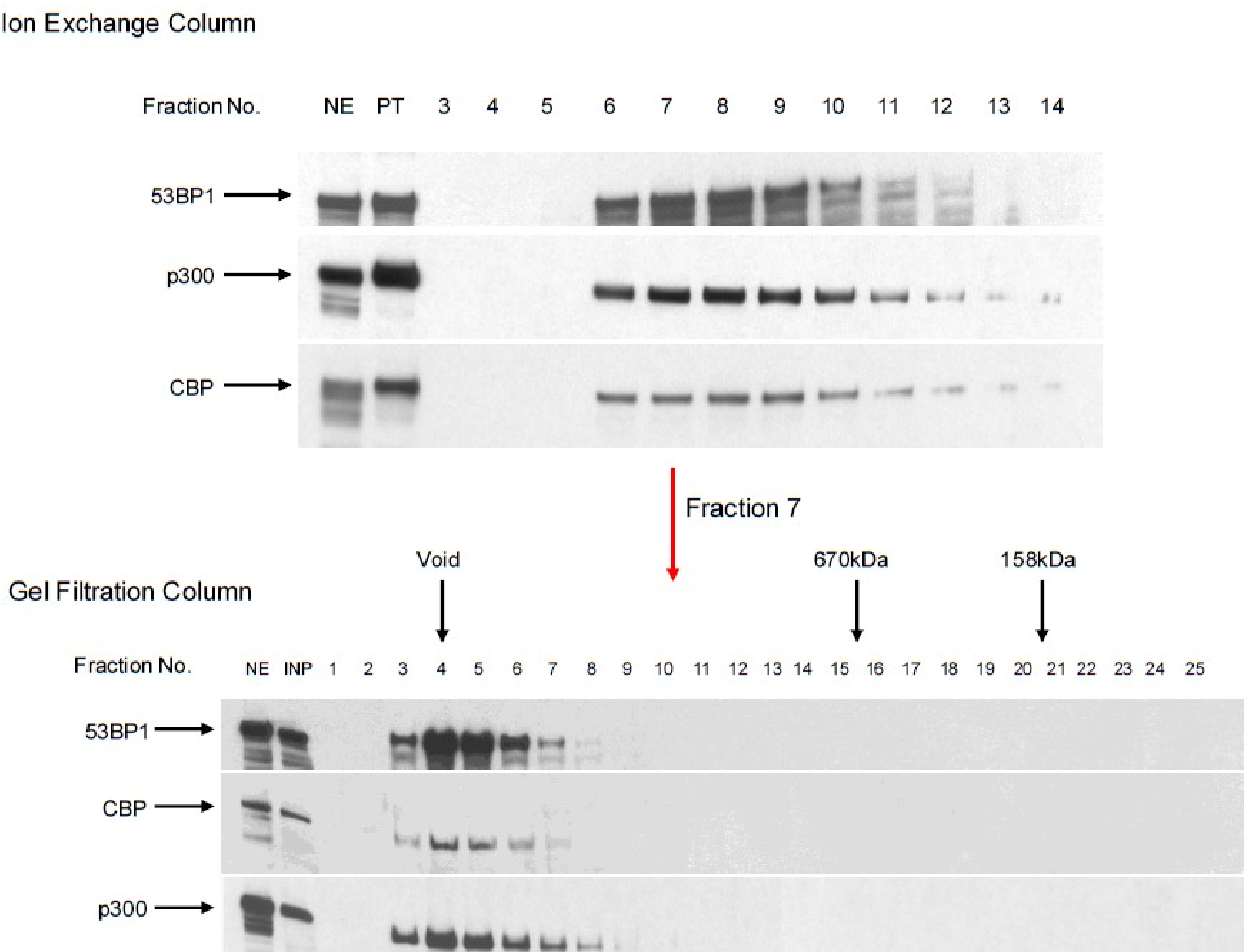


Figure 3.3 ***53BP1, CBP and p300 reside in large mega Dalton complexes.*** *HeLa nuclear extracts (NE) were fractionated and step eluted using KCL on a DEAE column. The majority of 53BP1, CBP and p300 co-eluted in fraction 7, which was fractionated further on a Superose 6 gel filtration column. 53BP1, CBP and p300 were detectable in the same fractions. The peak fraction was in the void volume (>2 MD).*

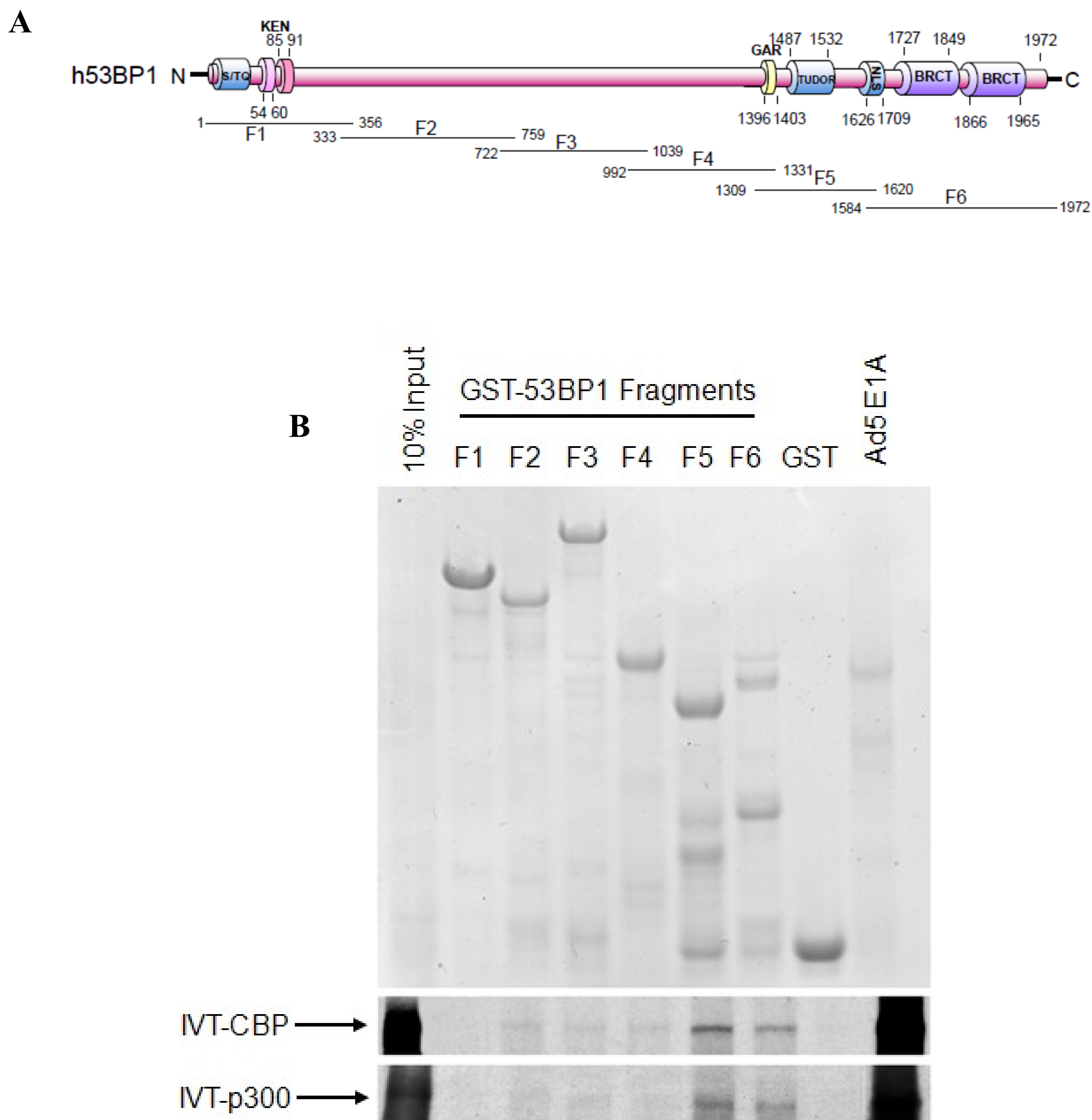


Figure 3.4 ***CBP and p300 bind to the C-terminal region of 53BP1.*** *(A) Diagrammatic representation of 53BP1 with the regions encompassed by the six GST-fusion proteins indicated. (B) 30µg GST fused overlapping fragments covering the entire open reading frame of 53BP1 were mixed with either CBP or p300, which had been in vitro translated and labelled with [^{35}S] L-α-methionine. Protein complexes were isolated using Glutathione-Sepharose beads and separated by SDS-PAGE. Radiolabelled proteins were visualised by fluorography and autoradiography. Coomassie blue stained version of the gel is shown in the top panel. GST and Adenovirus type 5 E1A proteins were used as a negative and positive control respectively.*

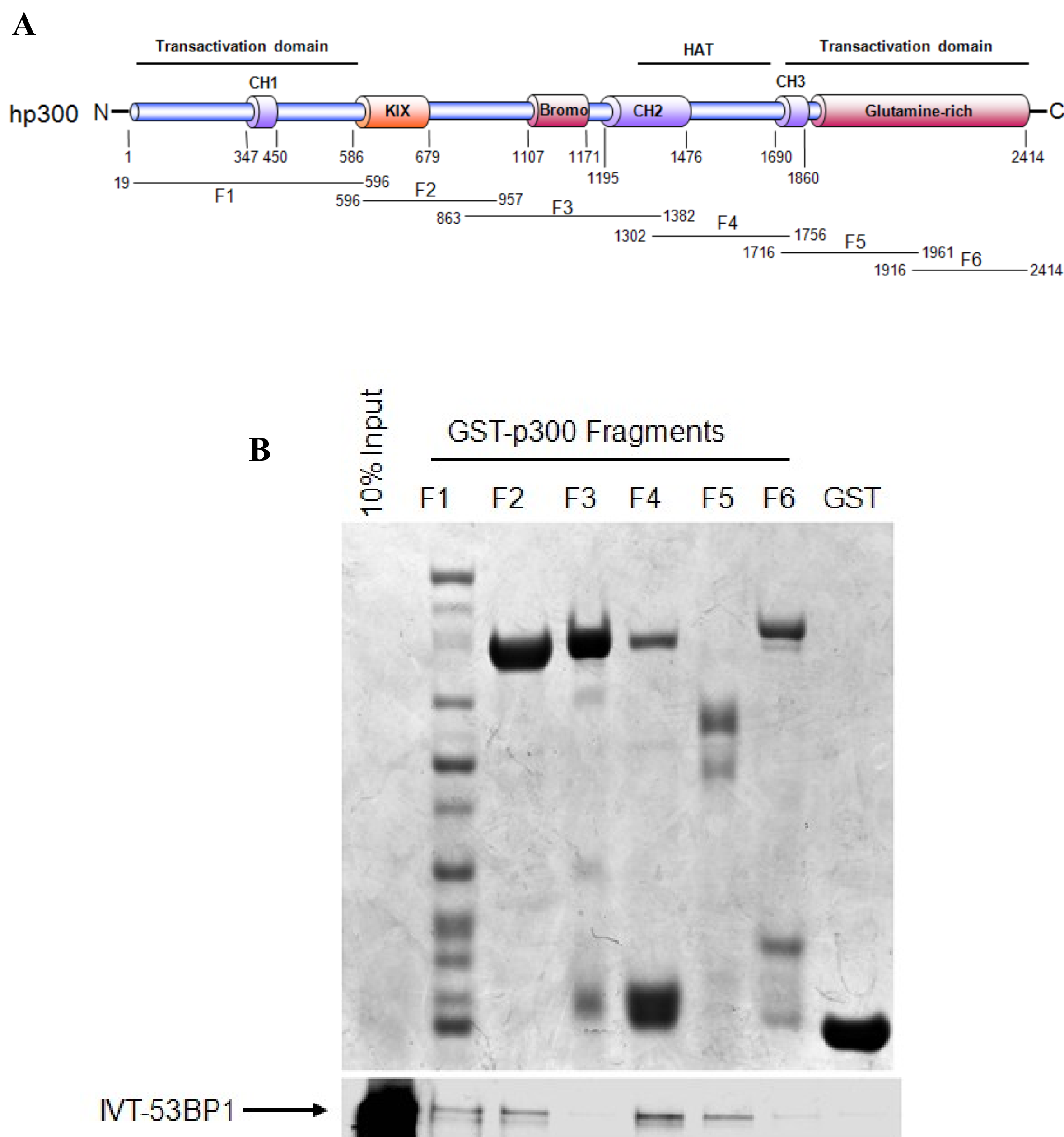


Figure 3.5 ***53BP1 binds to multiple regions of p300***. *(A) p300 protein is represented diagrammatically, and the regions encompassed by the six GST-fusion proteins are indicated. (B) 30μg GST fused overlapping fragments covering the entire open reading frame of p300 were purified and mixed with [35S] methionine-labelled in vitro translated 53BP1. GST-bound proteins were purified with Glutathione-Sepharose beads and separated by SDS-PAGE. Binding was assessed using fluorography and autoradiography. Coomassie blue stained gel is shown in the top panel. GST was used as a negative control.*

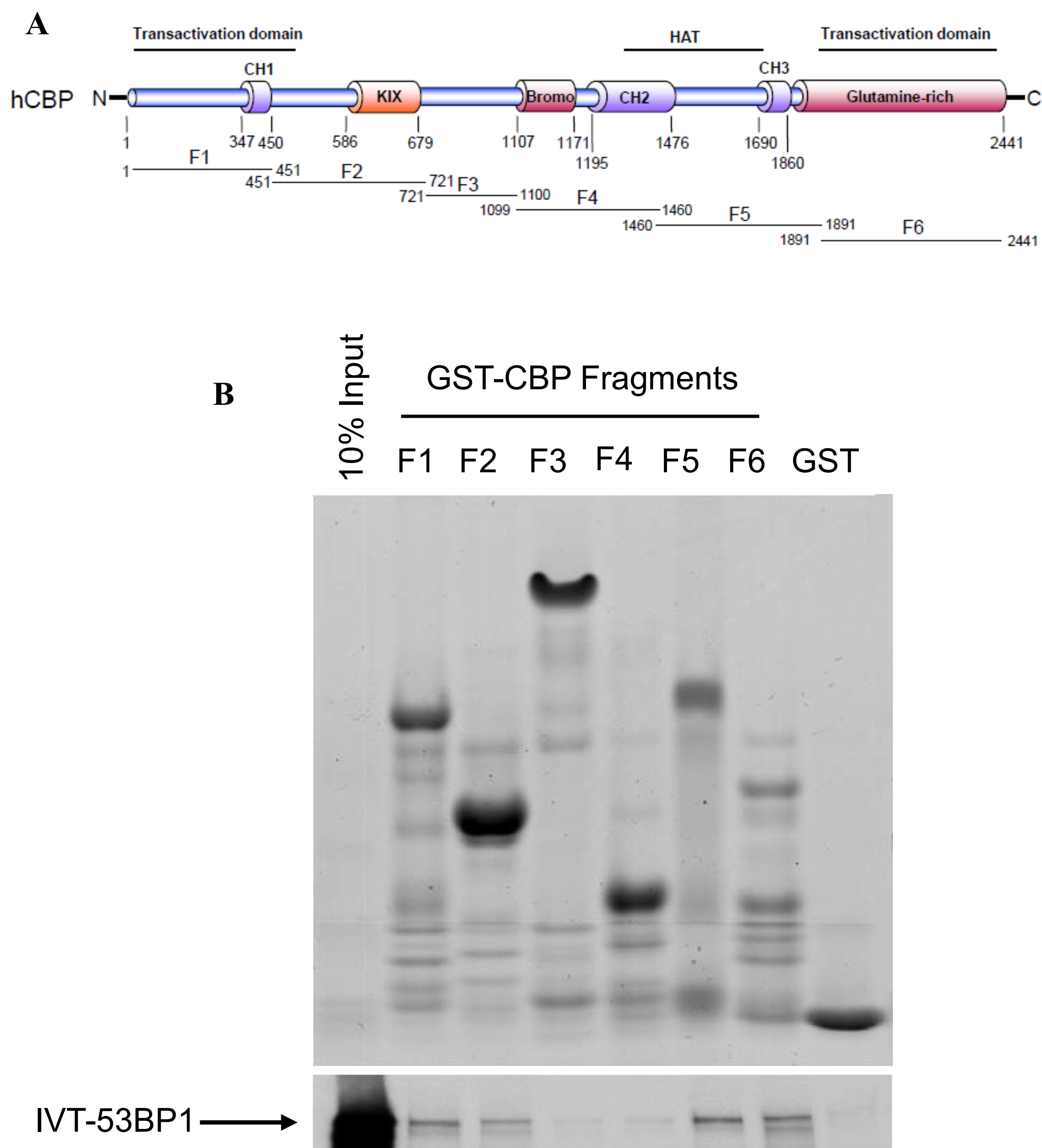


Figure 3.6 ***53BP1 binds to the N- and C-terminals of CBP****. (A) Diagrammatic representation of CBP with the six GST-fusion proteins indicated. (B) [^{35}S] methionine-labelled 53BP1 was incubated with 30µg GST-fused overlapping fragments covering the entire open reading frame of CBP. Complexes were isolated with Glutathione-Sepharose beads and fractionated by SDS-PAGE. Radiolabelled proteins were identified by fluorography and autoradiography. Coomassie stained version of the gel is shown in the top panel. GST was used as a negative control.*

3.6). Despite the observation that 53BP1 bound to different fragments of CBP and p300, it should be noted that these fragments encompass the same functional domains. These binding regions contain the KIX domain and the CH1 and CH3 binding domains, which are important for mediating protein-protein interactions (Goodman and Smolik 2000, Ponting *et al* 1996, Radhakrishnan *et al* 1997), the HAT domain that catalyses acetylation of both histones and non-histone proteins (Ogryzko *et al* 1996, Yuan and Giordano 2002) and the glutamine rich region at the C-terminal end, which is implicated in forming contacts with other co-activators, most notably those involved in nuclear hormone receptor signalling (Xu *et al* 1999a). Interestingly, these binding regions of CBP and p300 identified that mediate an interaction with 53BP1 have been shown to function as transactivation domains and are able to activate transcription, suggestive of a possible role of 53BP1 in modulating CBP and p300-dependent transcription (Lee *et al* 1996b, Swope *et al* 1996).

3.2.3 53BP1 transactivates p300

Given that CBP and p300 share extensive homology, it is not surprising that they have been shown to play similar roles, particularly being able to co-activate the same transcription factors (Chan and La Thangue 2001). In light of this and the fact that 53BP1 binds to the same functional domains on each protein, a functional link between 53BP1 and p300 was investigated further. Since 53BP1 bound to the regions of p300 that contain the two transactivation domains, luciferase reporter assays were performed to determine if 53BP1 could regulate the transcriptional activity of p300. Given that p300 cannot bind DNA itself and can only be recruited to promoters indirectly by its ability to bind to various transcription factors. To analyse the effect of 53BP1 on p300 activity, a construct was used in which full length p300 had been fused to the DBD of the yeast Gal4 transcription factor (Snowden *et al* 2000). Co-transfection of this Gal4-p300 fusion construct in conjunction with a reporter construct containing 5 Gal4 DNA binding sites upstream of the E1B TATA box and firefly luciferase gene directly allows the transcriptional regulatory function of p300 to be determined via the Gal4 portion of the molecule mediating DNA binding to the promoter element of the reporter plasmid. Co-transfection of cells with the construct containing the Gal4 DBD alone does not induce any transcriptional activity indicating that the gene expression is mediated by the p300 component of the hybrid Gal4-p300 transcription factor (Figure 3.7). Therefore, this system allows the effect of 53BP1 on the ability of p300 to

activate transcription to be studied without complications arising from the recruitment of p300 to DNA via interaction with a separate DNA-bound transcription factor, which may itself be modulated by 53BP1 or other proteins. As expected, co-transfection of Gal4-p300 and the Gal4-E1B luciferase reporter construct into p53 proficient U2OS cells stimulated luciferase activity (Figure 3.8a). Interestingly, when increasing amounts of 53BP1 were additionally co-transfected into the cells, an increase in p300 transcriptional activity was observed as shown by the increase in luciferase expression. This was not seen in the cells transfected with Gal4 alone and indicates that 53BP1 can promote p300-dependent transactivation. Figure 3.8b demonstrates that the increase in p300 transcriptional activity was not due to an increase in Gal4-p300 protein levels.

To ensure that the stimulation of p300 by 53BP1 was not cell type specific, the luciferase reporter assay was also conducted in H1299 cells. Co-transfection of increasing amounts of 53BP1 together with Gal4-p300 resulted in an increase in p300 transactivation, whilst transfection of Gal4 alone was unable to stimulate p300 transcriptional activity (Figure 3.9a). This is consistent with the effect observed in U2OS cells and suggests that 53BP1 can modulate p300 transcriptional activity. Again, the increase in p300 transcriptional activity seen was not due to an increase in Gal4-p300 protein levels as shown in figure 3.9b.

3.2.4 BRCT domains of 53BP1 are important for the interaction with p300

The GST pull-down assays revealed that p300 and CBP could interact with 53BP1 via its C-terminal region, so to identify if this region of 53BP1 was required for p300 transcriptional activity, various 53BP1 deletion mutants were used in the p300 luciferase reporter assay (Figure 3.10). 53BP1 is a nuclear protein (Schultz *et al* 2000), so before the luciferase assay was conducted, the localisation of each of the 53BP1 deletion constructs was ascertained by immunofluorescence microscopy to ensure that each construct localised to the nucleus. As shown in figure 3.11, all the deletion constructs localised to the nucleus except for construct 7, which was found to be cytoplasmic. This construct has the C-terminal half of 53BP1 deleted, which contains the nuclear localisation signal. Despite the nuclear localisation signal being moved to the beginning of the 53BP1 protein, it does not appear to function correctly, so this construct was not used in any further experiments. All the other 53BP1 deletion mutants were transfected into U2OS cells along with either Gal4 alone or Gal4-p300 and the Gal4 E1B luciferase reporter construct (Figure 3.12a). As previously shown increasing the

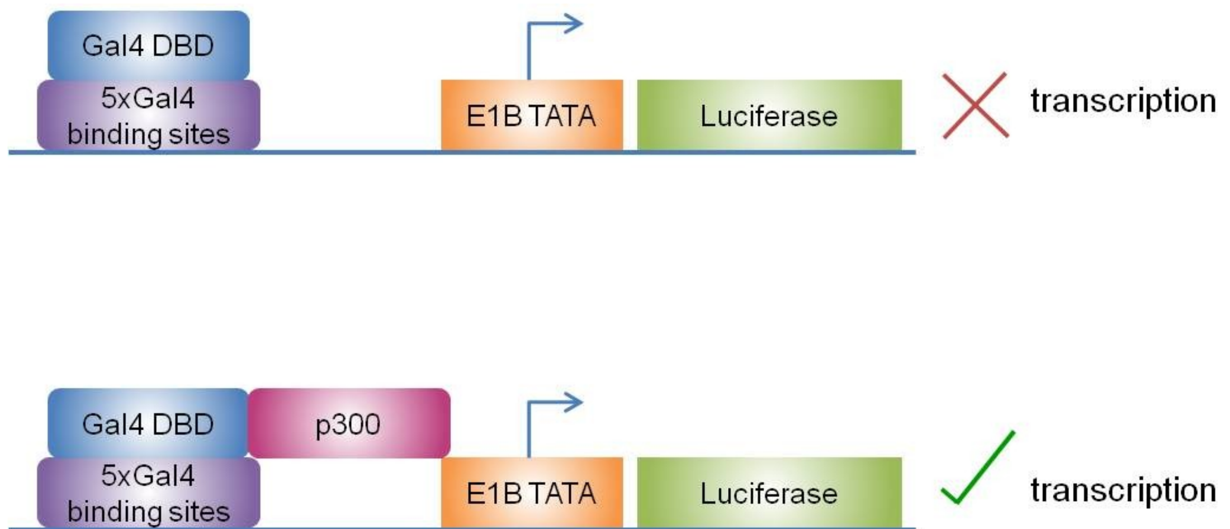


Figure 3.7 ***p300 luciferase assay***. *Schematic representation of how the Gal4 luciferase assay functions.*

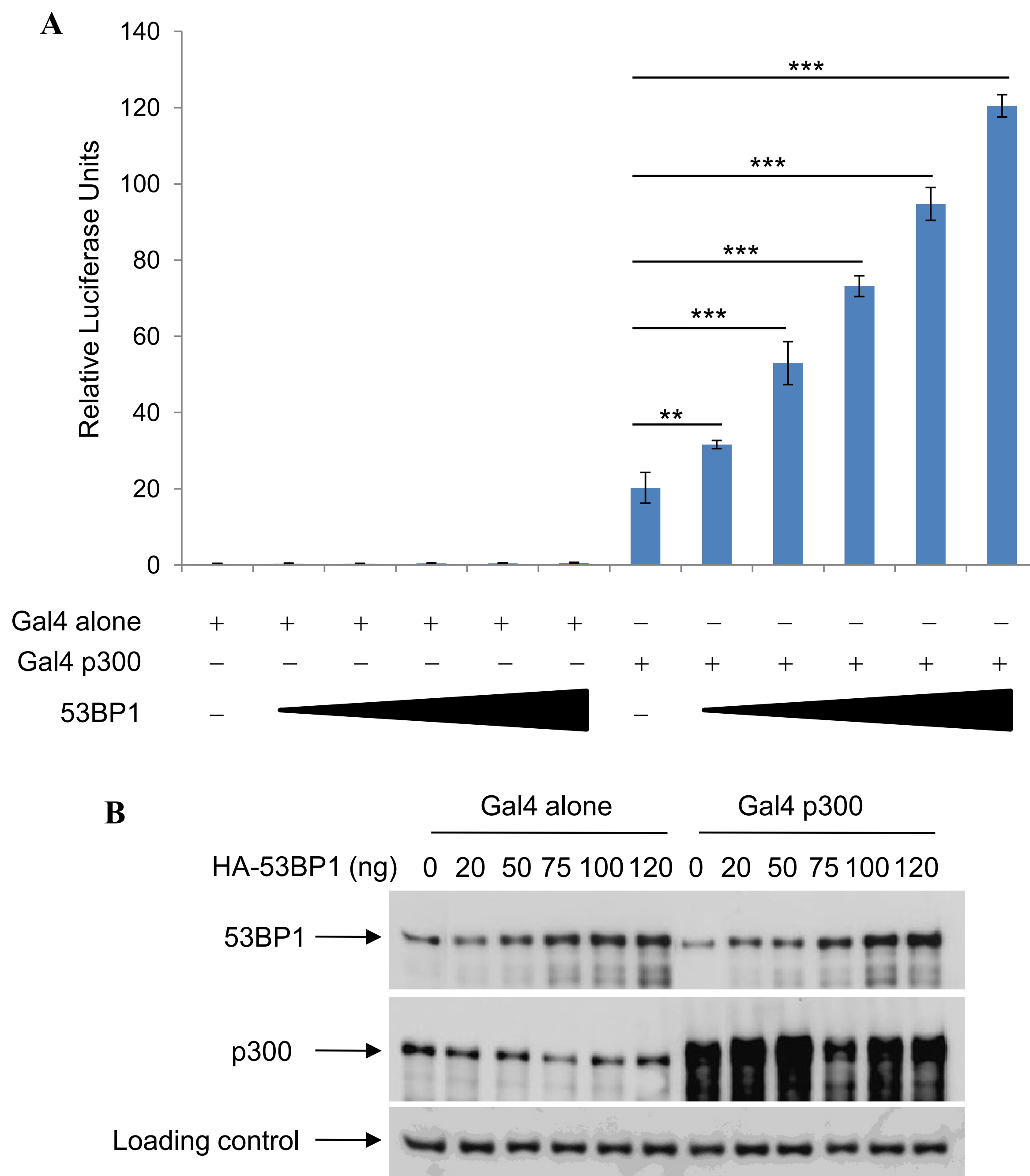


Figure 3.8 ***53BP1 stimulates p300 transcriptional activity in U2OS cells****. (A) Cells were transfected with 300ng Gal4 E1B luciferase reporter construct, 50ng Renilla, 30ng Gal4-p300 or Gal4 alone and 20, 50, 75, 100 and 120ng 53BP1. Luciferase activity was normalised to Renilla activity. Data from three independent experiments is presented ± standard deviations. Significant differences in relative luciferase units in samples with ectopic expression of 53BP1 compared to samples with endogenous expression of 53BP1 ** p< 0.01, *** p<0.001. (B) U2OS cells were transfected as in A, whole cell extracts were resolved by SDS-PAGE and the protein levels of 53BP1 and p300 were analysed by Western blot.*

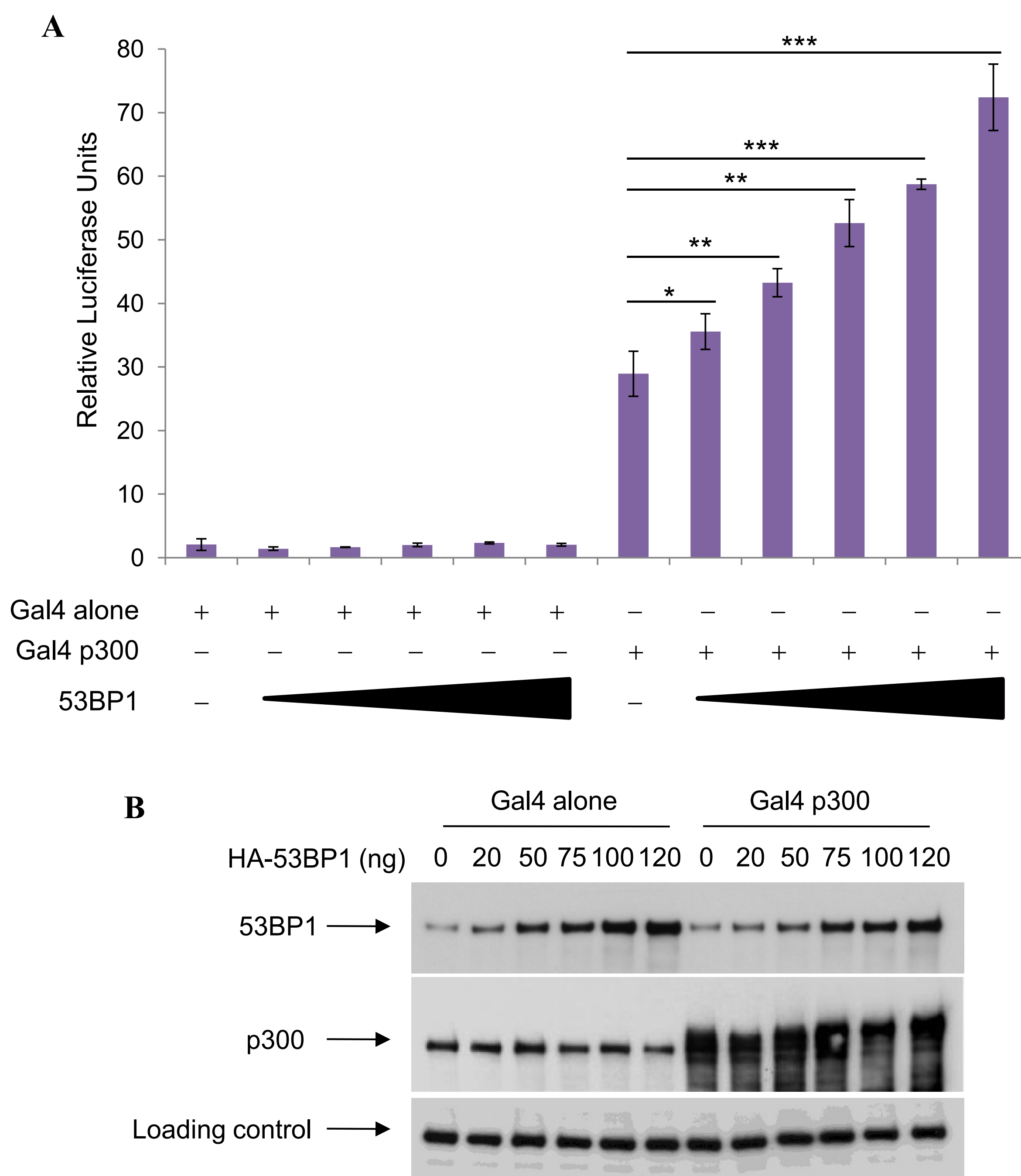


Figure 3.9 ***53BP1 stimulates p300 transcriptional activity in H1299 cells***. *(A) Cells were transfected with 300ng Gal4 E1B luciferase reporter construct, 50ng Renilla, 30ng Gal- p300 or Gal4 alone and 20, 50, 75, 100 and 120ng 53BP1. Luciferase activity was normalised to Renilla activity. Data from three independent experiments is presented ± standard deviations. Significant differences in relative luciferase units in samples with ectopic expression of 53BP1 compared to samples with endogenous expression of 53BP1 * p< 0.05, ** p< 0.01, *** p<0.001. (B) H1299 cells were transfected as in A, whole cell lysates were prepared, resolved by SDS-PAGE and immunoblotted with anti-53BP1 and anti-p300 antibodies. SMC1 was used as a loading control.*

amounts of full length 53BP1 (construct 1) resulted in an increase in luciferase expression indicating an increase in p300 transcriptional activity. In accordance with the observation that p300 binds to the C-terminal region of 53BP1 (Figure 3.4), deleting residues 1710-1972 (construct 2), which contains the BRCT domains, had a profound effect on p300 transactivation when compared to wild type. Surprisingly, deleting the N-terminal residues 1-1052 or 1-1710 (construct 5 and 6) also caused a similar reduction in luciferase expression demonstrating that these mutants can only weakly stimulate p300 transcriptional activity. However, the mutant in which residues 1235-1616 were deleted (construct 3) only mildly compromised p300 transcriptional activity when compared to the other 53BP1 mutants indicating that the GAR domain and Tudor domains are not essential for the 53BP1 mediated stimulation of p300 transactivation. Interestingly, deletion of residues 1-1052 in addition to 1710-1972 (construct 4) stimulated luciferase expression at a similar level to that of Gal4-p300 alone, again highlighting that residues 1235-1616 are not crucial for transactivation of p300. Overall these data suggest that the N-terminal region (residues 1-1052) and the BRCT domains of 53BP1 are required for p300 transcriptional activity. Western blot analysis shows the protein expression levels for each of the mutants used in the luciferase assay (Figure 3.12b).

To substantiate the luciferase data and the interaction data, a co-immunprecipitation was conducted to determine if deleting the BRCT domains altered the interaction between 53BP1 and p300. H1299 cells were transfected with either HA-tagged wild type (construct 1) or the ΔBRCT mutant (construct 2), p300 was immunoprecipitated and co-precipitation of HA-53BP1 was analysed by Western blot analysis. Figure 3.13 revealed that deleting the BRCT domains of 53BP1 reduced the interaction between 53BP1 and p300. The deletion mutant studies demonstrate that the BRCT domains of 53BP1 are important for binding and regulating the transcriptional activity of p300.

3.2.5 53BP1 modulates p53-mediated transcriptional activity

Taken together these data suggest that 53BP1 is acting as a cofactor for p300 because 53BP1 can interact and regulate p300 transcriptional activity. However, 53BP1 was originally identified as a p53 binding protein and despite Iwabuchi *et al* (1994) showing that 53BP1 can activate p53 transcriptional activity, there is still controversy in the field about whether 53BP1

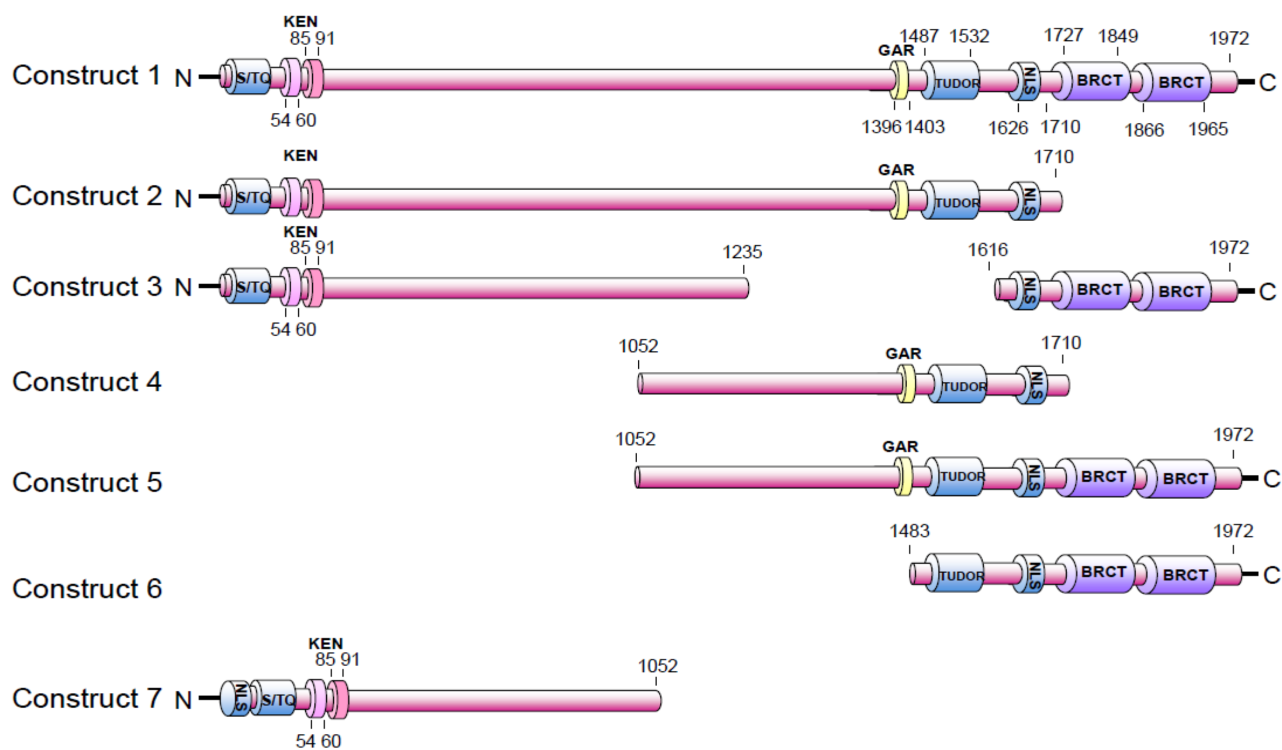


Figure 3.10 ***Schematic representation of 53BP1 and its derivatives fused to a HA tag.*** *The constructs are numbered 1-7.*

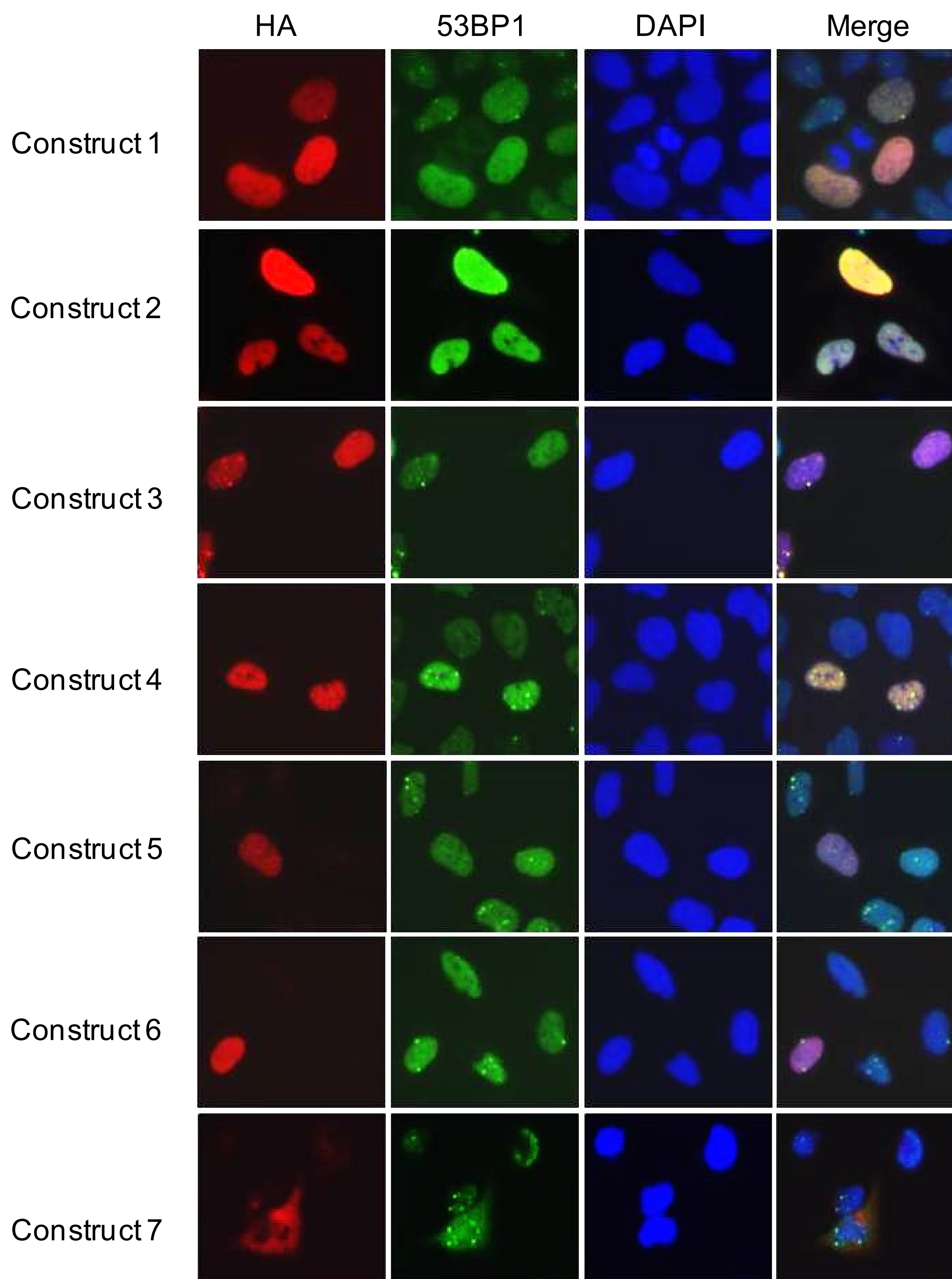


Figure 3.11 ***Localisation of 53BP1 and its deletion constructs.*** *U2OS cells were transfected with 100ng of each construct and 24 hours later cells were fixed and the localisation of each construct was analysed by immunofluorescence using the indicated antibodies.*

Figure 3.12 ***The N-terminal and BRCT domains of 53BP1 are important for p300 transcriptional activity****. (A) U2OS cells were transfected with 300ng Gal E1B luciferase reporter construct, 50ng Renilla, 30ng Gal4-p300 and varying amounts of HA-53BP1 constructs as indicated in (B). For the control experiments, 300ng Gal4 E1B luciferase reporter construct was transfected with 50ng Renilla, 30ng Gal4 alone and either 150ng construct 1, 150ng construct 2, 120ng construct 3, 50ng construct 4, 50ng construct 5 or 50ng construct 6. Luciferase activity was normalised to Renilla activity. Data from four independent experiments is presented ± standard deviations. (B) U2OS cells were transfected with the indicated amounts of HA-53BP1 fusions, whole cell extracts were subjected to SDS-PAGE and immunoblotted with indicated antibodies.*

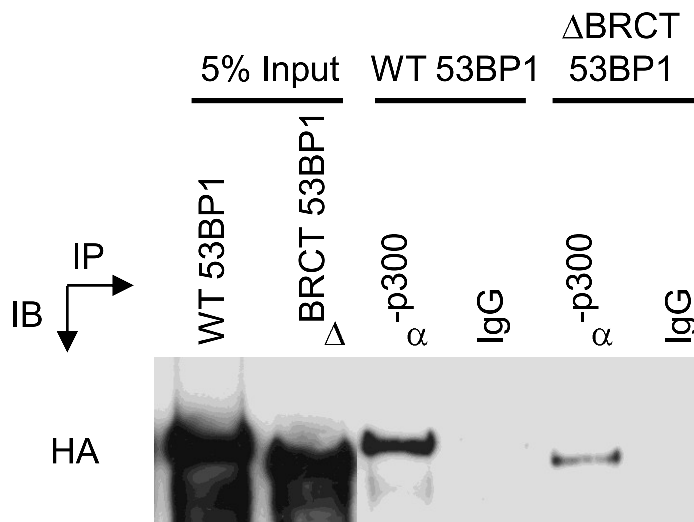


Figure 3.13 ***BRCT domains of 53BP1 are important for binding to p300.*** *p300 was immunoprecipitated from H1299 whole cell extracts transfected with 1µg of construct 1 and 2 for 24 hours, separated by SDS-PAGE and the binding of p300 to HA-53BP1 was assessed by Western blotting.*

regulates p53 transcriptional activity (Adams and Carpenter 2006, Mochan *et al* 2004). Therefore, to establish if 53BP1 could regulate p53 transcriptional activity, a p53 luciferase reporter assay was performed. The luciferase gene under the control of 13 consensus p53 DNA binding sites was transfected into the p53 null H1299 cell line together with either wild type p53 or mutant p53 (R175H) and increasing amounts of 53BP1. The p53-R175H mutant is a structural mutant that destabilises the tertiary structure of the p53 DNA binding domain, therefore preventing p53 from binding to DNA (Bullock *et al* 2000). In the presence of wild type p53, luciferase expression increased in a dose dependent manner with increasing amounts of 53BP1 indicating that 53BP1 can stimulate p53 transcriptional activity. In contrast, p53 transcriptional activity was not stimulated in the presence of mutant p53 (Figure 3.14a). This result is consistent with previous observations suggesting that 53BP1 can transactivate p53 in a dose dependent manner in the absence of DNA damage (Iwabuchi *et al*, 1998) and that this transactivation is dependent on p53 being transcriptionally competent. Western blot analysis showed that the observed co-activation of p53 by 53BP1 was not due to ectopic expression of 53BP1 affecting p53 protein levels (Figure 3.14b).

The CDK inhibitor, p21 is an important transcriptional target of p53 because it is the main effector of p53-mediated cell cycle arrest (el-Deiry *et al* 1993, Waldman *et al* 1995). Therefore to examine if 53BP1 could stimulate p53-dependent transcriptional activity from a more biologically relevant promoter element, H1299 cells were transfected with a plasmid containing the luciferase gene under the control of the p53 responsive p21 promoter along with wild type p53 and increasing amounts of 53BP1. Surprisingly, ectopic expression of 53BP1 significantly repressed p21 transcriptional activity in a concentration dependent manner in the presence of p53 when compared with induction of p21 by p53 alone (Figure 3.15a). This repression is dependent on p53 because in the absence of p53, 53BP1 was unable to repress p21 expression. Western blot analysis demonstrated that increasing the amounts of 53BP1 had no significant effect on protein levels of p53 (Figure 3.15b). These data suggests that 53BP1 can suppress p53-mediated transactivation of p21 in the absence of DNA damage.

To probe the functional role of 53BP1 in p53 regulation in a physiological context, wild type p53 U2OS cells were depleted of 53BP1 using siRNA and either mock irradiated or exposed to 3 Grays of IR. The protein expression levels of p53 and its target genes HDM2, PUMA and p21 were analysed by Western blot analysis (Figure 3.16). Following DNA damage, the levels of HDM2 and PUMA were reduced in 53BP1 depleted cells compared to control cells

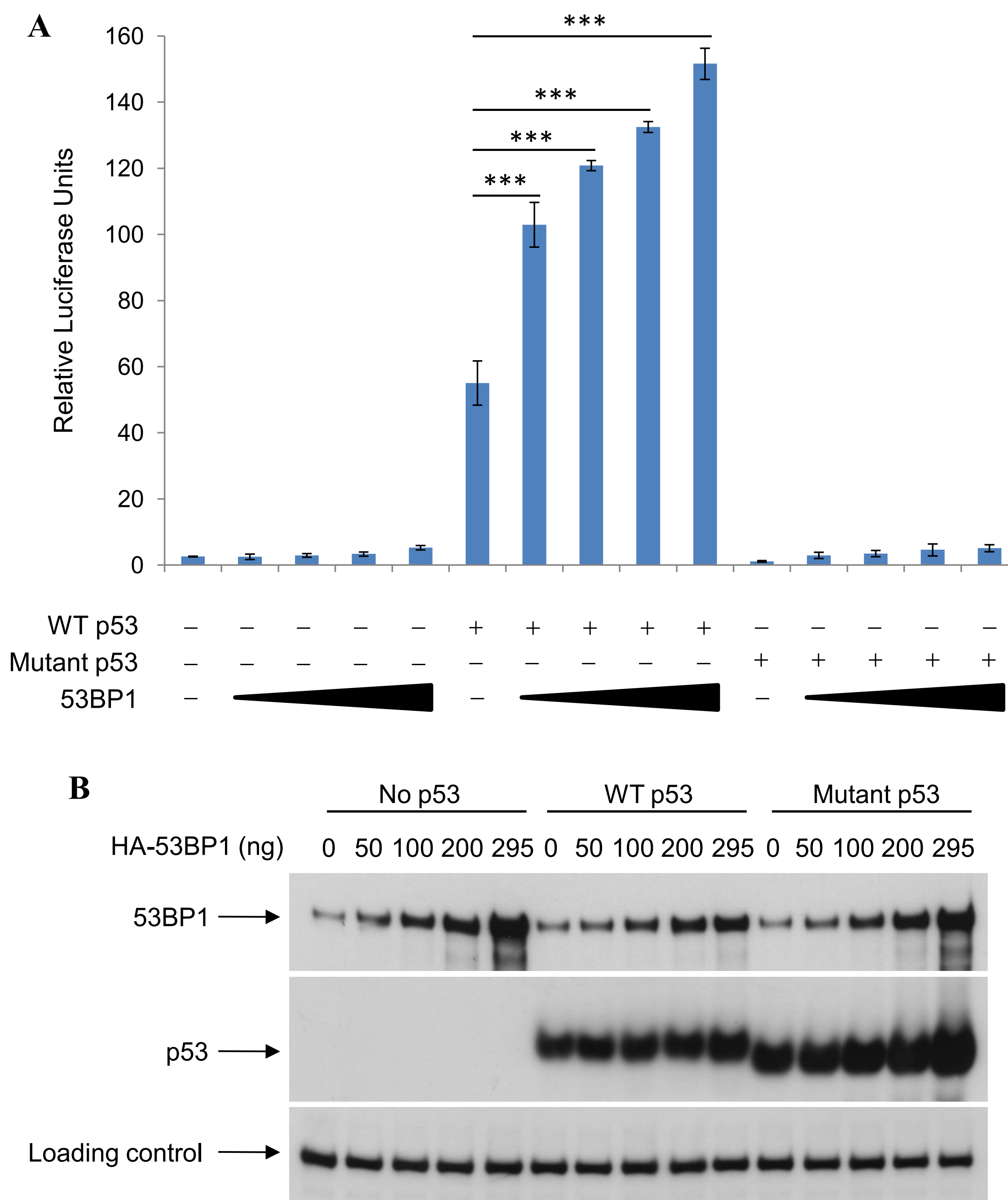


Figure 3.14 ***53BP1 stimulates p53 transcriptional activity****. (A) H1299 cells were transfected with 150ng of the PG13 luciferase reporter construct, 50ng Renilla, 5ng wild type (WT) or mutant p53 expression constructs and 50, 100, 200, 295ng of an expression plasmid encoding 53BP1. Luciferase activity was normalised to Renilla activity. Data from three independent experiments is presented ± standard deviations. Significant differences in relative luciferase units in samples with ectopic expression of 53BP1 compared to samples with endogenous expression of 53BP1 *** $p<0.001$. (B) H1299 cells were transfected as in A, whole cell lysates were prepared, resolved by SDS-PAGE and immunoblotted with anti-53BP1 and anti-p53 antibodies. SMC1 was used as a loading control.*

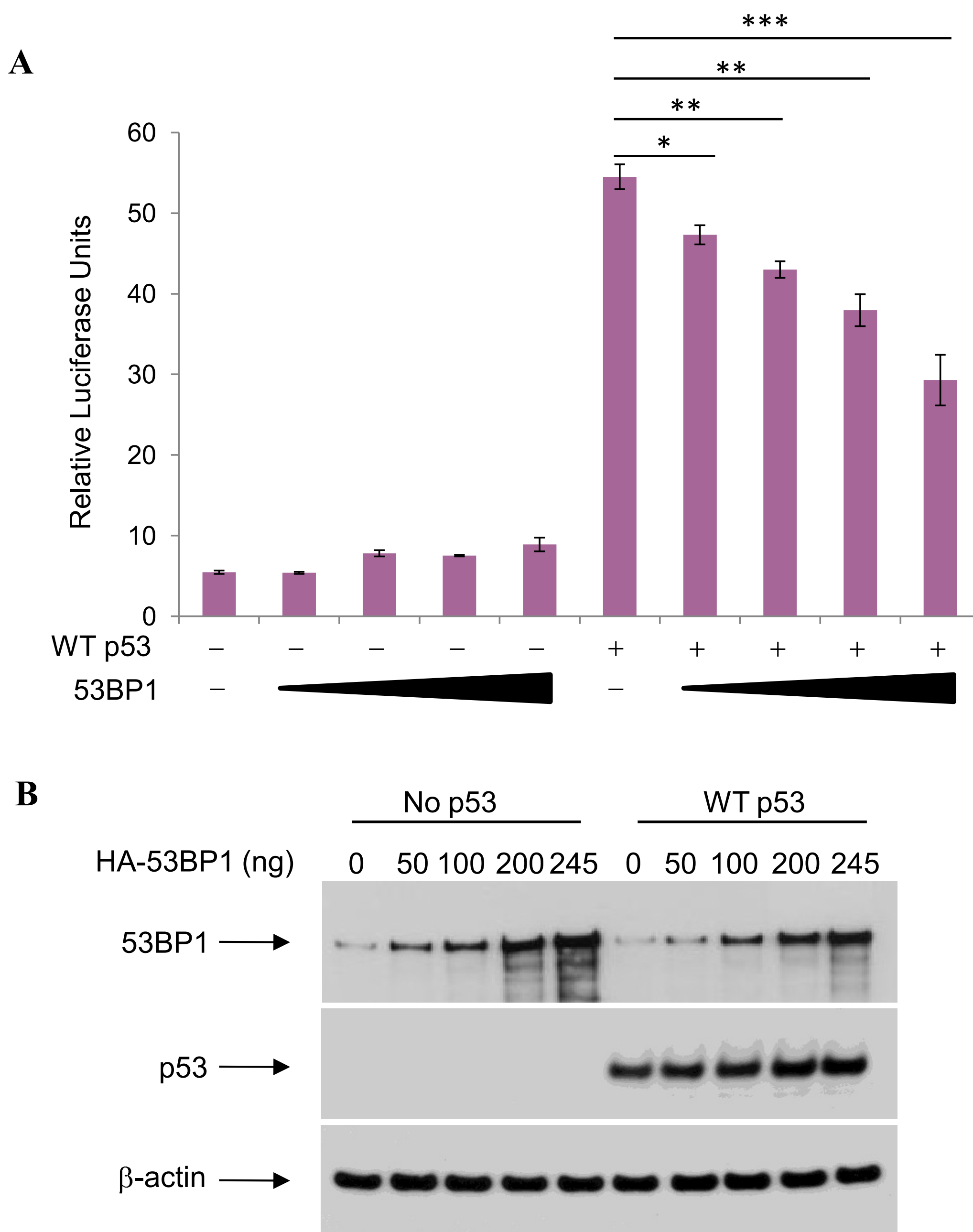


Figure 3.15 ***53BP1 represses p53-dependent p21 transcriptional activity****. (A) H1299 cells were transfected with 200ng of the WWP (p21) luciferase reporter construct, 50ng Renilla, 5ng wild type (WT) or mutant p53 expression constructs and 50, 100, 200, 245ng of an expression plasmid encoding 53BP1. Luciferase activity was normalised to Renilla activity. Data from three independent experiments is presented ± standard deviations. Significant differences in relative luciferase units in samples with ectopic expression of 53BP1 compared to samples with endogenous expression of 53BP1* $p<0.05$, ** $p< 0.01$, *** $p<0.001$. (B) H1299 cells were transfected as in A, whole cell lysates were prepared, resolved by SDS-PAGE and protein levels of 53BP1 and p53 were assessed by immunoblotting.*

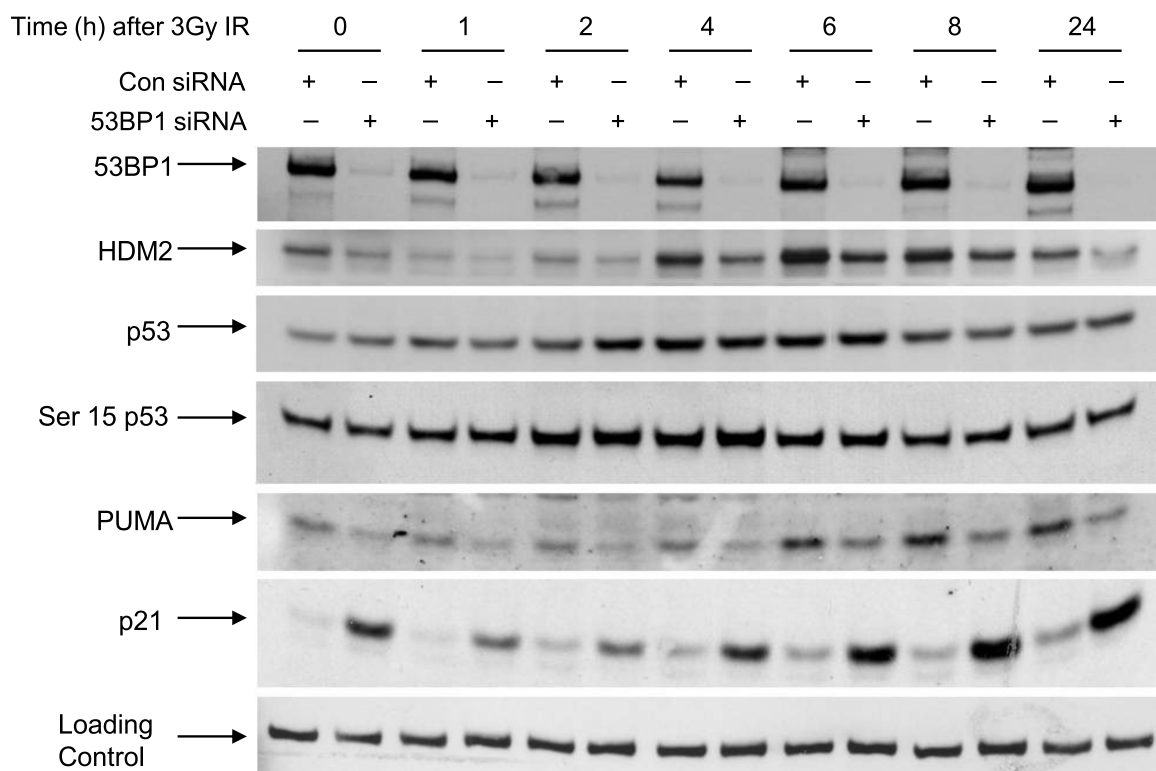


Figure 3.16 ***53BP1 differentially regulates p53 target genes.*** *U2OS cells were transfected with either control or 53BP1 siRNA, 72 hours later the cells were either mock irradiated or irradiated with a 3 Gray dose and harvested at the times indicated. Whole cell extracts were separated by SDS-PAGE and immunoblotting used to analyse the protein levels of 53BP1, p53, serine 15 phosphorylation of p53 and p53 target genes, HDM2, PUMA and p21. SMC1 was used as a loading control.*

whereas the levels of p21 were increased in the absence of 53BP1. However, in contrast 53BP1 depletion did not affect IR-induced p53 stabilisation or serine 15 phosphorylation of p53. Therefore, these data indicate that 53BP1 may be differentially regulating the expression of p53 responsive genes and as a result provide specificity to the p53 response.

To determine if the increase in p21 protein expression observed in unstimulated cells was due to loss of 53BP1 enhancing p53-dependent p21 transcriptional activity, 53BP1 depleted cells were transfected with p53-reponsive p21 luciferase reporter construct together with wild type p53. As shown in figure 3.17, p21 transcriptional activity was significantly increased in a p53-dependent manner in cells lacking 53BP1, despite Western blot analysis showing that p53 protein levels were lower in the 53BP1 depleted cells when compared to control siRNA treated cells. Interestingly, a small increase in p21 transcriptional activity was also observed in the absence of p53 indicating that loss of 53BP1 could be affecting other proteins that regulate p21 transactivation. These findings reveal that the depletion of 53BP1 results in activation of p21 in a p53-dependent and possibly a p53-independent manner in the absence of overt DNA damage.

Taken together, these data indicate that that the ability of 53BP1 to modulate p53 target gene expression is complex, with the promoter sequence, surrounding chromatin, type of cellular stress and presence of additional regulatory proteins likely influencing whether 53BP1 had a positive or negative effect on the ability of p53 to activate or repress gene transcription.

3.3 DISCUSSION

Many signal transduction pathways converge at the level of transcriptional co-activators, which serve as signal integrators that process cellular signals and regulate gene expression (Naar *et al* 2001). CBP/p300 are two of the most extensively studied transcriptional co-activators, which interact with a wide variety of proteins, including many transcription factors and participate in a broad spectrum of biological activities (Goodman and Smolik 2000). The data reported here indicates that 53BP1 may function as a cofactor for CBP/p300.

Co-immunoprecipitation studies revealed that 53BP1, CBP and p300 interact with each other (Figure 3.2). This is consistent with the mass spectrometry data, which originally identified the interaction between 53BP1 and CBP/p300 (Figure 3.1). Interestingly, almost all the CBP and p300 interacting proteins that were identified by mass spectrometry were different

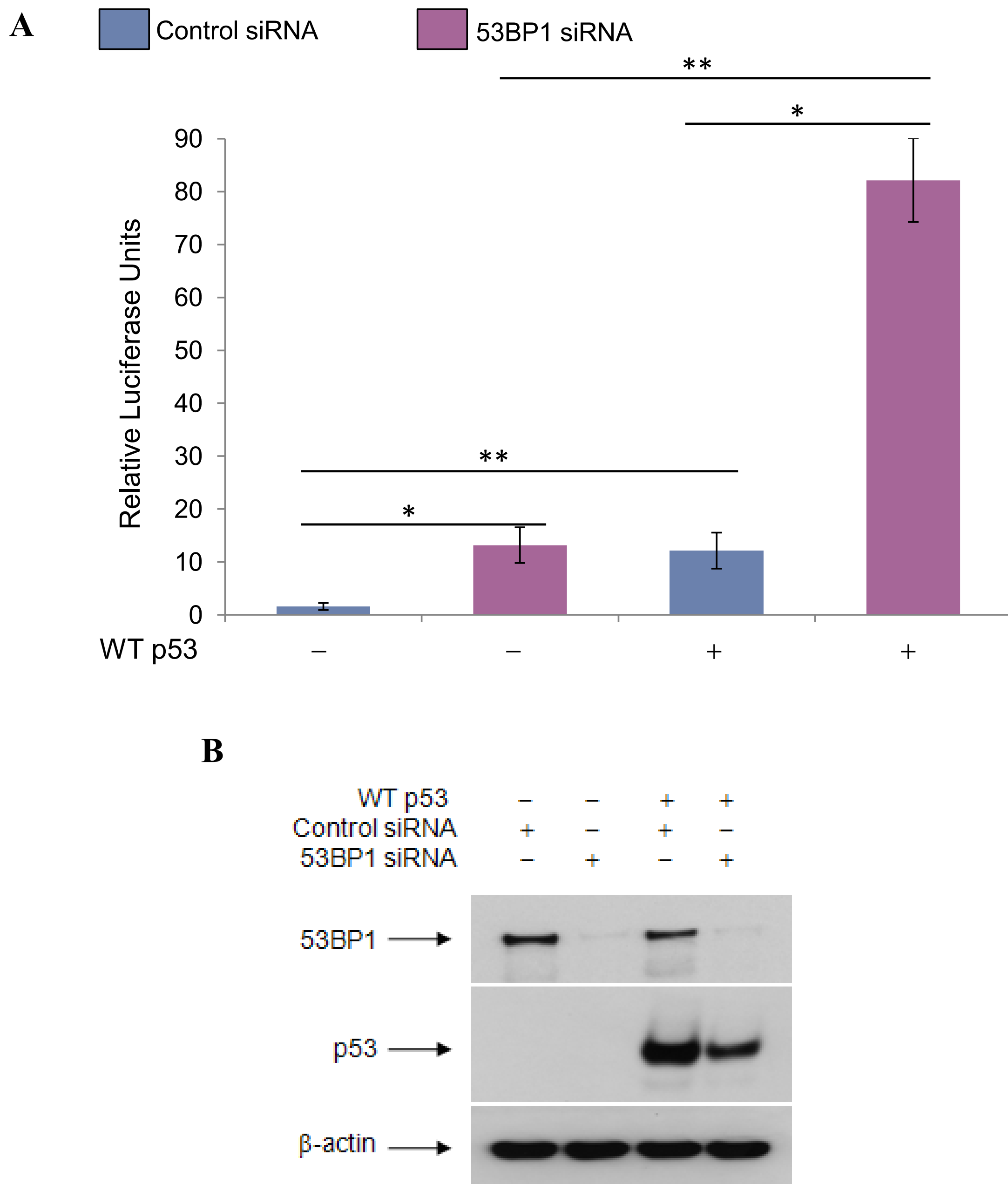


Figure 3.17 ***Depletion of 53BP1 enhances p53-dependent p21 transactivation***. *(A) H1299 cells were either mock treated or treated with 53BP1 siRNA, 48 hours later cells were transfected with 200ng WWP (p21) luciferase reporter construct, 50ng Renilla and 5ng wild type (WT) p53. Luciferase activity was normalised to Renilla activity. Data from three experiments is presented ± standard deviations. Significant differences in relative luciferase units in samples depleted of 53BP1 compared to control samples with and without p53* $p<0.05$, ** $p< 0.01$. (B) H1299 cells were transfected as in A, whole cell lysates were prepared, resolved by SDS-PAGE and protein levels of 53BP1 and p53 were assessed by immunoblotting.*

(Figure 3.1). This suggests that despite CBP and p300 being highly homologous, they may perform different functions, particularly in the DDR since the majority of proteins identified are involved in the DDR. When the interaction sites were mapped, CBP and p300 were found to bind to the C-terminal region of 53BP1 (residues 1309-1972) (Figure 3.4). This section of 53BP1 encompasses all the domains of 53BP1 including the tandem BRCT domains and the Tudor domain. Deletion of the BRCT domains of 53BP1 dramatically reduced the interaction between 53BP1 and p300, although the interaction was not completely abolished indicating that other regions N-terminal to the BRCT domains are also likely to be involved (Figure 3.12). Furthermore, the identification of two 53BP1 binding regions on CBP and p300 indicates that potentially multiple 53BP1 molecules may bind to a single p300 or CBP molecule at different sites (Figure 3.5 and 3.6). Alternatively, a single 53BP1 molecule maybe able to bind to both regions on CBP and p300, although this will depend on the structural conformation of CBP and p300. Another possibility is that like p53 (Grossman 2001), the functional significance of the two interaction sites on CBP and p300 is different and therefore, 53BP1 may influence CBP and p300 activity differentially depending on where 53BP1 binds.

Since, the regions on CBP and p300 that 53BP1 binds to encompass the transactivation domains of CBP/p300, luciferase reporter assays were performed. These demonstrated that 53BP1 can enhance transcription mediated by p300 when fused to the Gal4 DNA binding domain (Figure 3.8 and 3.9). This was abolished when residues 1-1052 and/or 1710-1972 of 53BP1 were deleted indicating that these two areas are necessary for the ability of 53BP1 to stimulate p300 transcriptional activity (Figure 3.12 and 3.13). This highlights the possibility that the BRCT domains of 53BP1 are not only important for 53BP1 to interact with p300, but also for the ability of p300 to stimulate transcription. Interestingly, since BRCT domains are phospho-protein binding domains, it is possible that the ability of 53BP1 to bind p300 may be modulated by phosphorylation (Manke *et al* 2003, Yu *et al* 2003). However, to address this directly, a point mutation in the BRCT domains of 53BP1 that ablates the phospho-peptide binding would have to be constructed and then the ability of 53BP1 to bind to p300 and potentiate its transactivation capability determined. Conceivably, it is also possible that regions of the 53BP1 fragment shown to bind to p300 that are outside the BRCT domains could also be mediating this interaction. For example, the Tudor domain of 53BP1 binds to methylated lysines and arginines, therefore methylation of p300 could also facilitate 53BP1 binding to p300 (Cote and Richard 2005, Kim *et al* 2006). Interestingly, BRCA1 has recently

been shown to interact with p300 via its BRCT domains and this interaction is mediated by CARM1-dependent methylation of arginine 754 in p300 (Lee *et al* 2011). This demonstrates that as well as binding to phosphorylated residues, BRCT domains can also interact with methylated arginine residues. Therefore, similar to BRCA1, the interaction between 53BP1 and CBP/p300 may be mediated by the BRCT domains of 53BP1 binding to methylated p300.

Interestingly, these data demonstrate that different regions of 53BP1 are important for binding to p300 and for regulating p300 transcriptional activity. Exactly how the N-terminal portion of 53BP1 is involved in mediating p300 transactivation is unclear since it does not contain any known functional domains. However, this region does contain several biological relevant amino acid motifs. Firstly, this region contains numerous potential phosphorylation target sites for PIKK family members such as ATM and ATR, which could play a role in regulating the ability of 53BP1 to potentiate p300-dependent transcription following DNA damage but would be unlikely to contribute to this activity in unstressed cells (Jowsey *et al* 2007, Traven and Heierhorst 2005, Ward *et al* 2006). Secondly, residues 54-60 and 85-91 of this region are two potential KEN boxes, which are sequences recognised by the APC/C activators, Cdc20 and Cdh1 (Peters 2006, Thornton and Toczyski 2006). Whilst there is currently no evidence that links 53BP1 with Cdc20 and/or Cdh1, Lergerski and colleagues have demonstrated that 53BP1 can interact with the Cdc27 subunit of the APC/C (Akhter *et al* 2004). Interestingly, CBP/p300 have also been shown to interact with components of the APC/C that can potentiate the transcriptional activity of both CBP and p300 (Turnell *et al* 2005). Therefore, it is also possible that the ability of 53BP1 to activate p300-mediated transcription may involve other proteins such as the APC/C that could interact with the N-terminus of 53BP1. To address this directly, N-terminal deletion mutants would need to be used to define the precise region required for p300-mediated transactivation, which could then be utilised for mass spectrometry to aid identification of any potential interacting proteins that could be involved in regulating p300-dependent transcriptional activity. However, taken together, these data suggest that 53BP1 can interact with CBP and p300 both *in vitro* and *in vivo* and stimulate p300-mediated transcription in the absence of DNA damage.

Since CBP/p300 both function as transcriptional co-activators for numerous transcription factors, it would be of interest to ascertain if 53BP1 was important for facilitating CBP/p300-mediated co-activation of any of these transcription factors. One approach to potentially identify these transcription factors would be to use protein-DNA array technology, which

allows activity profiling of multiple transcription factors simultaneously (Panomics). Subsequently, those transcription factors that were identified by the array and whose activity was also known to be potentiated by CBP/p300 would be investigated further to try to elucidate the role of 53BP1s ability to bind CBP/p300 in regulating the activity of these transcription factors and also how this influences the expression of their target genes.

Due to 53BP1 only possessing protein-protein interaction domains, it is considered to function as a scaffold protein during the DNA damage response and recently, it has been reported that 53BP1 is required for the recruitment of the checkpoint protein Rif1 and the chromatin remodelling protein EXPAND1 to the sites of DNA damage (Huen *et al* 2010a, Silverman *et al* 2004). Consequently, it is plausible to hypothesise that 53BP1 could be providing a scaffold with which to recruit CBP/p300 to the gene promoters/enhancers or stabilise them on the DNA once bound, where they can enhance transcription by acetylating histones in the vicinity of these genes. This would allow CBP/p300 to connect transcription factors bound to their response elements with the basal transcriptional machinery situated on gene core promoter sequences simultaneously and further augment transcription by acetylating the transcription factors.

Alternatively, in many cases, transcription factors bind to response elements, which can be some distance away from the core promoters where the general transcriptional machinery is bound (Bulger and Groudine 2010). To facilitate activation of gene expression the enhancers/transcription factor binding sites and core promoter sequences of genes need to be brought in close proximity to each other, which would involve the formation of a DNA loop (Miele and Dekker 2008). Recently, the Mediator co-activator complex and cohesin have been shown to be important for connecting the enhancers and core promoters of active genes in murine embryonic stem cells by promoting DNA loop formation and stabilisation (Kagey *et al* 2010). Interestingly, it has been reported that 53BP1 facilitates the joining of distant damaged DNA ends by its ability to increase the mobility of the local chromatin to bring together the two DNA ends (Difilippantonio *et al* 2008, Dimitrova *et al* 2008). Therefore, in addition to the Mediator co-activator complex and cohesin, 53BP1 could also be involved in bringing regulatory sequences bound by the transcription factor and its co-activators CBP/p300 and promoter elements into close proximity by altering the chromatin structure.

Before 53BP1 was identified as a DNA damage response protein, it was proposed to function as a transcriptional co-activator of p53 by Stanley Fields and colleagues (Iwabuchi *et al* 1998). Recent data from Shelley Berger and colleagues supports this function for 53BP1 and potentially resolves the controversy surrounding the ability of p53 to bind to DNA and 53BP1 simultaneously by suggesting that the interaction between 53BP1 and p53 is mediated by post-translational modifications of p53 (Huang *et al* 2007a). In contrast, when Chen and colleagues investigated the mRNA expression levels of p53 target genes in 53BP1 deficient murine thymocytes, they demonstrated that 53BP1 was not required for p53 transactivation. Moreover, they observed that loss of 53BP1 increased expression of p21 suggesting that 53BP1 was functioning as a co-repressor rather than a co-activator of p53 (Ward *et al* 2005). One hypothesis that accounts for both sets of conflicting data is the possibility that in fact 53BP1 may not strictly be a co-activator or co-repressor of p53, but rather a modulator of p53 that can differentially regulate p53 responsive genes. Consistent with this, the data presented here indicates that 53BP1 can both potentiate the transcriptional activity of p53, possibly to induce HDM2 and PUMA expression following DNA damage (Figure 3.14 and 3.16), and also repress it, in the p21 expression in unstressed cells (Figure 3.15 and 3.17). This apparent schizophrenic behaviour of 53BP1 as a regulator of transcription is not unusual and is very similar to that of the p52 NF-κB subunit and the Tip60/p400 co-activator proteins. In the case of Tip60 and p400 these proteins have been shown to repress p21 expression in unstimulated cells, but cooperate with p53 to induce apoptosis in response to UV DNA damage (Tyteca *et al* 2006). The NF-κB subunit, p52 has also been shown to be antagonistic to p53 by suppressing p21 expression in unstimulated U2OS cells, but again cooperating with p53 at other promoters such as PUMA, GADD45α and DR5 and succeeding UV damage (Schumm *et al* 2006). Therefore, 53BP1 is unlikely to be functioning as a strict co-activator or co-repressor of p53, as previously reported, but rather behave as a modulator of p53 function indicating that in some situations 53BP1 may influence p53 target gene selectivity and as a consequence the p53-dependent transcriptional response. To understand precisely the involvement of 53BP1 in modulating p53 function, the expression of other p53 target genes would need to be analysed to obtain a global view on which aspect of the p53-dependent transcriptional profile requires 53BP1 and whether this is influenced by specific cellular stresses. Furthermore, it would be of interest to determine whether 53BP1 binds directly to the promoters of specific p53-regulated genes or whether other indirect mechanisms are involved

to ascertain whether this differential regulation of p53 by 53BP1 was occurring at the sites of transcription or not. Since, it appears that 53BP1 regulates p21 and PUMA differently, the biological significance of these data would need to be investigated to establish the effect of 53BP1 on cell cycle progression and apoptosis.

In light of the fact that 53BP1 regulates the transactivation potential of p300 (Figure 3.8 and 3.9) and p53 (Figure 3.14), it would be interesting to determine if the increase in p53 transcriptional activity observed was dependent on the ability of 53BP1 to potentiate p300-dependent increases in gene expression or whether these two events are mutually exclusive. Interestingly, it has been shown that the p300 cofactors, JMY and Strap can increase p53 acetylation and consequently activate p53 pro-apoptotic target genes through interacting and enhancing p300 transcriptional activity (Demonacos *et al* 2001, Shikama *et al* 1999).

Collectively, these data have shown that 53BP1 plays a role in the regulation of p53 target gene expression, although the precise mechanism remains unclear.

CHAPTER 4

CHAPTER 4 USE OF GLOBAL GENE EXPRESSION PROFILING TO IDENTIFY 53BP1 REGULATED GENES

4.1 INTRODUCTION

53BP1 was originally identified in a yeast two-hybrid screen looking for novel p53 interacting proteins that could potentially modulate p53 transcriptional activity (Iwabuchi *et al* 1994, Iwabuchi *et al* 1998). Up until recently the ability of 53BP1 to bind to p53 and alter its transcriptional activity was unclear due to contradictory studies demonstrating that p53 could not bind to 53BP1 and DNA simultaneously (Derbyshire *et al* 2002, Joo *et al* 2002). However, data from Berger and colleagues showed that 53BP1 can recognise and bind to di-methylated lysine 370 of p53 and enhance p53 transactivation supporting a role for 53BP1 as a transcriptional regulator (Huang *et al* 2007a). Consistent with this notion, in addition to p53, 53BP1 can function as a positive transcriptional regulator of *BRCA1* gene expression by binding to a novel palinodromic DNA sequence in the BRCA1 promoter (Rauch *et al* 2005). Moreover, the murine homologue of 53BP1 has been shown to bind and inhibit the activity of the transcriptional regulator, p202 (Datta *et al* 1996).

Interestingly, the di-methylated lysine 20 of histone H4 and the di-methylated lysine 79 of histone H3 that have been shown to directly bind to 53BP1 via its Tudor domain are found in regions of chromatin that are transcriptionally active suggesting that 53BP1 can be localised to regions of high transcriptional activity (Botuyan *et al* 2006, Huyen *et al* 2004, Ng *et al* 2003). Furthermore, data presented in the previous chapter reveals that 53BP1 can interact and regulate the transactivation function of the transcriptional co-activator p300. Taken together, these observations suggest that 53BP1, as well as being a DSB repair protein, may also function as a transcriptional regulator. To address this hypothesis directly, a microarray approach was utilised to study the gene expression patterns in cells treated with and without 53BP1 siRNA, before and after IR to identify genes whose expression was dependent on 53BP1 and whether their expression was affected by genotoxic stress.

The aims of this study were to:

(i) Identify any differences in the transcriptional profiles of cells treated with control and 53BP1 siRNA.

(ii) Determine any specific differences in transcriptional responses between control and 53BP1 siRNA treated cells following IR-induced DNA damage.

(iii) Identify any cellular pathways that are different between unirradiated control and 53BP1 siRNA treated cells.

(iv) Ascertain any pathways that are different between control and 53BP1 siRNA treated cells in response to IR.

To accomplish this, two different analysis methods were used, single gene analysis (‘univariate’ analysis) and biological pathway analysis (‘multivariate’ analysis).

4.2 RESULTS

4.2.1 Gene expression profiling identified a link between 53BP1 and TNFR1 signalling, independent of DNA damage

In response to DNA damage, p53 becomes stabilised and activated, subsequently the expression of p53 target genes are altered. To determine the optimal time point to analyse the transcriptional differences between control and 53BP1 depleted samples following irradiation, the kinetics of p53 induction needed to be established. U2OS cells were exposed to 3 Grays of IR and the protein levels of p53, HDM2 and p21 were analysed by Western blot. As shown in figure 4.1 the induction of p53 peaked at 4 hours post-IR, which also coincided with maximal induction of the p53 responsive genes, p21 and HDM2. This demonstrates that p53-dependent transcription reaches a maximum at 4 hours post-IR and therefore, 4 hours was chosen as the time following the induction of DNA damage that the transcriptional profile was analysed by microarray.

To investigate the potential role of 53BP1 in regulating cellular transcription, U2OS cells were transfected with either control or 53BP1 specific siRNA and exposed to 3 Grays of IR, 72 hours after transfection. At 0 and 4 hours post-IR, cells were harvested for both RNA and protein. The RNA quality was checked by agarose gel analysis and the protein levels of 53BP1 were assessed by Western blot analysis to ensure 53BP1 had been efficiently knocked

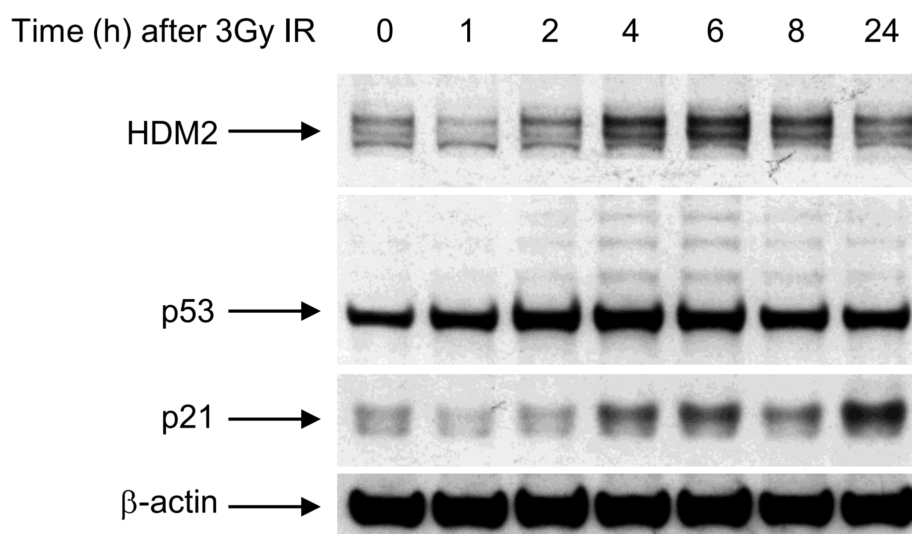


Figure 4.1 ***p53 transcriptional activity occurs at 4 hours post-IR****. U2OS cells were irradiated and harvested at 0, 1, 2, 4, 6, 8 and 24 hours. Whole cell lysates were prepared, resolved by SDS-PAGE and the protein expression levels of p53, HDM2 and p21 were analysed by immunoblotting.*

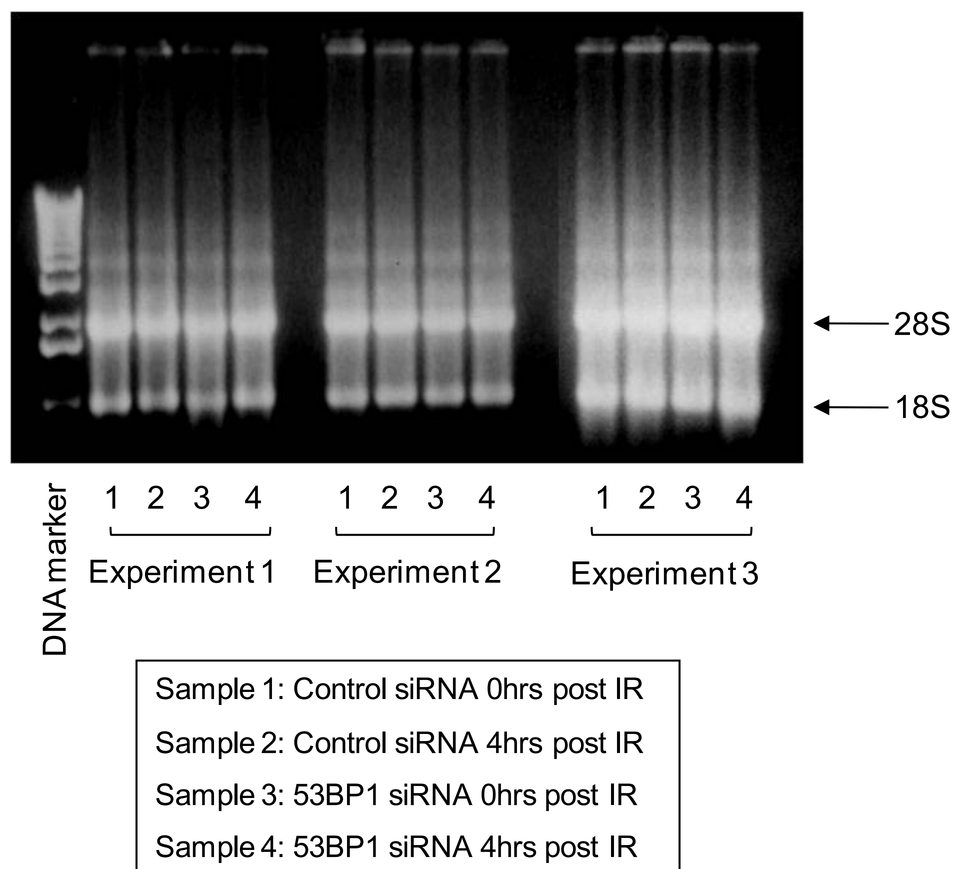


Figure 4.2 ***RNA hybridised to the Affymetrix GeneChips was good quality***. *U2OS cells were treated with either control or 53BP1 siRNA, 72 hours later cells were either exposed to 3 Grays of IR or left untreated. RNA was extracted from whole cell extract and 2µl of each sample was run on a 0.8% agarose gel to check the quality of the RNA. Data from the three independent experiments is shown.*

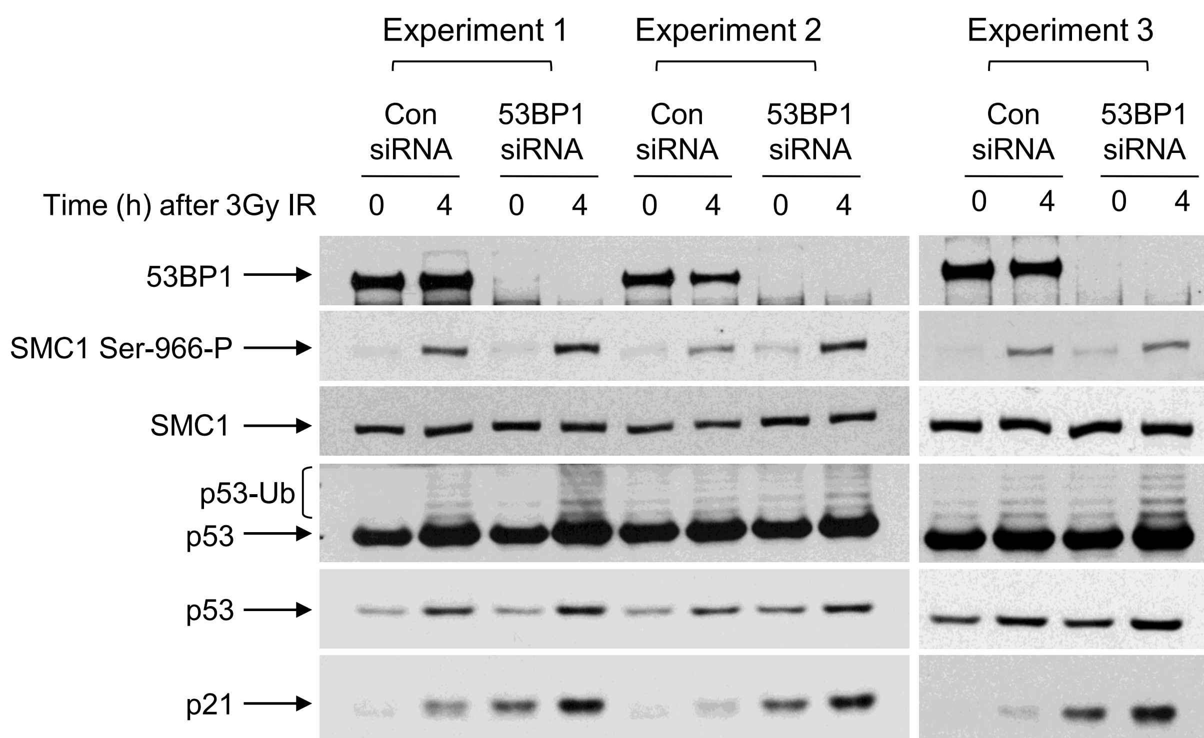


Figure 4.3 ***53BP1 was efficiently depleted using siRNA.*** *U2OS cells were transfected with either control or 53BP1 siRNA. 72 hours later, cells were exposed to 3 Grays of IR and harvested at 0 and 4 hours post-IR. Whole cell lysates were lysed, separated on an SDS-PAGE and immunoblotted with the indicated antibodies. SMC1 was used as a loading control. Data from the three independent experiments is shown.*

down (Figure 4.2 and 4.3). The RNA from each time point was hybridised to an Affymetrix U133 Plus 2.0 GeneChip for gene expression profiling. To ensure the validity of any changes in gene expression, three independent experiments were carried out (Allison *et al* 2006).

4.2.1.1 Univariate analysis of results

Univariate analysis of microarray data examines the expression of individual genes between two conditions and identifies the genes that have the greatest differential expression. Therefore, univariate analysis was used to identify those genes whose expression profiles had differed between the control and 53BP1 siRNA treated samples, and also to determine whether there was any effect of IR exposure.

4.2.1.1.1 Examination of pre-IR transcriptional differences

The baseline transcriptional differences between the control and 53BP1 depleted samples were examined to identify genes that were dependent on 53BP1, but independent of exposure to IR. After the data had been filtered and statistical analysis conducted, the expression profiles of 779 transcripts (674 genes) were found to differ significantly, 505 of these transcripts (435 genes) were differentially down-regulated (Figure 4.4) and 274 transcripts (239 genes) were up-regulated (Figure 4.5) in the 53BP1 depleted samples compared to the control samples. Analysis of the identified differentially regulated genes using GeneSpring highlighted a diverse array of cellular functions that did not specifically localise to any one biological process.

Many of the genes that were down-regulated in the 53BP1 siRNA treated samples functioned in metabolism including *ALDH2*, *GK*, *IDS*, *ME1* and *PGK1*, several genes had roles in transcription such as *ATF1*, *MED18, ZNF589* and *SP4* and other genes included those involved in ubiquitylation and sumoylation (*SH3MD2*, *UCHL5*, *UBE2W*, and *SUMO3*), DNA repair (*RAD23B*), cell cycle (*ESCO2*), and apoptosis (*TNFRSF9* and *AMID*). Genes that were found to be up-regulated in the 53BP1 siRNA treated cells also encode proteins involved in DNA repair such as *XPA*, *REV3L* and *XRCC4*, cell cycle progression including *CHES1*, *STAG2*, *CDKN1B*, and *LATS2*, transcription such as *SAP30*, *NR0B1*, *TC4* and *WWTR1*, as well as proteins involved in ubiquitylation and sumoylation (*PIAS3*, *SENP3*, *USP24*) and the apoptotic and antioxidant protein *TP53INP1*.

Interestingly, a subset of the genes that were differentially regulated in the 53BP1 depleted

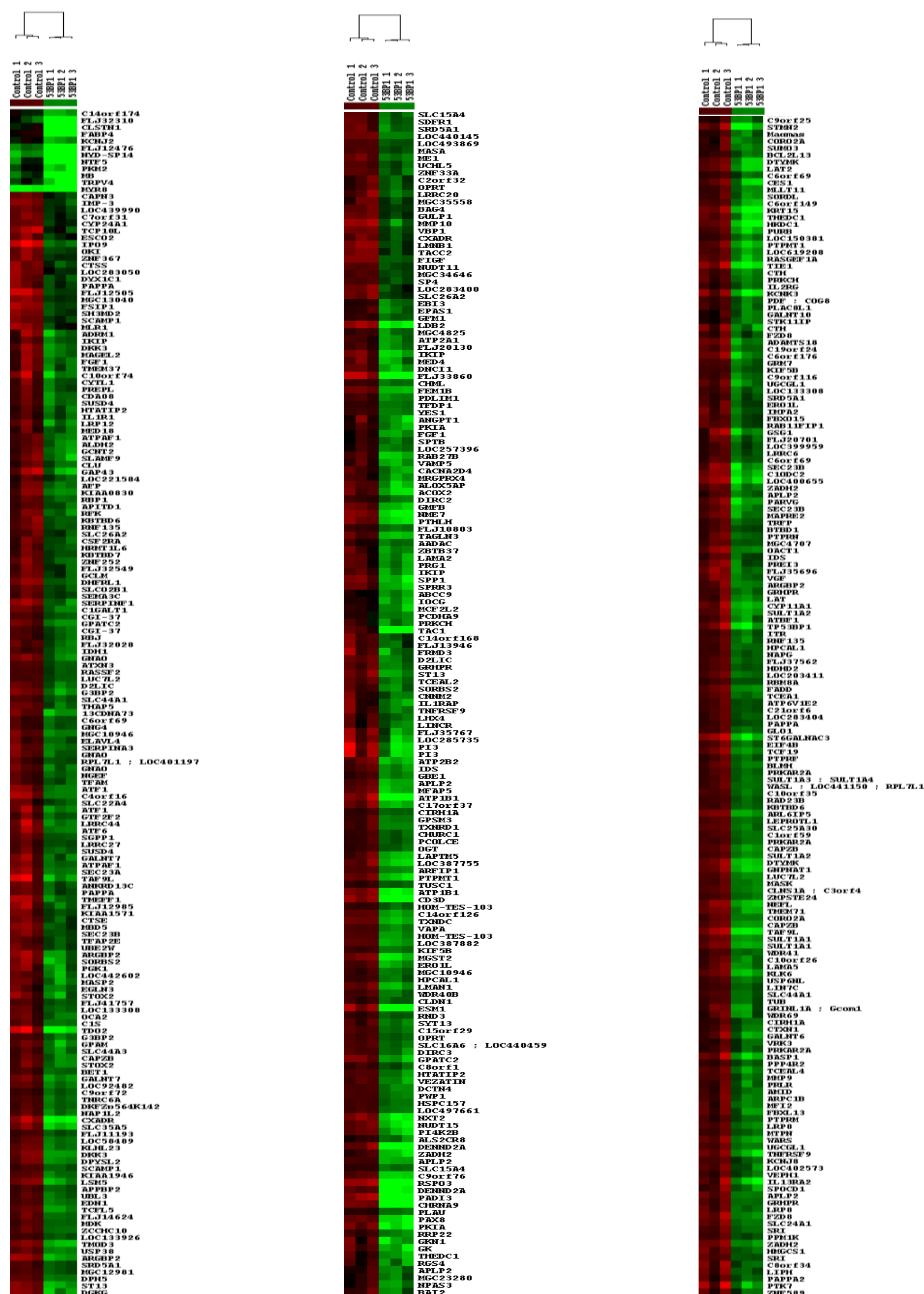


Figure 4.4 ***Heatmaps of genes down-regulated in 53BP1 siRNA treated samples before IR****. Hierarchical clustering of genes was performed using Cluster 3.0 program and heatmaps were generated using Treeview. Columns represent individual samples and rows correspond to genes. Colour changes within a row indicate expression levels. Red indicates up-regulation, green indicates down-regulation*

Figure 4.5 ***Heatmaps of genes up-regulated in 53BP1 siRNA treated samples before IR****. Hierarchical clustering of genes was performed using Cluster 3.0 program and heatmaps were generated using Treeview. Columns represent individual samples and rows correspond to genes. Colour changes within a row indicate expression levels. Red indicates up-regulation, green indicates down-regulation.*

samples compared to control samples encode proteins that have roles in TNFR1 signalling pathways. The expression of *SH3MD2, G3BP2, EDN1, IKIP, FADD, MMP9, BAG4/SODD* and *PLAU* were reduced in cells lacking 53BP1 when compared to cells treated with control siRNA, whereas in contrast, the expression of *PI3KCA, PI3KR2, GAS6, BIRC4/XIAP, AKT2, PIAS3, PAK1, SMAD7, STAT3* and *RPS6KAI/RSK1* were increased (Figure 4.6). Activation of TNFR1 has been demonstrated to occur after a wide variety of stimuli including pro-inflammatory cytokines and genotoxic stress, which induces the activation of the NF-κB, JNK and p38 pathways to promote either an inflammatory response and/or survival depending on the stimuli. Under some circumstances, stimulation of TNFR1 can also induce apoptosis. However, this is not usually observed since the activation of NF-κB induces expression of its anti-apoptotic genes (Chen and Goeddel 2002, Karin and Lin 2002, Van Antwerp *et al* 1996). The majority of the proteins encoded by this subset of genes identified by the microarray analysis that were differentially expressed in 53BP1 knockdown cells were either involved in the NF-κB pathway or in pathways that integrated into the NF-κB pathway, suggesting that 53BP1 may be modulating certain NF-κB-dependent transcriptional responses (Figure 4.7, Table 4.1 and 4.2).

In summary, examination of pre-IR transcriptional differences between control and 53BP1 depleted samples identified a wide variety of genes that were dependent on 53BP1 for their expression. These genes were involved in a broad range of cellular processes, but a significant number of these were involved in metabolism and transcription. However, a small selection of the differentially expressed genes either functioned in signalling pathways that influenced the activity of NF-κB or were NF-κB target genes.

4.2.1.1.2 Analysis of differential responses to DNA damage at 4 hours post-IR

In order to compare the relative expression values between the irradiated samples, the post-IR samples were normalised to the pre-IR equivalent before comparison. This excluded any baseline differences and ensured that only those genes whose expression altered in response to IR were identified. From the analyses, significant differences in the expression of 285 genes were identified between cells treated with control and 53BP1 siRNA. The expression levels of 147 genes were identified as down-regulated and 138 genes were found to be up-regulated in the 53BP1 siRNA treated cells compared to the control siRNA treated cells (Figure 4.8). The

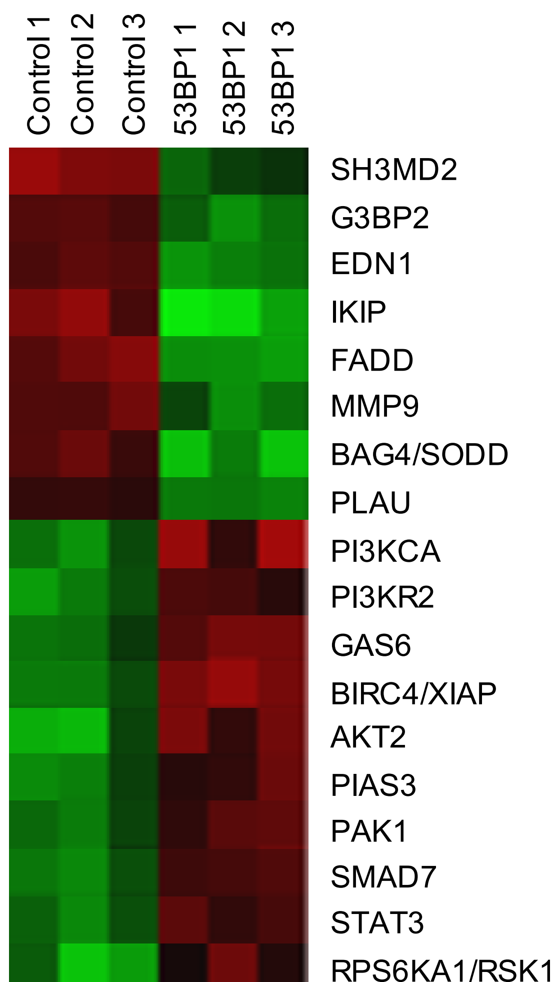


Figure 4.6 ***Subset of genes differentially expressed before IR involved in TNFR1 signalling pathways.*** *Heat map showing the 8 genes that were significantly down-regulated and the 10 genes significantly up-regulated in the 53BP1 depleted samples compared to control samples. Columns represent individual samples and rows correspond to genes. Colour changes within a row indicate expression levels. Red indicates up-regulation, green indicates down-regulation.*

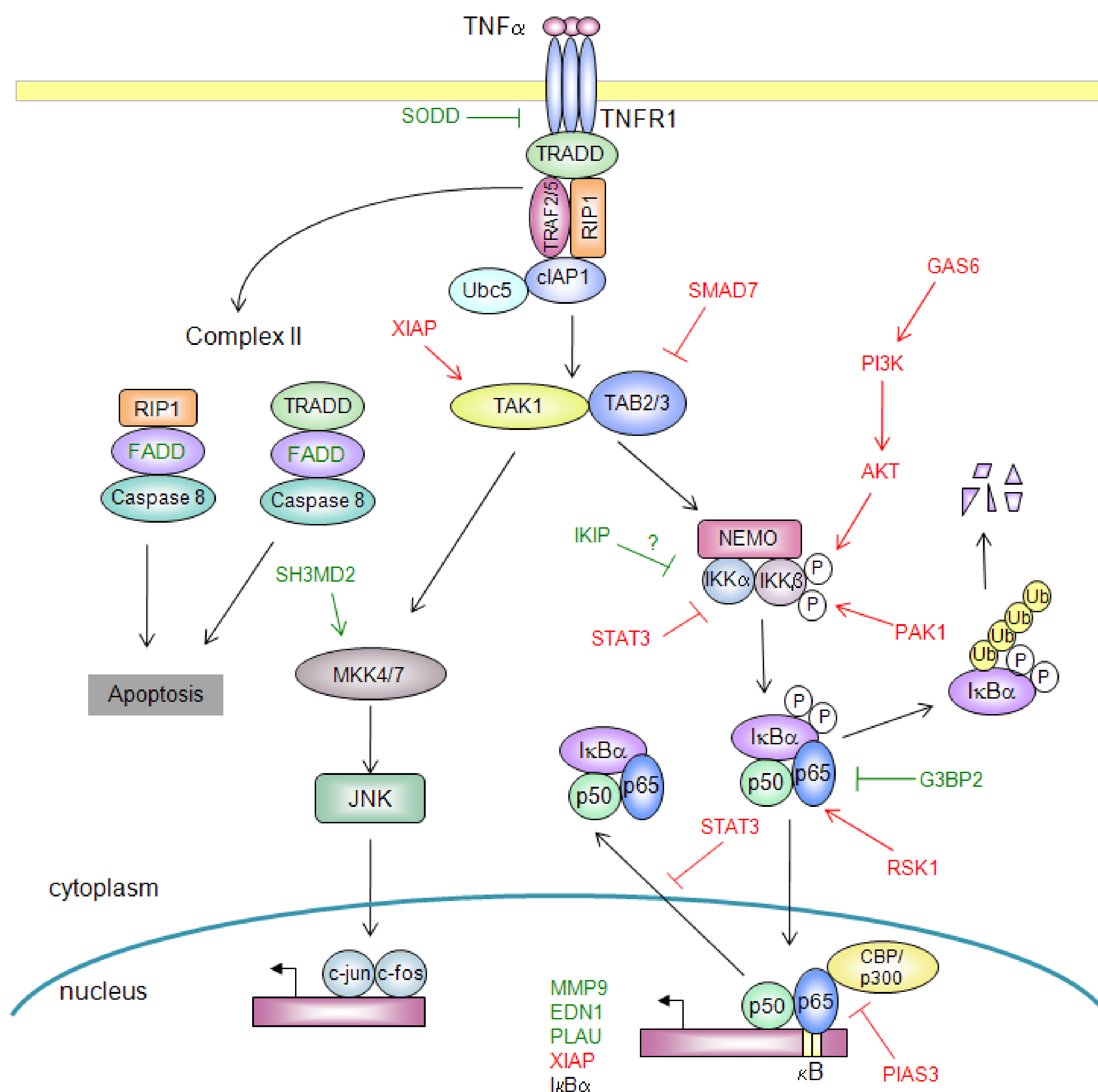


Figure 4.7 ***The involvement of the 18 genes in TNFR1 signalling pathways****. Engagement of TNF with its cognate receptor TNFR1 results in the release of SODD and formation of a proximal signalling complex composed of TRADD, TRAF2 and RIP1. RIP1 recruits the TAK1/TAB2/3 complex thereby promoting activation of the IKK complex. Activated IKK phosphorylates 1κBα at serine 32 and 36 leading to ubiquitylation by the SCF$^{\beta TrCP}$ E3 ligase and subsequent degradation by the 26S proteasome. NF-κB is released and enters the nucleus where it can activate its target genes following various post-translational modifications. 1κBα is resynthesised, dissociates NF-κB from the DNA and exports NF-κB back to the cytoplasm. TNFR1 activate JNK kinase via recruitment of MKK4/7, which activates transcription factors such as AP-1 and ATF2. TNFR1 can also initiate events that lead to apoptosis by forming another complex containing FADD and either RIP1 or TRADD. This activates caspase 8/10, which activates effector caspases triggering apoptosis. The genes highlighted in red are up-regulated whereas the genes in green are down-regulated in the 53BP depleted samples compared to control samples.*

Gene	Role in TNFR1 signalling pathways	Reference
BAG4/SODD	Inhibitor of TNFR1. Binds to the intracellular domains of TNFR1 preventing TNFR1 from being constitutively activated.	(Jiang *et al* 1999)
FADD	Involved in TNFR1-mediated apoptosis. FADD is an adaptor protein that binds to the death domain in TRADD via its death domain resulting in recruitment and activation of procaspase 8/10.	(Micheau and Tschopp 2003)
SH3MD2	E3 ubiquitin ligase that acts as a scaffold protein in the JNK pathway	(Xu *et al* 2003)
PLAU	NF-κB target gene that is important for cell migration as it degrades the extracellular matrix	(Smith and Marshall 2010, Wang *et al* 2000)
MMP9	NF-κB target gene that is important for cell migration as it degrades the extracellular matrix	(Gum *et al* 1996)
EDN1	NF-κB target gene that functions in vascular homeostasis	(Quehenberger et al 2000)
IKIP	Identified in a yeast-two hybrid screen using IKKβ as bait. Function in NF-κB signalling is unknown, however it was found to promote p53-dependent apoptosis suggesting that it may have an inhibitory role in NF-κB because NF-κB and p53 can antagonise each other.	(Ak and Levine 2010, Hofer-Warbinek et al 2004)
G3BP2	Cytoplasmic protein that interacts with IκBα and IκBα/NF-κB complexes through its ability to recognise the cytoplasmic retention sequence in IκBα. Therefore, retains NF-κB in the cytoplasm	(Prigent *et al* 2000)

Table 4.1 ***Genes differentially down-regulated in 53BP1 siRNA treated samples involved in TNFR1 signalling pathways.*** *Table explains the involvement of the proteins encoded by these genes in the TNFR1 signalling pathways.*

Gene	Role in TNFR1 signalling pathways	Reference
BIRC4/XIAP	Anti-apoptotic protein that is not only an NF-κB target gene, but also interacts and activates TAK1 resulting in activation of IKK complex and therefore NF-κB.	(Hofer-Warbinek et al 2000, Stehlik et al 1998)
SMAD7	Inhibitory SMAD of transforming growth factor beta (TGF-β). Recently, been shown to function as negative regulator of NF-κB by binding to TAB2/3 and preventing TAK1 activation. In addition, SMAD7 can inhibit the expression of anti-apoptotic NF-κB target genes and therefore sensitise cells to TNF-induced apoptosis	(Hong *et al* 2007a, Hong *et al* 2007b)
PI3KCA, PI3KR2 and AKT2	PI3KCA (also known as p110α) and PI3KR2 (also known as p85β) are the catalytic and regulatory subunits of PI3K respectively. Activation of PI3K by receptor tyrosine kinases results in recruitment and subsequent activation of AKT. AKT can stimulate NF-κB transcriptional activity, although this has been reported to occur by different mechanisms. One mechanism involves the stimulation of the transactivation potential of p65 via activation of IKKβ. In contrast, other reports have shown that AKT activates NF-κB through activation of IKKα rather than IKKβ	(Vanhaesebroeck et al 2010, Vivanco and Sawyers 2002, Madrid *et al* 2001, Sizemore et al 1999, Ozes *et al* 1999, Romashkova and Makarov 1999)
GAS6	Anti-apoptotic protein. Activates NF-κB via the PI3K pathway.	(Demarchi *et al* 2001)
PAK1	PAK1 is a serine/threonine protein kinase activated by multiple signalling pathways. Activates NF-κB through IKKβ.	(Foryst-Ludwig and Naumann 2000, Frost *et al* 2000)
RPS6KA1/RSK1	Serine/threonine protein kinase that can stimulate NF-κB activity by phosphorylating IκBα on Ser32 and inducing its degradation. In response to DNA damage RSK1 is phosphorylated by p53 resulting in activation of pro-apoptotic NF-κB response via an IKK and IκBα independent mechanism.	(Ghoda *et al* 1997, Schouten *et al* 1997, Bohuslav *et al* 2004)
STAT3	Interacts with NF-κB at several levels in response to inflammation. Can inhibit IKK activity and thereby reduce NF-κB inflammatory response. In tumours, STAT3 directly interacts with p65 preventing nuclear export of NF-κB and contributing to constitutive NF-κB activation. Several NF-κB target gens such as interleukin 6 are important activators of STAT3	(Welte *et al* 2003, Lee *et al* 2009, Yu *et al* 2009)
PIAS3	Represses NF-κB activity by interacting with the N-terminal region of p65 and preventing CBP and p300 from binding to p65.	(Jang *et al* 2004)

Table 4.2 ***Genes differentially up-regulated in 53BP1 siRNA treated samples involved in TNFR1 signalling pathways.*** *Table explains the involvement of the proteins encoded by these genes in the TNFR1 signalling pathways.*

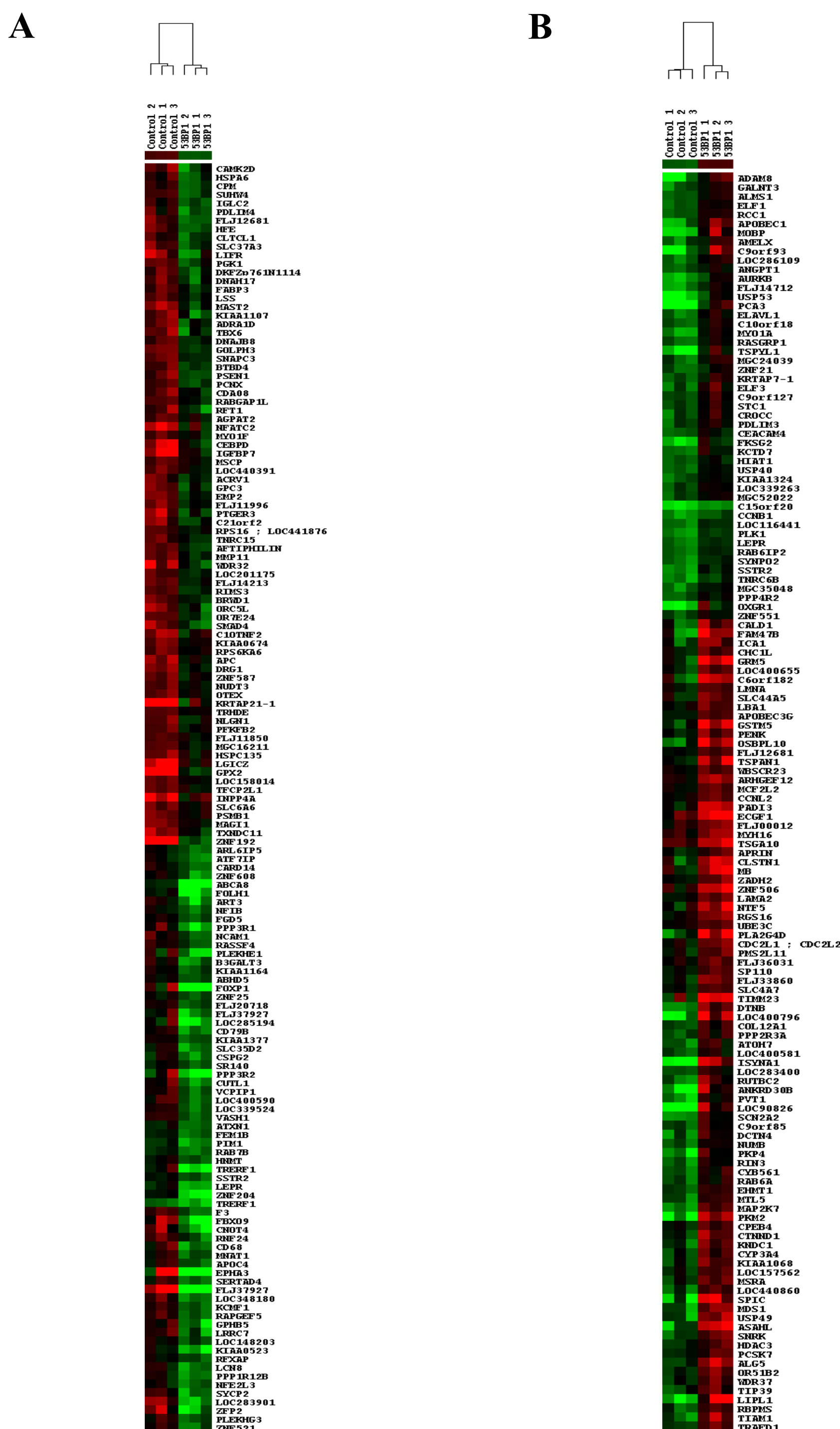


Figure 4.8 ***Heatmaps of genes differentially expressed in response to IR****. Hierarchical clustering of genes was performed using Cluster 3.0 program and heatmaps were generated using Treeview. (A) Genes significantly down-regulated in 53BP1 siRNA treated samples compared to control siRNA treated samples post-IR. (B) Genes significantly up-regulated in 53BP1 siRNA treated samples compared to control siRNA treated samples post-IR. Columns represent individual samples and rows correspond to genes. Colour changes within a row indicate expression levels. Red indicates up-regulation, green indicates down-regulation.*

function of these genes again varied with many of them being involved in metabolism such as *GPX2*, *PGK1*, *PKM2* and *ECGF1*, transcriptional regulation including *C/EBPδ, CUX1, FOXP1* and *ELF1* and also cell cycle regulation, for example *AURKB*, *CCNB1*, *PLK1* and *CROCC*.

In conclusion, there were a large variety of genes whose expression was affected in response to IR and these were mainly involved in metabolism and transcription, although several of the genes that were up-regulated in the 53BP1 depleted samples were involved in cell cycle progression suggesting that the cell cycle could be affected in response to IR when 53BP1 is depleted. Surprisingly, despite IR being a potent trigger of p53 transcriptional activity and data presented in chapter 3 indicating that 53BP1 could be regulating p53 target gene expression in response to DNA damage, no p53 responsive genes were found to be differentially regulated by 53BP1 following IR exposure. This suggests that 53BP1 may not be playing a role in modulating the expression of p53 target genes induced 4 hours after DNA damage. However, it is possible that 53BP1 may regulate the expression of p53 target genes activated at other times following DNA damage.

4.2.1.2 Multivariate analysis of results

Multivariate analysis is designed to assess the impact that multiple gene expression changes may have on single biological pathways and processes by taking into account interactions between functionally related genes, since small coordinated changes in gene expression within a pathway can have a major biological effect even if these changes are not significant for any individual gene. This method of analysis was used to identify any biological pathways that may be affected by depletion of 53BP1 before and after exposure to IR.

Gene Set Enrichment Analysis (GSEA) (Subramanian *et al* 2005) is an analytical method that focuses on expression changes within 'gene sets' rather than on individual genes. The gene sets used in this study are groups of genes that share a similar biological function i.e. those that function in, or act on, the same cellular signalling pathways. The GSEA software compares samples from two distinct classes (in this analysis these were control siRNA treated samples and 53BP1 siRNA treated samples) using genome wide expression profiles and ranks the genes within each gene set according to the differences in expression between the two classes. An enrichment score is then calculated, which reflects the level at which a gene set is over-represented at the top or bottom of the ranked gene list. Results are considered

significant on the basis of a false discovery rate (FDR) of less than 0.25, or on a p-value lower than 0.05. The FDR is slightly more stringent, so was used as the main gauge of significance in this analysis. An FDR indicates the likelihood that pathways identified have been chosen by chance with an FDR score of <0.25 meaning that less than one in four pathways is a false positive result.

4.2.1.2.1 GSEA analysis of differentially expressed pathways before IR.

247 gene sets were used for the analysis, 128 of which were found to be up-regulated in the control samples compared to the 53BP1 depleted samples. Based on the FDR score, 8 of these pathways were significantly up-regulated in the control siRNA treated samples compared to the 53BP1 siRNA treated samples. These pathways were involved in metabolism, highlighted in blue in the table and TNF/NF-κB signalling highlighted in yellow in the table (Table 4.3). In addition, several pathways had significant p-values but not FDR scores and some of these pathways were linked to the pathways that had significant FDR values (Table 4.3). In comparison, 119 genes sets were identified as being up-regulated in the 53BP1 siRNA treated samples compared to control samples, although none of the gene sets were found to have an FDR score of <0.25. However, 18 gene sets had significant p-values <0.05 and were predominantly involved in cell cycle progression and DNA damage response as shown in red in the table, transcription as highlighted in green and PI3K signalling shown in purple in the table (Table 4.4).

Consistent with the univariate analysis, the multivariate analysis highlighted that the TNFR1 signalling pathways were being affected before IR. In contrast to this, some of the pathways up-regulated in the absence of 53BP1 were linked to the cell cycle and DNA damage response suggesting that loss of 53BP1 is affecting the expression of genes involved in the DNA damage response and cell cycle arrest.

4.2.1.2.2 GSEA analysis of differentially expressed pathways at 4 hours post IR

As with the univariate analysis, the post-IR samples were normalised to their pre-IR equivalents to identify those pathways, which were differentially regulated between control and 53BP1 depleted cells in response to IR. This makes the expression values relative to 1 and therefore, any pathways identified are due to responses to IR and not due to pre-IR expression levels.

	GS	Size	NOM p-val	FDR q-val
1	MAP00620_PYRUVATE_METABOLISM	18	0.002	0.148
2	VALINE_LEUCINE_AND_ISOLEUCINE_ DEGRADATION	17	0.000	0.138
3	NFKB_INDUCED	89	0.002	0.189
4	BUTANOATE_METABOLISM	15	0.003	0.189
5	ELECTRON_TRANSPORTER_ACTIVITY	88	0.001	0.143
6	STARCH_AND_SUCROSE_METABOLISM	17	0.016	0.139
7	TNFR1PATHWAY	21	0.01	0.209
8	ANDROGEN_AND_ESTROGEN_METABOLISM	15	0.011	0.198
9	GLYCOLYSIS_GLUCONEOGENESIS	34	0.015	0.285
10	CELL_SURFACE_RECEPTOR_LINKED_SIGNAL_ TRANSDUCTION	118	0.002	0.258
11	BILE_ACID_BIOSYNTHESIS	17	0.027	0.310
12	NFKBPATHWAY	17	0.037	0.311
13	TUMOUR_NECROSIS_FACTOR_PATHWAY	22	0.035	0.301
14	IL17PATHWAY	15	0.056	0.287
15	FASPATHWAY	21	0.048	0.346
16	EMT_UP	43	0.047	0.326
17	FATTY_ACID_METABOLISM	36	0.046	0.322
18	VIPPATHWAY	21	0.058	0.317
19	COMPPATHWAY	16	0.073	0.331
20	INFLAMMATORY-RESPONSE-PATHWAY	24	0.048	0.323
21	NFKB_REDUCED	18	0.078	0.373
22	TRYTOPHAN_METABOLISM	39	0.054	0.395
23	CSKPATHWAY	21	0.096	0.436
24	AMIPATHWAY	21	0.104	0.453
25	NITROGEN_METABOLISM	18	0.102	0.442

Table 4.3 ***GSEA analysis shows TNFR1 signalling pathways were differentially up-regulated in control compared to 53BP1 depleted cells****. Analysis of the pathways before IR revealed that multiple pathways related to metabolism (highlighted in blue) were significantly over-expressesd in control cells along with 4 pathways associated with TNFR1 and the transcription factor NF-kB (highlighted in yellow). NF-kB INDUCED and TNFR1 PATHWAY were significant at FDR <25%, whereas NF-kB PATHWAY and TUMOUR NECROSIS FACTOR PATHWAY had significant p-values (<0.05). The pathways have been ranked in order of their normalised enrichment score.*

	GS	Size	NOM p-val	FDR q-val
1	CELL_CYCLE	55	0.003	0.922
2	G2PATHWAY	15	0.013	0.505
3	MEF2DPATHWAY	15	0.020	0.462
4	EIF4PATHWAY	23	0.022	0.474
5	HDACPATHWAY	26	0.029	0.503
6	RAC1PATHWAY	15	0.041	0.548
7	PGC1APATHWAY	18	0.044	0.472
8	CARM-ERPATHWAY	20	0.049	0.433
9	PPARAPATHWAY	45	0.025	0.385
10	HTERT_DOWN	49	0.028	0.426
11	MTORPATHWAY	20	0.048	0.406
12	CR_CELL_CYCLE	63	0.022	0.395
13	ST_DIFFERENTIATION_PATHWAY_IN_PC12_CELLS	39	0.028	0.409
14	G1PATHWAY	25	0.051	0.429
15	ERKPATHWAY	26	0.064	0.407
16	GLUT_DOWN	162	0.012	0.384
17	DNA_DAMAGE_SIGNALLING	63	0.023	0.384
18	GSK3PATHWAY	19	0.083	0.370
19	ECMPATHWAY	19	0.083	0.399
20	LEU_DOWN	93	0.019	0.395
21	RACCYCDPATHWAY	20	0.108	0.410
22	SIG_PIP3SIGINCARDIACMYOCTES	50	0.033	0.401
23	PAR1PATHWAY	19	0.069	0.397
24	INSULIN_SIGNALLING	79	0.033	0.414
25	SIG_INSULINRECEPTORPATHWAYINCARDIOMYOCTES	40	0.080	0.413

Table 4.4 ***GSEA pathways up-regulated in 53BP1 siRNA treated cells compared to control siRNA treated cells.*** *Analysis of pathways before IR revealed up-regulation of cell cycle and DNA damage response pathways (highlighted in red) in the absence of 53BP1, as well as PI3K signalling pathways (highlighted in purple) and transcriptional regulation pathways (highlighted in green). Although, none of the pathways were significant at FDR <25%, they all had significant p-values (<0.05). The pathways have been ranked in order of their normalised enrichment score.*

Following exposure to IR, 113 out of the 247 gene sets used in this analysis were found to be up-regulated in the control cells compared to 53BP1 depleted cells. However, none of the gene sets had significant FDR scores of <0.25 and only 1 gene set had a significant p-value (<0.05), which was the GSK3 pathway, which is highlighted in blue in the table (Table 4.5). 134 out of 247 gene sets were up-regulated in the 53BP1 depleted cells compared to control cells in response to IR. Again there were no pathways that had a significant FDR value, but there were 6 pathways that had significant p-values of <0.05. The majority of these were metabolism pathways highlighted in orange in the table, although the PI3K pathway was amongst these, as shown in green in the table (Table 4.6). Overall in response to IR, there were only a few pathways that were significantly differentially regulated between the control and 53BP1 siRNA treated samples.

4.2.1.3 DNA damage response was activated by IR

Due to the lack of genes involved in the DNA damage and p53 responses being identified by the microarray analysis following IR, the irradiated control and 53BP1 depleted samples were compared with their unirradiated equivalents (i.e. the control 4 hours IR samples were compared with control 0 hours IR samples and the same comparison was conducted for the 53BP1 knockdown samples) to ensure that a DNA damage response had been activated in response to IR. Identification of any genes that were known to be involved in the DNA damage response or were p53 responsive genes, as well as any pathways that were related to the DNA damage response or p53 signalling would confirm that a DNA damage response had occurred following IR.

Univariate analysis identified several DNA damage response associated changes in gene expression in both control and 53BP1 deficient cells with p53 dependent genes such as CDKN1A, BTG2, FAS, MDM2, ATF3 and SESN1 being up-regulated in both wild type and 53BP1 depleted cells in response to IR. Multivariate analysis of the irradiated and unirradiated control samples revealed that 171 of the 247 gene sets used for this analysis were up-regulated in response to IR. Out of these, DNA damage response and p53 pathways highlighted in blue were significantly up-regulated based on an FDR score <0.25. An additional p53 pathway that was not significant by FDR score, but had a significant p-value was also up-regulated following IR exposure (Table 4.7). Analysis of the 53BP1 depleted unirradiated and irradiated samples showed that out of the 247 gene sets used for this analysis,

	GS	Size	NOM p-val	FDR q-val
1	GSK3PATHWAY	19	0.034	1.000
2	CHREBPATHWAY	18	0.051	1.000
3	NKTPATHWAY	24	0.062	1.000
4	TUMOR_SUPPRESSOR	18	0.075	1.000
5	PGC1PATHWAY	18	0.097	1.000
6	WNT_SIGNALING	49	0.063	1.000
7	GLUCOSE_UP	31	0.078	1.000
8	ELECTRON_TRANSPORT_CHAIN	48	0.053	1.000
9	ANDROGEN_UP_GENES	46	0.116	1.000
10	WNTPATHWAY	22	0.135	1.000
11	AR_MOUSE_PLUS_TESTO_FROM_NETAFFIX	50	0.124	1.000
12	HTERT_UP	70	0.123	1.000
13	SIG_CHEMOTAXIS	33	0.179	1.000
14	GLUT_DOWN	162	0.081	1.000
15	ST_WNT_BETA_CATENIN_PATHWAY	25	0.188	1.000
16	LEU_UP	87	0.109	1.000
17	FETAL_LIVER_HS_ENRICHED_TF_JP	60	0.149	1.000
18	NKCELLSPATHWAY	15	0.217	1.000
19	CELL_GROWTH_AND_OR_MAINTENANCE	53	0.190	1.000
20	INFLAMPATHWAY	27	0.205	1.000
21	FRUCTOSE_AND_MANNOSE_METABOLISM	16	0.225	1.000
22	LEU_DOWN	93	0.149	1.000
23	MTORPATHWAY	20	0.244	1.000
24	RAC1PATHWAY	15	0.263	1.000
25	ELECTRON_TRANSPORTER_ACTIVITY	88	0.224	1.000

Table 4.5 **GSEA pathways up-regulated in control cells compared to 53BP1 depleted cells in response to IR.** *Analysis of pathways post-IR revealed significant up-regulation of GSK3 PATHWAY in presence of 53BP1 (highlighted in blue). Although, none of the pathways were significant at FDR <25%, this pathway had a significant p-value (<0.05).*

	GS	Size	NOM p-val	FDR q-val
1	PYRUVATE_METABOLISM	18	0.014	0.796
2	CERAMIDEPATHWAY	18	0.004	0.589
3	GLYCOLYSIS_GLUCONEOGENESIS	34	0.006	0.801
4	ST_G_ALPHA_I_PATHWAY	30	0.030	0.861
5	ST_PHOSPHOINOSITIDE_3_KINASE_PATHWAY	25	0.028	0.712
6	NO1PATHWAY	23	0.042	0.650
7	BCL2FAMILY_AND_REG_NETWORK	16	0.084	1.000
8	ANDROGEN_AND_ESTROGEN_METABOLISM	15	0.099	1.000
9	SHH_LISA	16	0.110	1.000
10	GPCRS_CLASS_A_RHODOPSIN-LIKE	129	0.054	1.000
11	CELL_CYCLE_ARREST	25	0.112	1.000
12	CHEMICALPATHWAY	15	0.147	1.000
13	P53_SIGNALING	74	0.070	1.000
14	PURINE_METABOLISM	60	0.098	1.000
15	BILE_ACID_BIOSTNTHESIS	17	0.165	1.000
16	S1P_SIGNALING	18	0.122	1.000
17	FATTY_ACID_METABOLISM	36	0.134	1.000
18	ST_GA12_PATHWAY	19	0.157	1.000
19	SPRYPATHWAY	17	0.195	1.000
20	ECMPATHWAY	19	0.171	1.000
21	INTEGRINPATHWAY	27	0.176	1.000
22	ARGININE_AND_PROLINE_METABOLISM	25	0.179	0.967
23	NITROGEN_METABOLISM	18	0.197	0.946
24	STARCH_AND_SUCROSE_METABOLISM	17	0.192	0.907
25	SIG_IL4RECEPTOR_IN_B_LYPHOCYTES	21	0.211	0.944

Table 4.6 ***GSEA pathways up-regulated in 53BP1 siRNA treated cells compared to control siRNA treated cells in response to IR.*** *Analysis of pathways post-IR revealed significant up-regulation of metabolism pathways (highlighted in orange) and the PI3K pathway (highlighted in green) in the absence of 53BP1. Although, none of the pathways were significant at FDR <25%, they had significant p-values (<0.05).*

	GS	Size	NOM p-val	FDR q-val
1	P53_UP	30	0.000	0.004
2	DNA-DAMAGE-SIGNALLING	63	0.001	0.125
3	IL1RPATHWAY	23	0.011	0.438
4	NKTPATHWAY	24	0.013	0.424
5	ST_GA13_PATHWAY	27	0.012	0.390
6	P53_SIGNALLING	74	0.001	0.363
7	MRNA_SPLICING	24	0.027	0.690
8	GSK3PATHWAY	19	0.038	0.672
9	SIG_CHEMOTAXIS	33	0.021	0.599
10	WNTPATHWAY	22	0.039	0.550
11	CR_DNA_MET_AND_MOD	18	0.047	0.537
12	TOLLPATHWAY	26	0.068	0.751
13	IL12PATHWAY	18	0.082	0.718
14	INFLAMPATHWAY	27	0.058	0.679
15	KREBS-TCA_CYCLE	17	0.054	0.690
16	CHREBPPATHWAY	18	0.089	0.700
17	GATA1_WEISS	18	0.091	0.744
18	P38MAPKPATHWAY	32	0.078	0.704
19	HTERT_UP	70	0.051	0.699
20	ST_GAQ_PATHWAY	20	0.101	0.686
21	CR_IMMUNE_FUNCTION	46	0.065	0.701
22	DEATHPATHWAY	26	0.106	0.675
23	NTHIPATHWAY	18	0.113	0.649
24	CR_CAM	86	0.051	0.640
25	ST_WNT_BETA_CATENIN_PATHWAY	25	0.117	0.665

Table 4.7 ***GSEA pathways up-regulated in control siRNA treated cells in response to IR.*** *Analysis of pathways between unirradiated and irradiated control samples revealed significant up-regulation of DNA damage response pathways in response to IR (highlighted in blue). p53 UP and DNA DAMAGE SIGNALLING pathways were significant at FDR <0.25, whereas p53 SIGNALLING pathway had a significant p-value (<0.05).*

	GS	Size	NOM p-val	FDR q-val
1	P53_UP	30	0.000	0.001
2	P53_SIGNALLING	74	0.000	0.002
3	DNA-DAMAGE-SIGNALLING	63	0.000	0.049
4	ARAPPATHWAY	18	0.006	0.208
5	ST_GA13_PATHWAY	27	0.013	0.320
6	ELECTRON_TRANSPORT	61	0.004	0.377
7	ANDROGEN_AND_ESTROGEN_METABOLISM	15	0.037	0.658
8	CELL_PROLIFERATION	170	0.002	0.592
9	RADIATION_SENSITIVITY	22	0.031	0.527
10	ANDROGEN_GENES_FROM_NETAFFIX	48	0.018	0.499
11	DRUG_RESISTANCE_AND_METABOLISM	78	0.013	0.497
12	GLYCEROLIPID_METABOLISM	35	0.035	0.556
13	CR_DNA_MET_AND_MOD	18	0.044	0.532
14	TRYPTOPHAN_METABOLISM	39	0.032	0.532
15	INSULIN_2F_DOWN	29	0.041	0.563
16	NTHIPATHWAY	18	0.061	0.553
17	KERATINOCYTEPATHWAY	35	0.051	0.564
18	STARCH_AND_SUCROSE_METABOLISM	17	0.060	0.540
19	HISTIDINE_METABOLISM	15	0.092	0.579
20	BCL2FAMILY_AND_REG_NETWORK	16	0.076	0.556
21	NO1PATHWAY	23	0.072	0.530
22	GO_0005739	100	0.032	0.586
23	NFKB_INDUCED	89	0.046	0.563
24	SIG_PIP3_SIGNALING_IN_B_LYMPHOCYTES	27	0.086	0.541
25	ST_PHOSPHOINOSITIDE_3_KINASE_PATHWAY	25	0.091	0.570

Table 4.8 ***GSEA pathways up-regulated in 53BP1 siRNA treated cells in response to IR.*** *Analysis of pathways between unirradiated and irradiated 53BP1 depleted samples revealed significant up-regulation of p53 and DNA damage response pathways in response to IR (highlighted in purple). p53 UP and p53 SIGNALLING pathways were significant at FDR <0.25, whereas DNA DAMAGE SIGNALLING, CELL PROLIFERATION AND RADIATION SENSITIVITY pathways had significant p-values (<0.05).*

169 were up-regulated in response to IR. Consistent with the control samples, p53 and DNA damage response were found to be significantly up-regulated in response to IR in cells lacking 53BP1 as highlighted in purple in Table 4.8.

Taken together the univariate and multivariate analyses both demonstrate that a DNA damage response was activated by IR in the control samples and in the 53BP1 depleted samples.

4.2.1.4 Gene expression profiling data was validated by qRT-PCR and Western blot analysis

Univariate analysis indicated a large number of genes that were differentially expressed between control and 53BP1 siRNA treated cells both before and after IR. To confirm the differences identified by the univariate analysis, 20 genes were selected to be verified at the mRNA level by qRT-PCR. These genes were chosen because they had a relative fold change in expression of >2 and they were of functional interest. Genes that were functionally interesting were those involved in TNFR1 signalling pathways, cell cycle, apoptosis, DNA damage response and/or ubiquitylation (Table 4.9 and 4.10). To ensure the responses identified by the microarray were real, RNA that had not being used for the microarray was used for qRT-PCR. Figure 4.9 demonstrates that the qRT-PCR data confirmed the microarray data at the mRNA level, as the alterations in mRNA levels for each gene correlated with the differences in gene expression identified by the microarray analysis, although the differences for some genes identified by the univariate analysis as being more than 2 fold between control and 53BP1 depleted cells were found to be lower than expected such as *ESCO2* and *GAS6*.

To determine if the transcriptional changes could be translated into altered protein levels, five genes were chosen from the qRT-PCR results to be verified at the protein level. These genes were *FADD*, *BAG4/SODD, IKIP1*, *G3BP2* and *BIRC4*/*XIAP*, which have all been shown to be involved in the TNFR1 signalling pathways (Figure 4.7). U2OS cells were treated with either control or 53BP1 siRNA, exposed to 3 Grays of IR and then the protein levels of the above genes were assessed by Western blot analysis. The levels of FADD, BAG4/SODD, IKIP1 and G3BP1 protein were decreased in the 53BP1 depleted cells compared to control whereas BIRC4/XIAP protein levels were increased in 53BP1 siRNA treated cells compared to control prior to IR. The levels of all 5 proteins were unaffected following IR exposure (Figure 4.10). These data are in agreement with the qRT-PCR data and demonstrate that the changes in protein expression for each gene correlated with the changes in gene expression identified

Gene Name	Description	Biological Function	Fold Difference
BAG4/SODD	BCL2-associated athanogene 4	Inhibitor of TNFR1. Apoptosis. anti-apoptosis	2.05
TNFRSF9/ CD137/4-1BB	tumour necrosis factor receptor superfamily, member 9	Apoptosis, immune response	4.243, 2.584
FADD	Fas (TNFRSF6)-associated via death domain	cell surface receptor linked signal transduction, apoptosis via death domain receptors, positive regulation of I-kappaB kinase/NF-kappaB cascade	3.175
IKIP	IKK interacting protein	Apoptosis, regulation of NF-κB cascade	4.219, 3.076, 2.33
G3BP2	Ras-GTPase activating protein SH3 domain-binding protein 2	mRNA processing, mRNA export from nucleus, cytoplasmic sequestering of NF-kappaB, Ras protein signal transduction	3.427, 2.349
LMNB1	lamin B1	Nuclear structure, mitosis, apoptosis	2.632
UBE2W	ubiquitin-conjugating enzyme E2W (putative)	regulation of transcription, ubiquitin cycle	2.18
RAD23B	RAD23 homolog B (S. cerevisiae)	nucleotide-excision repair , response to DNA damage stimulus	2.119
SH3MD2/ POSH	SH3 multiple domains 2	protein ubiquitination, JNK signalling cascade	2.462
ESCO2	establishment of cohesion 1 homolog 2 (S. cerevisiae)	cell cycle	2.068
UCHL5	ubiquitin carboxyl-terminal hydrolase L5	ubiquitin cycle	2.217
SUMO3	SMT3 suppressor of mif two 3 homolog 3 (yeast)	ubiquitin cycle	2.503

Table 4.9 ***Candidate genes chosen for validation by qRT-PCR.*** *These genes were significantly down-regulated in the 53BP1 depleted samples compared to control samples. The fold differences highlighted were from the univariate analysis.*

Gene Name	Description	GO Biological Function	Fold Difference
BIRC4/XIAP	baculoviral IAP repeat-containing 4	anti-apoptosis, protein ubiquitination,	-2.806
XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4	DNA repair	-2.05
CHES1	checkpoint suppressor 1	DNA damage checkpoint, G2 phase of mitotic cell cycle	-2.631, -2.013
CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	regulation of cyclin dependent protein kinase activity, cell cycle arrest	-2.598
GAS6	growth arrest-specific 6	regulation of cell growth	-2.367
STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)	negative regulation of transcription from RNA polymerase II promoter , cell motility, JAK-STAT cascade	-2.093
REV3L	REV3-like, catalytic subunit of DNA polymerase zeta (yeast)	DNA replication, DNA repair	-2.848
TP53INP1	tumor protein p53 inducible nuclear protein 1	apoptosis	-4.697
XPA	xeroderma pigmentosum, complementation group A	nucleotide-excision repair	-2.014

Table 4.10 ***Candidate genes chosen for validation by qRT-PCR.*** *These genes were significantly up-regulated in the 53BP1 knockdown samples compared to control samples. The fold differences highlighted were from the univariate analysis.*

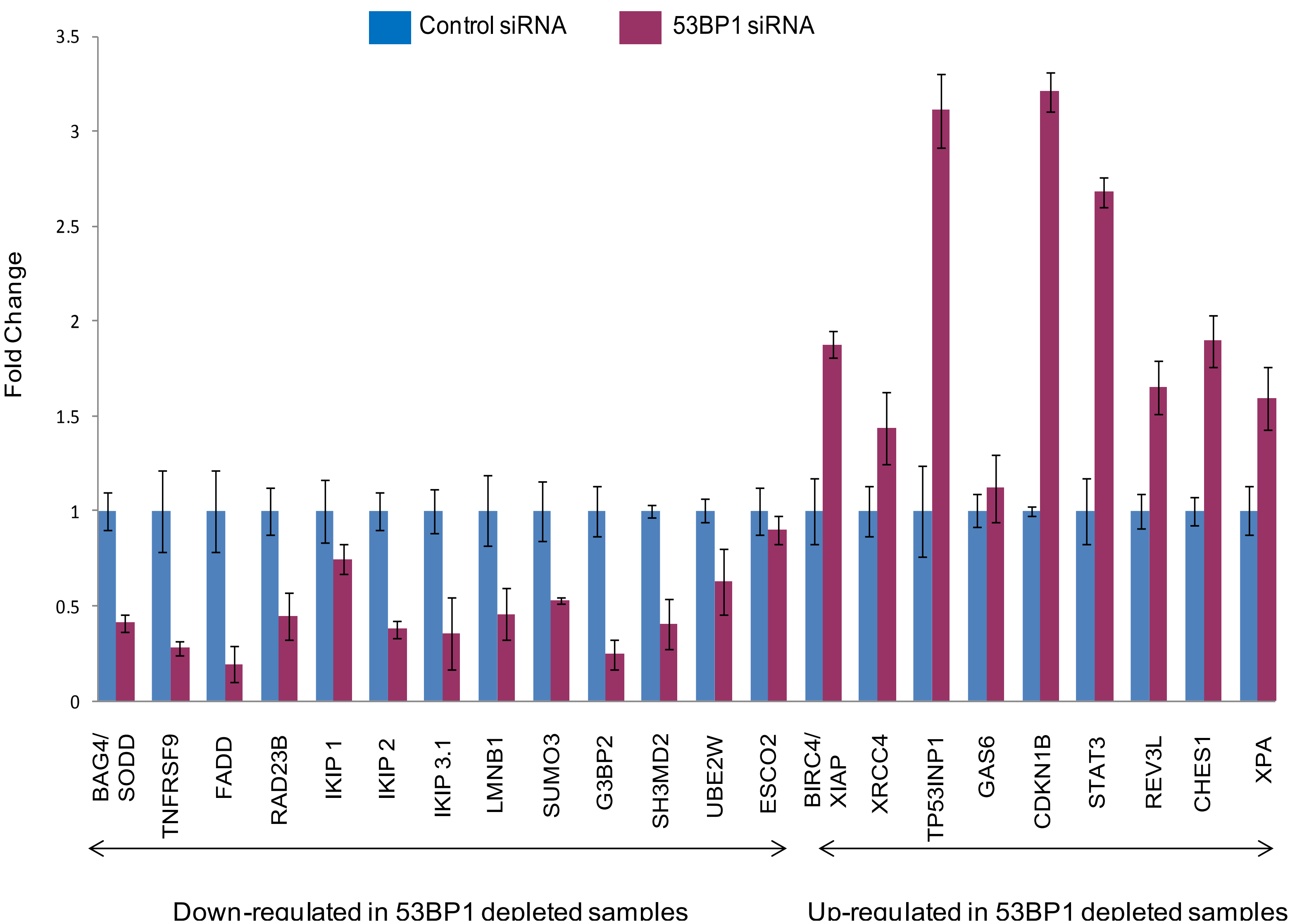


Figure 4.9 ***Changes in gene expression were confirmed at the mRNA level by qRT-PCR***. *RNA was extracted from unirradiated cells treated with control or 53BP1 siRNA, reverse transcribed into cDNA and the mRNA levels of each of the genes was quantified using qRT-PCR. Transcript levels were normalised to the 18S mRNA levels to determine relative expression levels. Data is represented as fold change where the unirradiated control sample was set to 1. All results are a mean of three separate experiments ± standard deviation.*

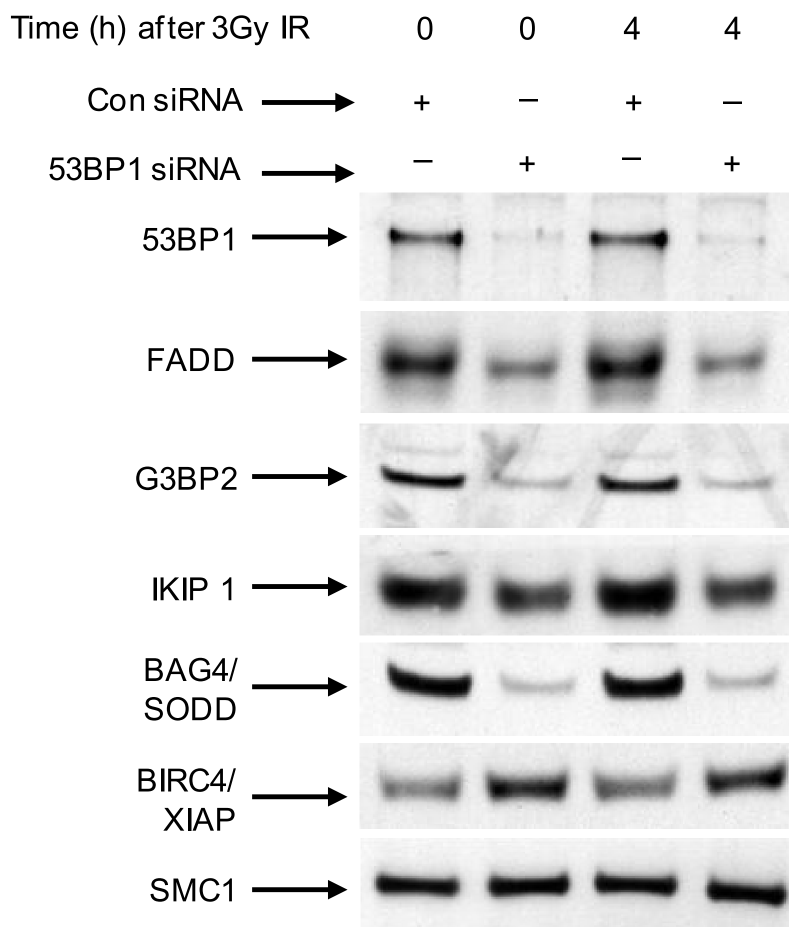


Figure 4.10 ***Changes in gene expression were confirmed at the protein level by Western blotting****. U2OS cells were transfected with either control or 53BP1 siRNA. 72 hours later, cells were either mock-irradiated or irradiated with 3 Grays of IR and harvested at 4 hours post-IR. Whole cell lysates were separated on an SDS-PAGE gel and Western blots were probed for 53BP1, FADD, G3BP2, IKIP1, BAG4/SODD and BIRC4/XIAP. SMC1 was used as a loading control.*

by the microarray analysis.

Together these data confirmed the results from the microarray analysis and also demonstrated that in the case of FADD, BAG4/SODD, IKIP, G3BP2 and BIRC4/XIAP, the changes in gene expression observed from the microarray could be translated into changes at the protein level.

4.3 DISCUSSION

In conclusion, the microarray data analysis revealed that there were significant differences in the expression of single genes and biological pathways between the control and 53BP1 depleted samples before IR. Interestingly, both types of analysis showed that before DNA damage, TNFR1-induced intracellular signalling pathways, in particular the NF-κB pathway, as well as pathways that are linked with NF-κB such as the PI3K pathway were affected suggesting that 53BP1 may be involved in modulating NF-κB signalling (Figure 4.6, 4.7 and Table 4.3). Currently there are no reports of 53BP1 playing a role in regulating NF-κB activity. However, another DNA damage response protein, ATM has been shown to be important for activating NF-κB in response to a variety of DNA damaging agents including IR, camptothecin, etoposide and doxorubicin (Wu *et al* 2006b). In addition to NF-κB pathways, pathways induced by PI3K, were up-regulated in cells lacking 53BP1 (Table 4.4). Both NF-κB and PI3K are pro-survival factors, therefore it is possible that 53BP1 may be influencing cell survival (Karin and Lin 2002, Kennedy *et al* 1997). Moreover, the univariate analysis identified four NF-κB target genes, three of which were down-regulated in 53BP1 depleted cells and one which was up-regulated suggesting that like with p53, 53BP1 could be differentially regulating the expression of NF-κB target genes (Figures 4.6, 4.7 and 3.16).

53BP1 is involved in the DNA damage response where it acts as a mediator protein that facilitates the recruitment of repair/checkpoint proteins to sites of damaged chromatin. However, analysis of the microarray data suggests that in the absence of damage 53BP1 may also be affecting the expression of genes involved in responding to genotoxic stress, although only the expression of a few genes involved in these processes were identified (Table 4.4). This suggests that 53BP1 could be repressing the expression of DNA damage-responsive genes under normal cellular conditions.

In the response to IR, there were a large number of genes that were significantly differentially expressed, with the majority being involved in metabolism and transcription (Figure 4.8). Although, there were no significant differences in the expression of biological pathways by FDR, there were a few pathways that were significant by p-value, but these were mainly involved in metabolism apart from two pathways, which were pro-survival (Table 4.5 and 4.6). These pro-survival pathways were the GSK3 pathway, which was up-regulated in control samples and the PI3K pathway, which was up-regulated in 53BP1 depleted samples. In light of the fact that both these pathways were up-regulated in response to IR, this suggests that the cell is promoting survival following DNA damage. Surprisingly, no genes or pathways that were linked to the DNA damage response or p53 response were identified when the control and 53BP1 depleted samples were compared, even though the microarray analysis indicated that these responses were occurring in control and 53BP1 depleted samples following IR (Figure 4.8, Table 4.5, 4.6, 4.7 and 4.8). This could be because some differences were observed in the expression of DNA damage response and cell cycle genes before IR in 53BP1 depleted cells (Table 4.4). Therefore, any effects seen in response to IR were likely to have been removed by the normalisation process. Taken together these data indicate that 53BP1 does not play a major role in regulating gene transcription induced by DNA damage.

Interestingly, data presented in chapter 3 indicated that 53BP1 may be modulating p53 function by differentially regulating a subset of its target genes before and after IR. However, no p53 responsive genes were identified between control and 53BP1 depleted samples in response to IR, despite a small selection of p53 inducible genes being differentially expressed following IR (Figure 4.8). An explanation for why this could be is that p53 responsive genes are expressed at different times following IR because p53 has different binding affinities for different promoters (Espinosa *et al* 2003). Initially, p53 transactivates genes involved in cell cycle arrest such as p21, GADD45 and 14-3-3σ, whereas pro-apoptotic genes including PUMA, FAS and BAX are induced at later stages of the p53 response (Zhao *et al* 2000). Therefore, it is likely that 4 hours may not have been sufficient to detect the expression of some of the p53 genes induced late in the response such as PUMA. In addition, the fold differences in the expression of some p53 responsive genes may have been <1.5 between the control and 53BP1 depleted samples at 4 hours post-IR, so consequently, these genes would have been removed by the filtration process. Furthermore, it is also plausible that there were p53 responsive genes that had >1.5 fold difference, however due to biological variations

between the three replicates they were not significant and therefore would have been removed by the rigorous statistical analysis that was conducted to reduce false positive results. To gain a better understanding of which p53 target genes are regulated by 53BP1 in response to DNA damage, a microarray experiment would need to be performed in control and 53BP1 depleted samples exposed to IR over a 24 hour period because this would alleviate the problems surrounding the differential expression of p53 target genes at certain times following IR exposure. Alternatively, qRT-PCR could be performed for a range of p53 target genes that are known to be activated in response to DNA damage in control and 53BP1 siRNA treated samples treated with IR over a period of 24 hours. In addition, this would also allow identification of those p53 responsive genes that may have been either excluded from the microarray analysis due to having <1.5 fold change or because they were not statistically significant. Consistent with the observation that 53BP1 can modulate p53 target gene expression in the absence of DNA damage, the p53 responsive genes, *AMID* and *TP53INP1* were shown to be differentially regulated by 53BP1 prior to IR (Riley *et al* 2008) (Figure 4.4). In contrast, previous data from chapter 3 indicated that 53BP1 was being antagonistic to p53 by repressing p21 expression in the absence of DNA damage. Despite p21 not being identified by the univariate analysis as being differentially expressed, it was found in all the cell cycle and DNA damage response gene sets identified by the multivariate analysis in the unirradiated 53BP1 deficient cells (Table 4.4). This suggests that it had a fold change of >1.5, but that it was not statistically significant, therefore it would have been removed by the statistical analysis performed in the univariate analysis process.

53BP1 has been shown to be a transcriptional regulator of BRCA1 in the absence of DNA damage. However, surprisingly BRCA1 was not identified as being significantly differentially expressed by 53BP1 in the unirradiated samples (Rauch *et al* 2005). Although, it is possible that there may have been a difference in BRCA1 expression, if the difference was only small or not statistically significant then it will have been excluded from the analysis.

Both types of analysis suggest a role for 53BP1 in regulating metabolism because there were numerous metabolic genes and pathways that were differentially regulated by 53BP1 before and after DNA damage. At present there are no reports of 53BP1 being involved in metabolism. However, recently, ATM has been shown to play an important role in metabolism. Loss of ATM has been linked to the development of insulin resistance and cardiovascular disease, two contributing factors to the development of metabolic syndrome

(Armata *et al* 2010, Schneider *et al* 2006). Furthermore, ATM has been shown to be an important sensor of oxidative stress and activation of ATM promotes an anti-oxidant response. Consequently, loss of ATM results in an increase in ROS, which are a known contributory factor to metabolic syndrome development (Cosentino *et al* 2010, Guo *et al* 2010, Roberts and Sindhu 2009). Therefore, it is also possible that 53BP1 may be playing a role in regulating the cellular response to oxidative stress and that cells lacking 53BP1 may exhibit metabolic deficiencies due to dysregulation of ROS resulting in increases/decreases of oxidative stress.

Overall, the microarray analysis demonstrated that the expression of a wide variety of genes are dependent on 53BP1, before and after DNA damage. Interestingly, the analysis indicated potential novel roles for 53BP1 in metabolism and TNFR1 signalling. In addition, the expression of the genes chosen to be verified at the mRNA level and the protein level were found to be consistent with the microarray data, therefore validating the microarray data (Figures 4.9 and 4.10).

CHAPTER 5

CHAPTER 5 53BP1 NEGATIVELY REGULATES NF-κB SIGNALLING PATHWAY

5.1 INTRODUCTION

The pro-inflammatory cytokine, TNFα signals through two distinct cell surface receptors, TNFR1 and TNFR2. However, TNFR1 initiates the majority of TNFα biological activities. The binding of TNFα to TNFR1 triggers several intracellular signalling pathways including those controlled by IκB kinase (IKK), JNK and p38, all of which ultimately result in the activation of two major transcription factors, NF-κB and AP-1. TNFα has also been demonstrated to induce a caspase cascade leading to apoptosis. However, this is normally inhibited by the activation of NF-κB-mediated anti-apoptotic signals (Chen and Goeddel 2002, Wajant *et al* 2003).

The transcription factor NF-κB plays a vital role in inflammation, immunity, cell proliferation and apoptosis. In mammalian cells, NF-κB is composed of 5 family members, p65 (RelA), RelB, c-Rel, p50/p105 and p52/p100, which form homo- and heterodimers. The most abundant form of NF-κB is the heterodimer composed of p50 and p65. In most cell types, NF-κB exists in a latent state in the cytoplasm and is prevented from activating transcription by the IκB proteins (Chen and Greene 2004). After stimulation of cells with a variety of stimuli such as TNFα or genotoxic stress, such as IR, the prototypical member of the IκB proteins, IκBα, is phosphorylated by the IKK complex, ubiquitylated and degraded by the 26S proteasome. NF-κB is subsequently released from its inhibitory restraints, whereby it translocates into the nucleus and binds to κB sites within the promoter/enhancer of target genes (Hayden and Ghosh 2008). Dysregulation of NF-κB expression is associated with a variety of diseases including chronic inflammation and cancer, therefore, NF-κB activity is tightly regulated (Courtois and Gilmore 2006, Karin 2006). This is achieved not only by the induction of IκBα re-synthesis by NF-κB, ultimately leading to nuclear export of NF-κB and termination of the NF-κB response, but also by post-translational modifications of NF-κB such as phosphorylation and acetylation (Arenzana-Seisdedos *et al* 1997, Perkins 2006). The full extent with which these different post-translational modifications of NF-κB regulate its

activity and localisation remains unclear. The majority of published literature has focused on the p65 subunit. However, it is clear that these regulatory events also have a functional impact on the other less well studied, subunits of this dimeric transcription factor.

p65 is phosphorylated at multiple sites by various kinases, which are induced by a wide range of different stimuli. Serine 276 is phosphorylated by PKAc and MSK1/2 (Vermeulen *et al* 2003, Zhong *et al* 1998, Zhong *et al* 2002), PKCζ phosphorylates serine 311 (Duran *et al* 2003) and serine 529 is phosphorylated by CK2 (Wang *et al* 2000) whereas, multiple kinases phosphorylate serine 536 including IKKα, IKKβ, IKKε, NAK and RSK1 (Buss *et al* 2004b, Jiang *et al* 2003, Sakurai *et al* 1999, Sizemore *et al* 2002). These post-translational modifications of NF-κB enhance its transcriptional activity and affect the ability of NF-κB to interact with the transcriptional co-activators, CBP/p300. By contrast, activation of CHK1 either by ARF or in response to cisplatin results in phosphorylation at threonine 505, which inhibits p65 transactivation through increasing the association of p65 with HDAC1 (Campbell *et al* 2006, Rocha *et al* 2005). Furthermore, serine 468 is inducibly phosphorylated by IKKε and IKKβ in response to T-cell stimulation and treatment with TNFα, IL-1β and the genotoxic agent etoposide resulting in stimulation of p65 transactivation, whereas in contrast, in unstimulated cells phosphorylation of this site by GSK3β inhibits the ability of p65 to transactivate gene expression (Buss *et al* 2004a, Mattioli *et al* 2006, Renner *et al* 2010, Schwabe and Sakurai 2005). In addition to kinases, several phosphatases have been identified that inhibit NF-κB activity such as PP2A and more recently WIP1, which has been shown to dephosphorylate serine 536 of p65 (Chew *et al* 2009, Yang *et al* 2001a).

p65 is also acetylated at lysines 218, 221 and 310 by CBP/p300. However, prior phosphorylation at serine 276 or serine 536 is required for lysine 310 to be acetylated by CBP/p300. Acetylation at lysine 221 has been shown to enhance the DNA binding activity of p65, which together with acetylated lysine 218, also functions to impair the assembly of p65 with newly synthesised IκBα, thereby preventing IκBα-dependent nuclear export of NF-κB complexes and prolonging the NF-κB-dependent transcriptional responses. CBP/p300-mediated acetylation of lysine 310 has been demonstrated to promote the transcriptional activity of NF-κB without altering the binding of p65 to DNA or IκBα (Chen *et al* 2002, Chen *et al* 2005).

In a similar manner to phosphorylation, p65 acetylation is reversible and has been shown to be important for regulating the duration of the NF-κB response (Chen *et al* 2001, Kiernan *et al* 2003). p65 is deacetylated through the interaction with HDAC co-repressor proteins, resulting in repression of the NF-κB response. HDAC3-mediated p65 deacetylation at lysine 221 and lysine 218 is involved in terminating the NF-κB response by promoting its interaction between IκBα and subsequently its nuclear export (Chen *et al* 2002). Lysine 310 can also be deacetylated by HDAC3 as part of the SMRT co-repressor complex or alternatively, by the histone deacetylase, SIRT1 again resulting in inhibition of NF-κB transcriptional activity. As a result this leads to a sensitisation of cells to apoptosis (Hoberg *et al* 2006, Yeung *et al* 2004). Moreover, it has been demonstrated that p65 can be deacetylated by HDAC1, which can physically associate with p65 leading to loss of p65 transactivation potential (Ashburner *et al* 2001). Interestingly, in contrast, there are additional sites on p65 known to be acetylated by CBP, p300 and PCAF, which have been proposed to have an inhibitory effect on NF-κB activity by reducing the DNA binding affinity of NF-κB and may be involved in terminating the NF-κB response (Kiernan *et al* 2003). This data indicates that CBP/p300 play a critical role in the NF-κB response and can both activate and repress the transcriptional capacity of NF-κB depending on the type of stimulus and cell type.

Interestingly, it has been found that ubiquitylation and proteasomal degradation of nuclear p65 is also required for efficient and prompt termination of NF-κB dependent transcription and that this can occur independently of IκBα (Saccani *et al* 2004). Recently, this has been shown to be triggered by the SOCS-1-containing ubiquitin ligase complex in association with the COMMD1 protein, as well as by the nuclear E3 ubiquitin ligase, PDLIM2. However, the lysine residues of p65 that are ubiquitylated have yet to be established (Maine *et al* 2007, Ryo *et al* 2003, Tanaka *et al* 2007a).

Since loss of 53BP1 was shown to affect the transcriptional regulation of genes involved in the TNFR1 signalling pathway, this suggests 53BP1 may be functioning to regulate the activity of the NF-κB transcription factor directly. Therefore the main aim of this study was to establish if 53BP1 was playing a role in the NF-κB signalling pathway and if so how was this achieved.

5.2 RESULTS

5.2.1 Depletion of 53BP1 increases NF-κB transcriptional activity in response to TNFα

The best characterised inducers of NF-κB are pro-inflammatory cytokines such as TNFα and IL-1. Therefore, in this study TNFα was predominantly used to stimulate NF-κB activity. However, before any experiments were conducted, a TNFα dose response was performed in all the cell lines used in this study to determine the lowest dose of TNFα that stimulated p65 phosphorylation. As shown in figure 5.1, 10ng/ml TNFα was sufficient to achieve this in each of the cell lines.

To investigate the involvement of 53BP1 in modulating the NF-κB signalling pathway, p53 proficient U2OS cells were depleted of 53BP1, stimulated with TNFα and the levels of the major NF-κB signalling pathway components were assessed by Western blot analysis. Interestingly, cells lacking 53BP1 exhibited elevated levels of basal p65, as well as serine 536 and serine 468 hyperphosphorylation, whereas the levels of the IKK complex, p50 and IκBα were unaffected (Figure 5.2). This data suggests that 53BP1 could be impacting on p65 stability and affecting NF-κB transcriptional activity. Interestingly, p65 phosphorylation was prolonged in the absence of 53BP1 indicating that 53BP1 may be affecting the termination of the NF-κB response. This experiment was also repeated in another p53 proficient cell line, A549 to determine if the response observed is cell type specific. As figure 5.3 shows this does not appear to be the case because the response seen in A549 cells is the same as that seen in U2OS cells.

It is known that there is significant crosstalk between p53 and NF-κB. Despite p65 having antagonistic effects on p53, it can also induce p53 expression as well as cooperate with p53 (Perkins 2007). Therefore, to assess whether p53 was involved in the NF-κB signalling abnormalities caused by loss of 53BP1, p53 null H1299 cells were depleted of 53BP1 using siRNA, stimulated with TNFα and the protein levels of NF-κB pathway components were assessed by Western blot analysis. The response observed in the H1299 cells was identical to that seen in the U2OS and A549 cells indicating that p53 is unlikely to be involved (Figure 5.4).

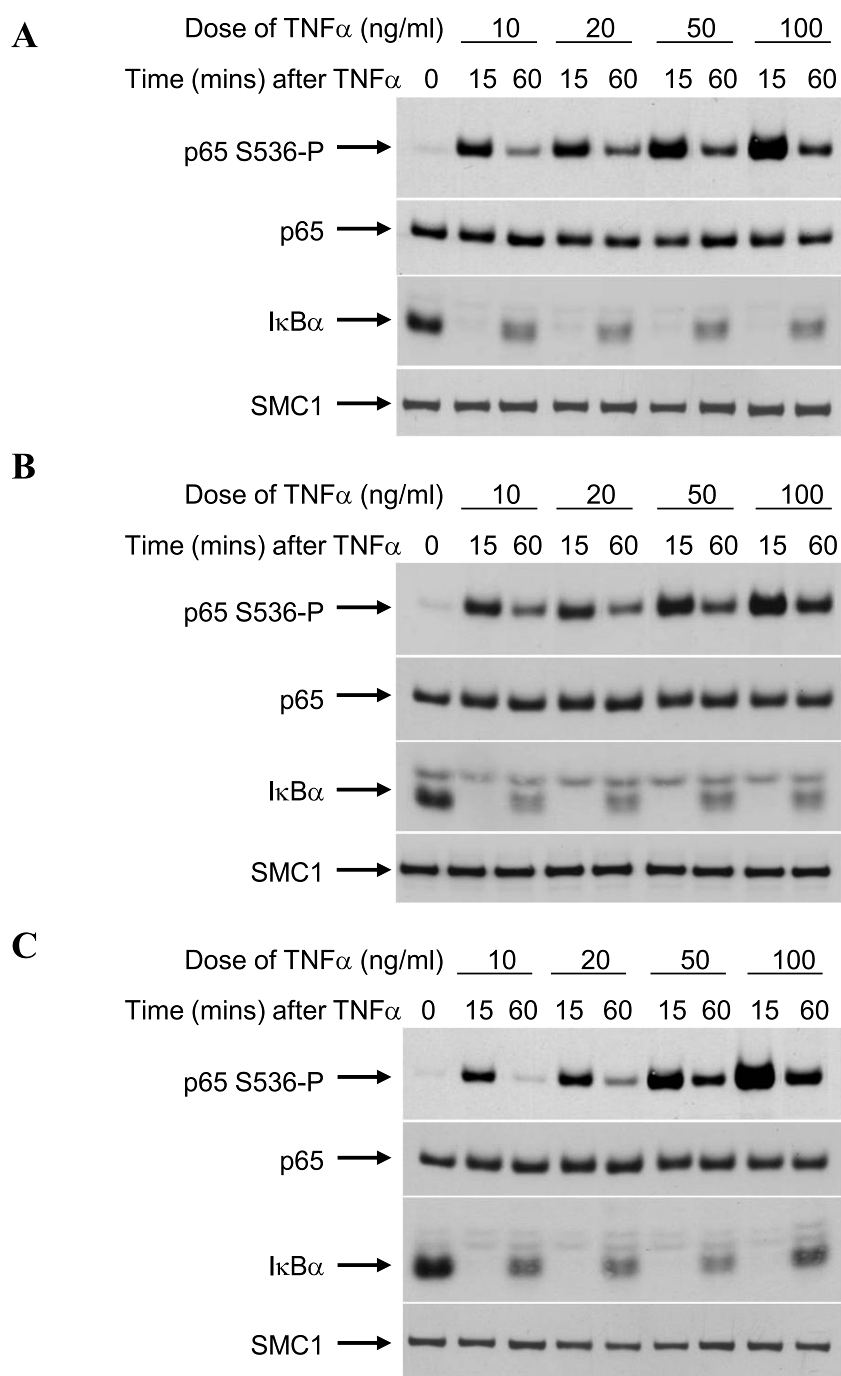


Figure 5.1 ***10ng/ml TNFα stimulates NF-κB response****. (A) U2OS (B) H1299 and (C) A549 cells were stimulated with TNFα and the cells were harvested at the times indicated. Whole cell lysates were prepared, resolved by SDS-PAGE and the NF-κB response was determined by Western blot analysis using antibodies to phospho-p65, IκBα and p65. SMC1 was used as a loading control.*

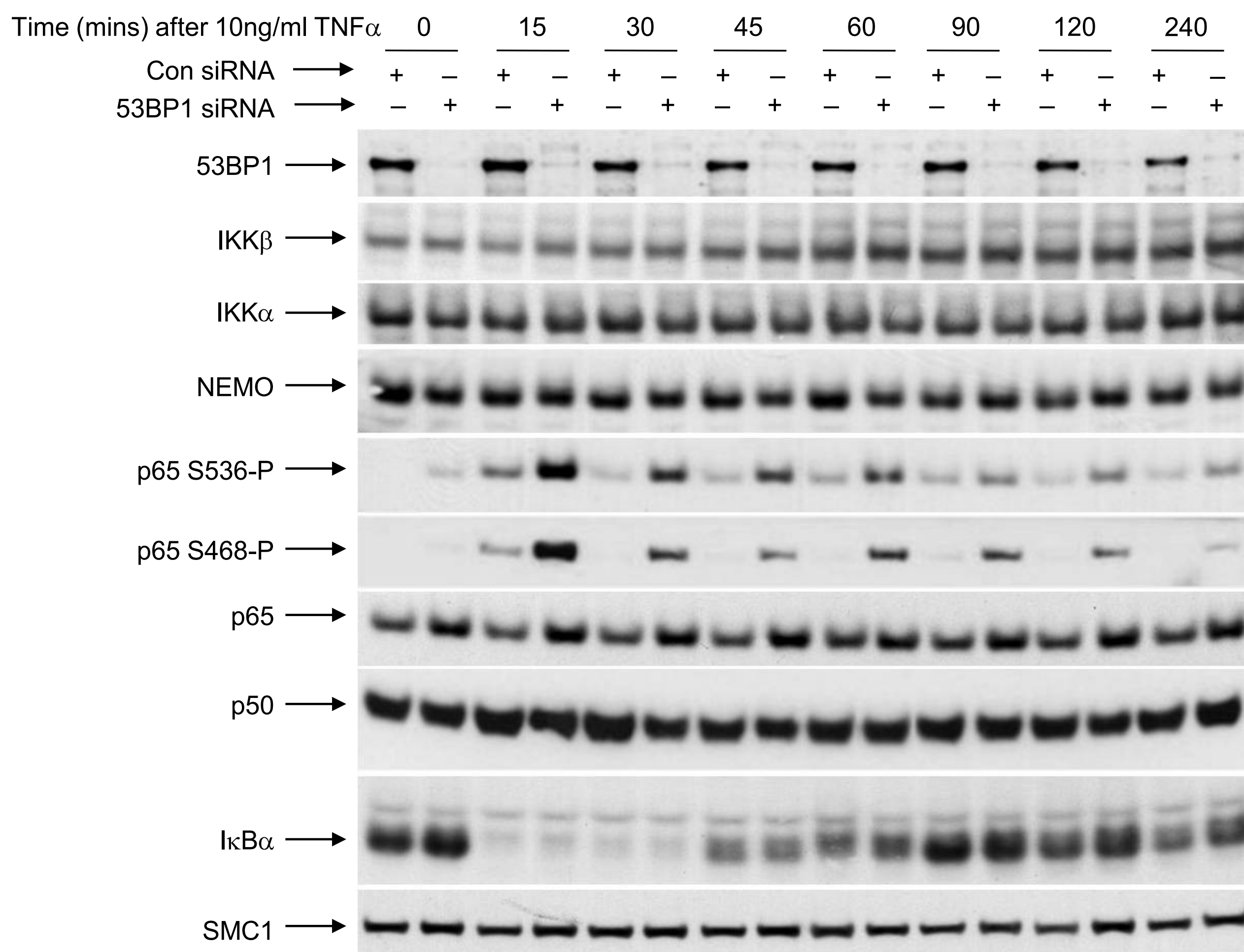


Figure 5.2 ***Loss of 53BP1 induces hyperphosphorylation of p65 in U2OS cells.*** *Cells were depleted of 53BP1 by siRNA, stimulated with TNFα and harvested at the times indicated. The protein levels of the major proteins in the NF-κB signalling pathway were analysed by Western blotting. SMC1 was used as a loading control.*

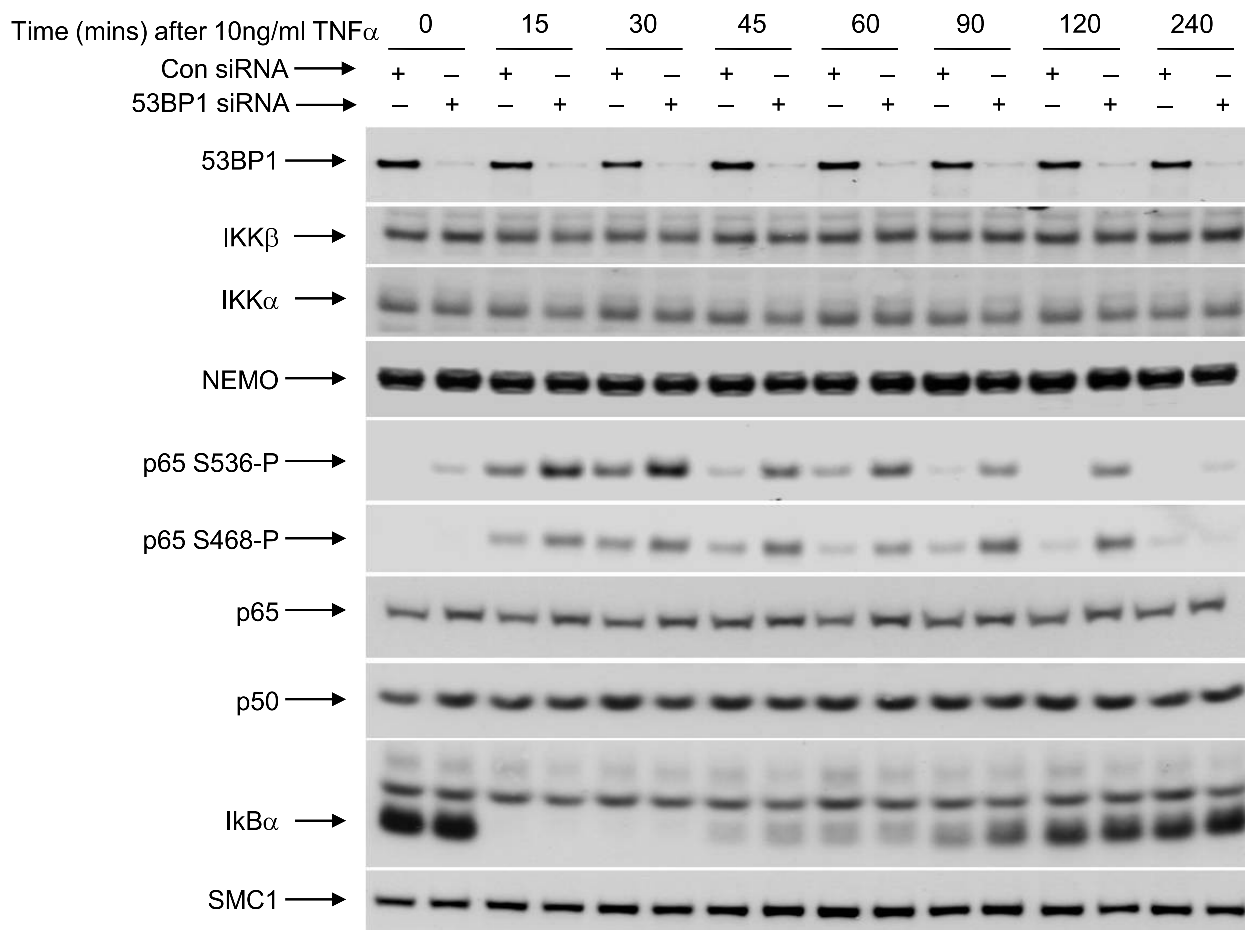


Figure 5.3 ***Loss of 53BP1 induces hyperphosphorylation of p65 in A549 cells.*** *Cells were depleted of 53BP1 by siRNA, stimulated with TNFα and harvested at the times indicated. The protein levels of the major proteins in the NF-κB signalling pathway were analysed by Western blotting. SMC1 was used as a loading control.*

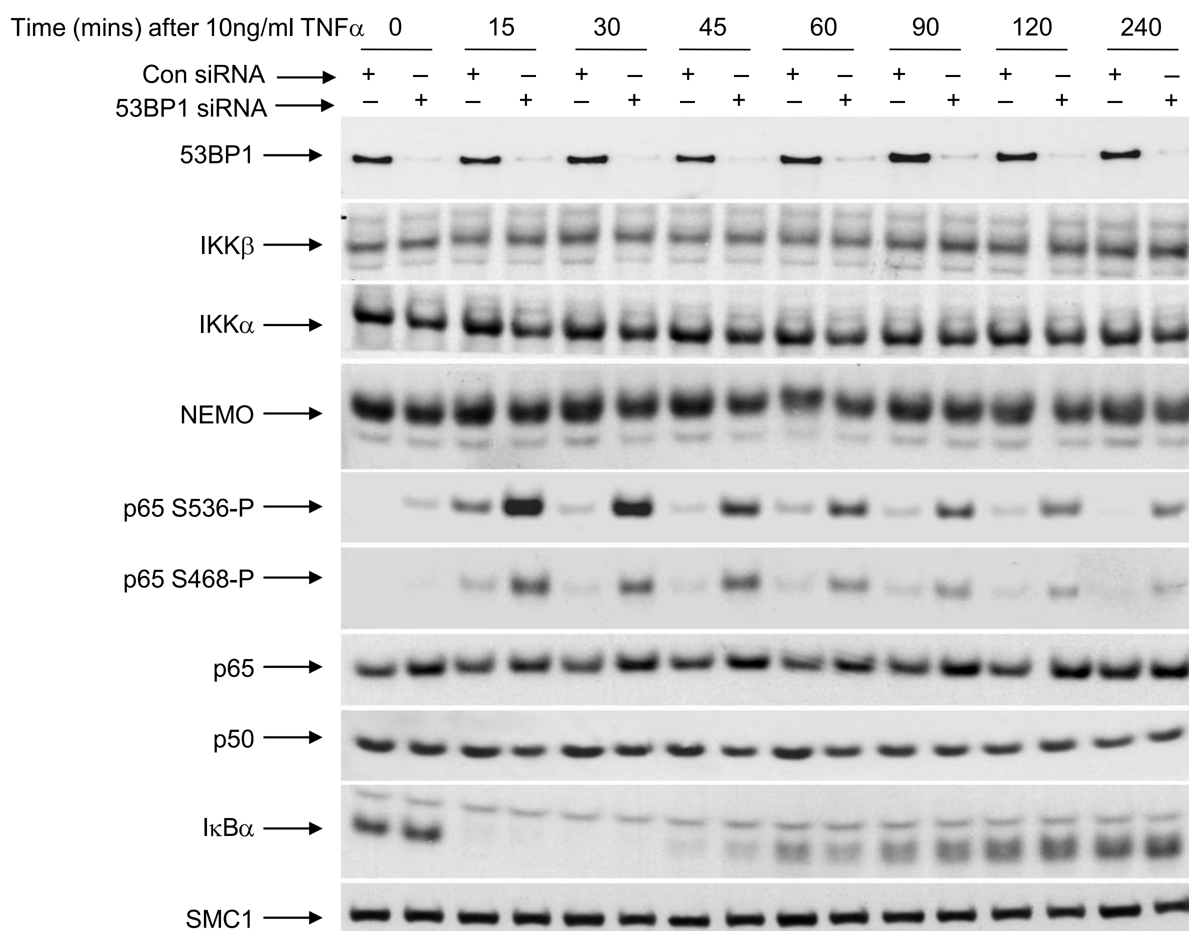


Figure 5.4 ***Loss of 53BP1 induces hyperphosphorylation of p65 independent of p53.*** *H1299 cells were depleted of 53BP1 by siRNA, stimulated with TNFα and harvested at the times indicated. The protein levels of the major proteins in the NF-κB signalling pathway were analysed by Western blotting. SMC1 was used as a loading control.*

To test whether 53BP1 was involved in regulating the transcriptional activity of NF-κB, U2OS cells depleted of 53BP1 were either transfected with the 3x κB luciferase reporter construct, which contains 3 κB response elements upstream of the luciferase gene or a control lacking κB elements and then stimulated with TNFα. Depletion of 53BP1 slightly increased the activity of the NF-κB luciferase reporter prior to TNFα stimulation when compared to control cells and this was further augmented following TNFα stimulation (Figure 5.5). These effects were dependent upon the κB elements present in this plasmid since the control reporter plasmid lacking the κB elements was not stimulated following TNFα treatment demonstrating that this effect results from the activity of NF-κB. Furthermore, a similar response was also observed in the p53 null H1299 cell line (Figure 5.6). Altogether, these data suggest that 53BP1 is repressing NF-κB transcriptional activity, independently of p53. This is consistent with the univariate analysis of the microarray data, which indicated that in the absence of overt DNA damage, depletion of 53BP1 could be activating NF-κB activity (Figure 4.6).

5.2.2 Localisation of p65 is not affected by 53BP1

The inhibitor of NF-κB, IκBα plays a key role in the localisation of NF-κB. In unstimulated cells, NF-κB is mainly cytoplasmic. However, following stimulation IκBα undergoes phosphorylation and subsequent ubiquitin-mediated proteasomal degradation, which allows NF-κB to translocate to the nucleus and activate its target genes. As part of its regulatory negative feedback loop, NF-κB upregulates the expression of IκBα, which dissociates NF-κB from the DNA and exports it back to the cytoplasm, thus terminating its transcriptional response. As observed in figures 5.2, 5.3 and 5.4, the degradation and resynthesis of IκBα induced by TNFα stimulation was unaffected by depletion of 53BP1 indicating that the increased phosphorylation of p65 was not due to defects in IκBα resynthesis. To ascertain whether the sustained serine 536 and serine 468 phosphorylation of p65 observed in 53BP1 depleted cells was due to a defect in the nuclear export of NF-κB, the localisation of p65 was analysed by immunofluorescence in cells treated with control or 53BP1 siRNA. Prior to TNFα stimulation in the control siRNA treated cells, p65 is localised mainly in the cytoplasm. Following 15 minutes of TNFα stimulation, a large proportion of p65 could be seen localised within the nucleus, which 90 minutes after TNFα stimulation had translocated back into the cytoplasm (Figure 5.7). In comparison, the TNFα-induced relocalisation of p65 in the 53BP1

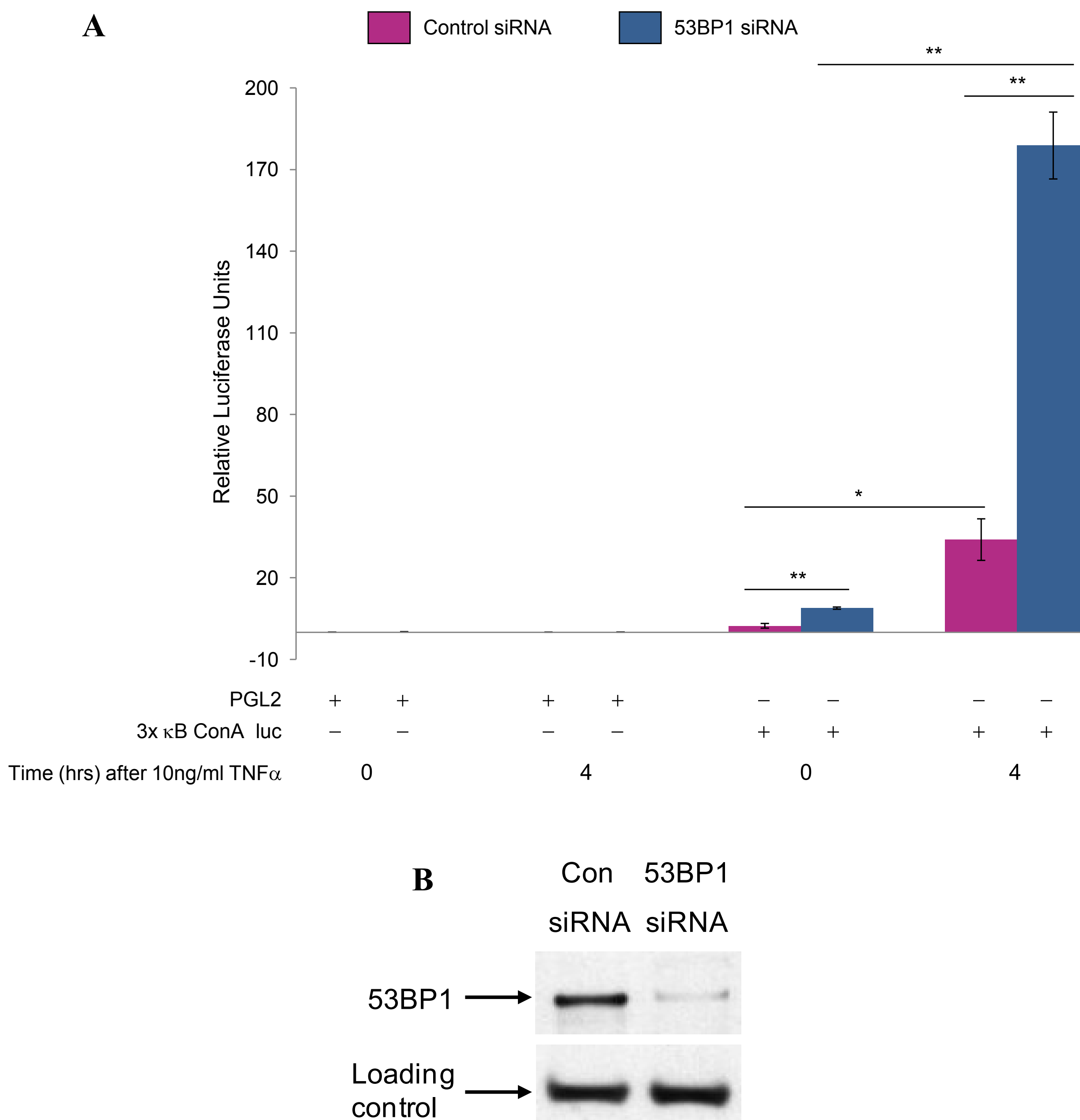


Figure 5.5 ***53BP1 represses NF-κB transcriptional activity****. (A) U2OS cells were first transfected with either control or 53BP1 siRNA, 48 hours later cells were transfected with either 300ng of 3x kB concanavalin A NF-κB luciferase reporter plasmid or PGL2, which lacks κB sites and 50ng Renilla. 24 hours later cells were either unstimulated or stimulated for 4 hours with 10ng/ml TNFα. Luciferase activity was normalised to Renilla activity. Data from three experiments is presented ± standard deviations. Significant differences in relative luciferase units in samples depleted of 53BP1 compared to control samples with and without TNFα stimulation * $p<0.05$, ** $p< 0.01$. (B) U2OS cells were transfected as in A, whole cell lysates were prepared, resolved by SDS-PAGE and immunoblotted with anti-53BP1 antibody. SMC1 was used as a loading control.*

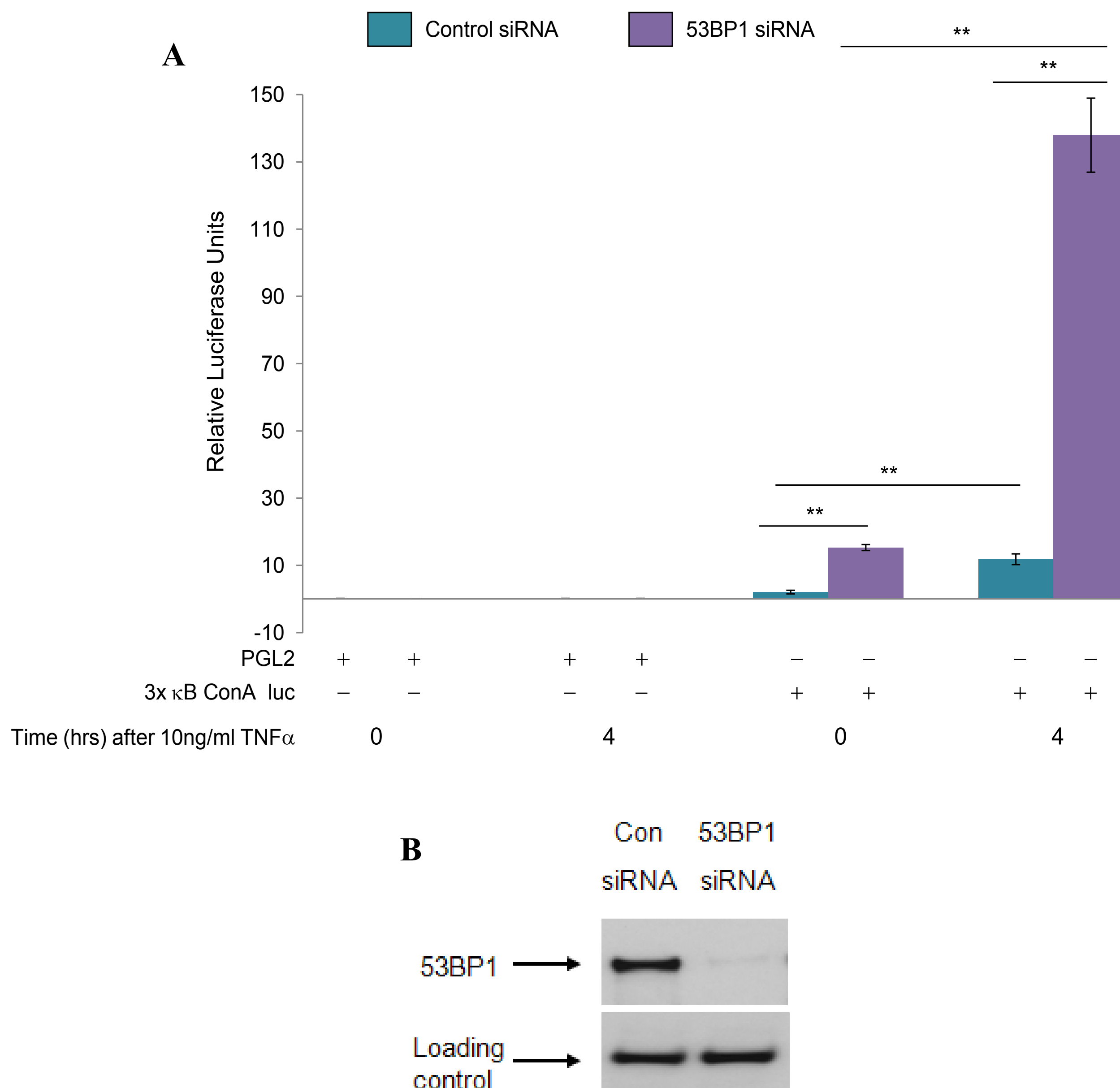


Figure 5.6 ***53BP1 represses NF-κB transcriptional activity, independent of p53.*** *(A) H1299 cells were first transfected with either control or 53BP1 siRNA, 48 hours later cells were transfected with either 300ng of 3x kB concanavalin A NF-κB luciferase reporter plasmid or PGL2, which lacks κB sites and 50ng Renilla. 24 hours later cells were either unstimulated or stimulated for 4 hours with 10ng/ml TNFα. Luciferase activity was normalised to Renilla activity. Data from three experiments is presented ± standard deviations. Significant differences in relative luciferase units in samples depleted of 53BP1 compared to control samples with and without TNFα stimulation * $p<0.05$, ** $p< 0.01$. (B) H1299 cells were transfected as in A, whole cell lysates were prepared, resolved by SDS-PAGE and immunoblotted with anti-53BP1 antibody. SMC1 was used as a loading control.*

depleted cells was identical to that observed in the control siRNA transfected cells indicating that the translocation of NF-κB in and out of the nucleus is unaffected by loss of 53BP1 and suggests that the termination of NF-κB activity by the IκB proteins is functional. Therefore, it is likely that the sustained NF-κB response observed when 53BP1 is depleted is due to alternative NF-κB termination mechanisms that are independent of the IκB proteins.

5.2.3 53BP1 does not interact with p65

To try and elucidate the mechanism with which 53BP1 is regulating NF-κB transcriptional activity, the interaction between 53BP1 and p65 was investigated. Endogenous p65 was immunoprecipitated from whole cell extracts prepared from U2OS cells before and after stimulation with TNFα using an anti-p65 antibody and Western blot analysis was used to determine whether p65 associated with 53BP1 *in vivo*. The data shows that p65 does not interact with 53BP1 (Figure 5.8). In the reciprocal experiment, endogenous 53BP1 was immunoprecipitated with an anti-53BP1 antibody and co-precipitation of p65 was analysed by Western blot analysis using an anti-p65 antibody. As shown by figure 5.8, p65 was not found in 53BP1 immunocomplexes. Together these data demonstrate that 53BP1 does not physically associate with p65 indicating that 53BP1 is influencing NF-kB transcriptional activity via an indirect mechanism.

5.2.4 TNFα does not induce a DNA damage response

It is well established that DNA damage can activate the NF-κB response, albeit via the atypical pathway, which can be either IKK-dependent or independent (Perkins 2007, Wu and Miyamoto 2007). Since 53BP1 plays a key role in the DNA damage response, it is conceivable that the increase in p65 basal and phosphorylation levels observed in TNFα stimulated 53BP1 depleted cells may be due to ATM and ATR being activated and as a consequence, activating NF-κB. To determine whether this was the case, U2OS cells were treated with either control or 53BP1 siRNA and then exposed to either caffeine to inhibit ATM and ATR function, and then stimulated with TNFα. Notably, neither the loss of ATM or ATR function caused by the caffeine treatment inhibited serine 536 phosphorylation of p65 following stimulation with TNFα (Figure 5.9). In addition, treatment with both caffeine and TNFα demonstrated that TNFα was not inducing a DNA damage response as illustrated by

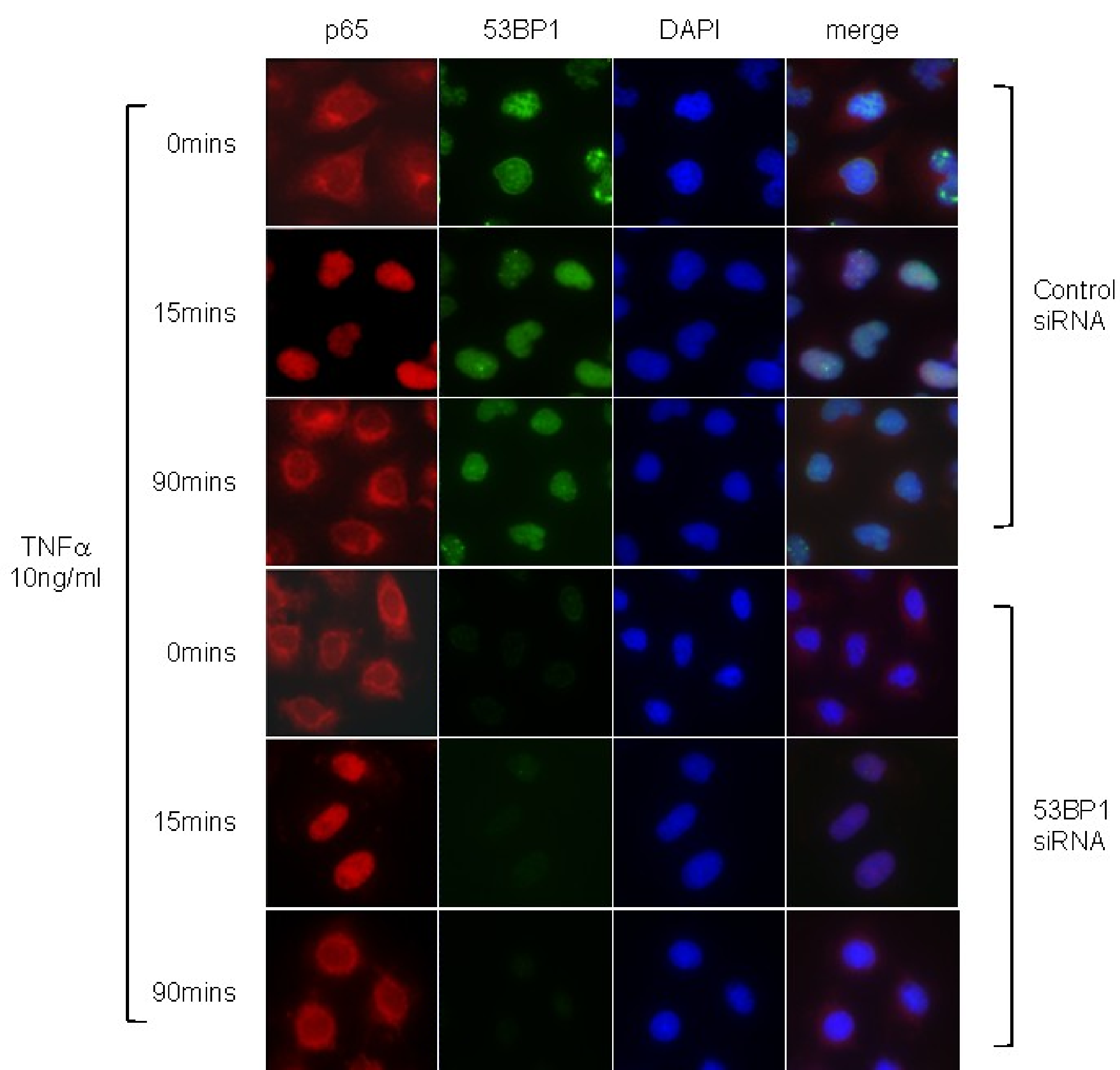


Figure 5.7 ***Depletion of 53BP1 does not affect p65 localisation****. A549 cells were transfected with either control or 53BP1 siRNA and 72 hours later, cells were stimulated with 10ng/ml TNFα for 0mins, 15mins and 90mins. Subsequently, cells were fixed and processed for immunofluorescence using the indicated antibodies.*

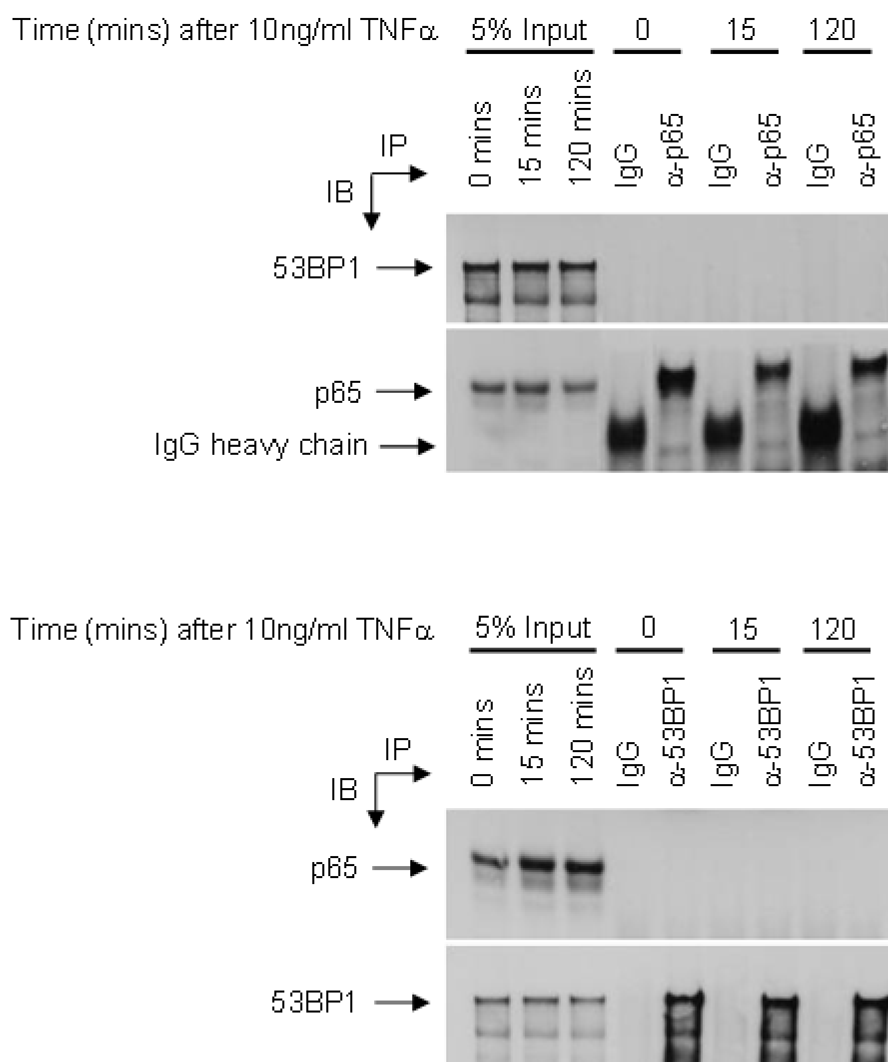


Figure 5.8 ***53BP1 does not interact with p65****. p65 and 53BP1 were immunoprecipitated from U2OS whole cell extract and Western blotting was used to assess the binding of 53BP1 and p65 respectively. IgG denotes immunoprecipitates performed using a non-specific IgG antibody as a control. IP and IB are abbreviations for immunoprecipitation and immunoblot respectively.*

the lack of phosphorylation of DNA damage response proteins. The lack of SMC1, NBS1 and H2AX phosphorylation in cells depleted of 53BP1 indicates that the increase in NF-κB transcriptional activity is unlikely to be caused by the loss of 53BP1 inducing DNA damage. Interestingly, despite ATM and ATR being inhibited, there is an increase in γ-H2AX and a slight increase in phosphorylation of SMC1 and NBS1 in the caffeine treated cells. However, this could be because endogenous DNA damage is activating the other PIKK family member, DNA-PK (An *et al* 2010, Stiff *et al* 2004). These data indicate that the DNA damage response is not activated in response to TNFα or following loss of 53BP1 function and therefore is unlikely to account for the increase in NF-κB transcriptional activity observed in cells lacking 53BP1.

5.2.5 Depletion of 53BP1 increases NF-kB transcriptional activity in response to IR

In addition to inflammatory cytokines, even though the signalling cascade leading to NF-κB activation in response to DSBs has yet to be fully characterised, it has been established that the downstream events following IKK activation are identical to those elicited by TNFα (Wu and Miyamoto 2007). Despite the fact that the functional consequence of DNA damage induced NF-κB activity can differ depending on the genotoxic agent used to generate the damage, treatment with IR has been shown to activate NF-κB in a similar manner to TNFα (Criswell *et al* 2003). To determine if the increase in NF-κB transcriptional activity observed in TNFα stimulated 53BP1 deficient cells was specific to TNFα, U2OS cells were treated with either control or 53BP1 siRNA, exposed to 10 Grays of IR and the protein levels and phosphorylation status of p65 were assessed by Western blot analysis along with components of the DNA damage response over a 24 hour period.

Consistent with the response observed following TNFα stimulation, there was no difference in IκBα protein levels between control and 53BP1 depleted cells whereas, p65 basal levels, as well as its phosphorylation on serine 536 were increased in cells lacking 53BP1 both prior to and in response to IR. In addition, p65 phosphorylation was also prolonged in the absence of 53BP1 following IR (Figure 5.10). Phosphorylation of NBS1, SMC1 and H2AX demonstrated that the DNA damage response had been activated following exposure to IR. Moreover, the levels of phosphorylated NBS1 and SMC1 were unaffected in irradiated 53BP1

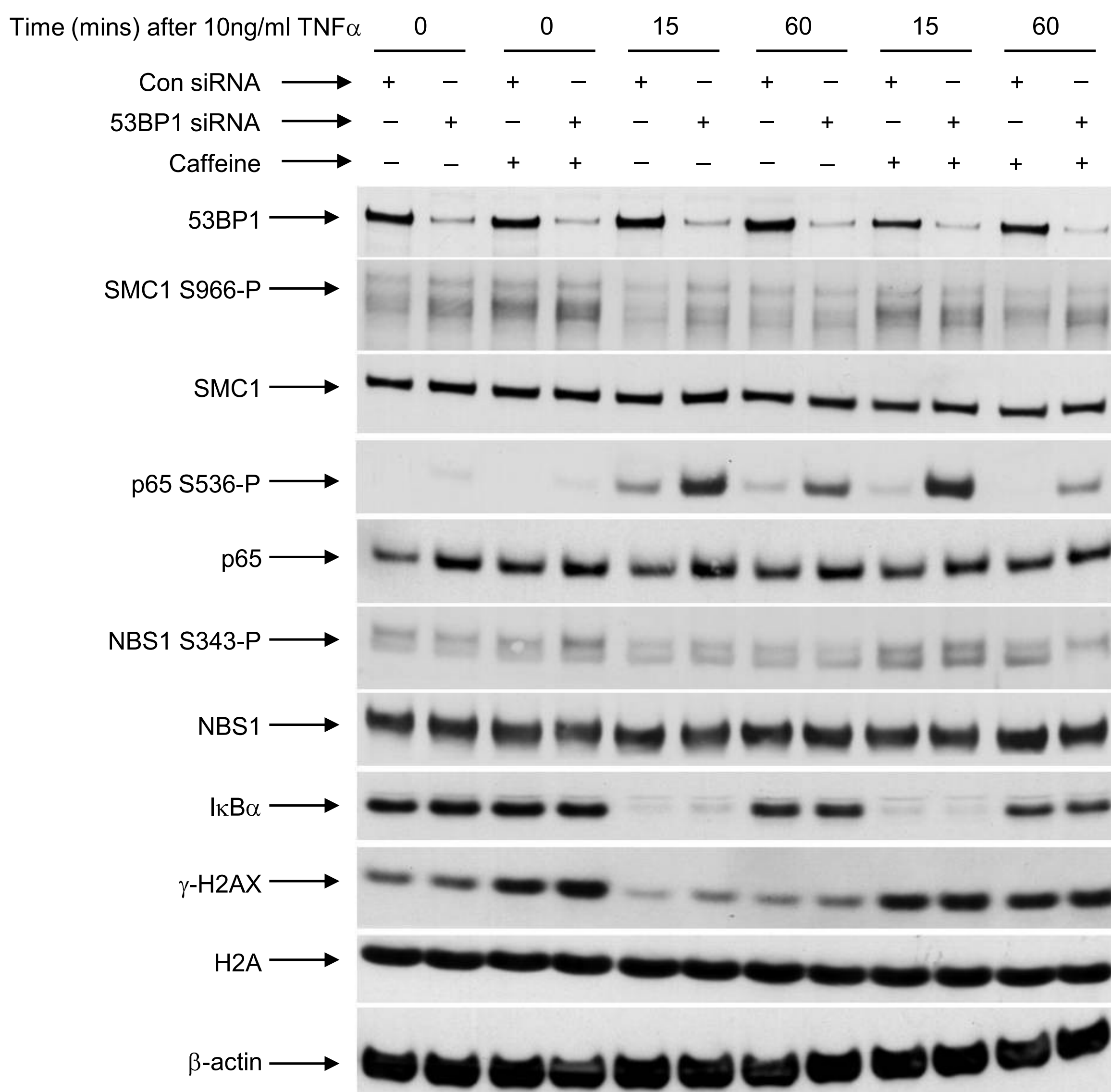


Figure 5.9 ***Depletion of 53BP1 does not activate the DDR****. U2OS cells were transfected with either control or 53BP1 siRNA, 72 hours after transfection, cells were either mock-treated, exposed to 8mM caffeine, stimulated with 10ng/ml TNFα or treated with both caffeine and TNFα. Cells were harvested and the NF-κB response was determined by Western blotting using antibodies to p65, phospho-p65 and 1κBα. Phospho-SMC1, phospho-NBS1 and phospho-H2AX antibodies were used as markers of the DNA damage response. β-actin was used as a loading control.*

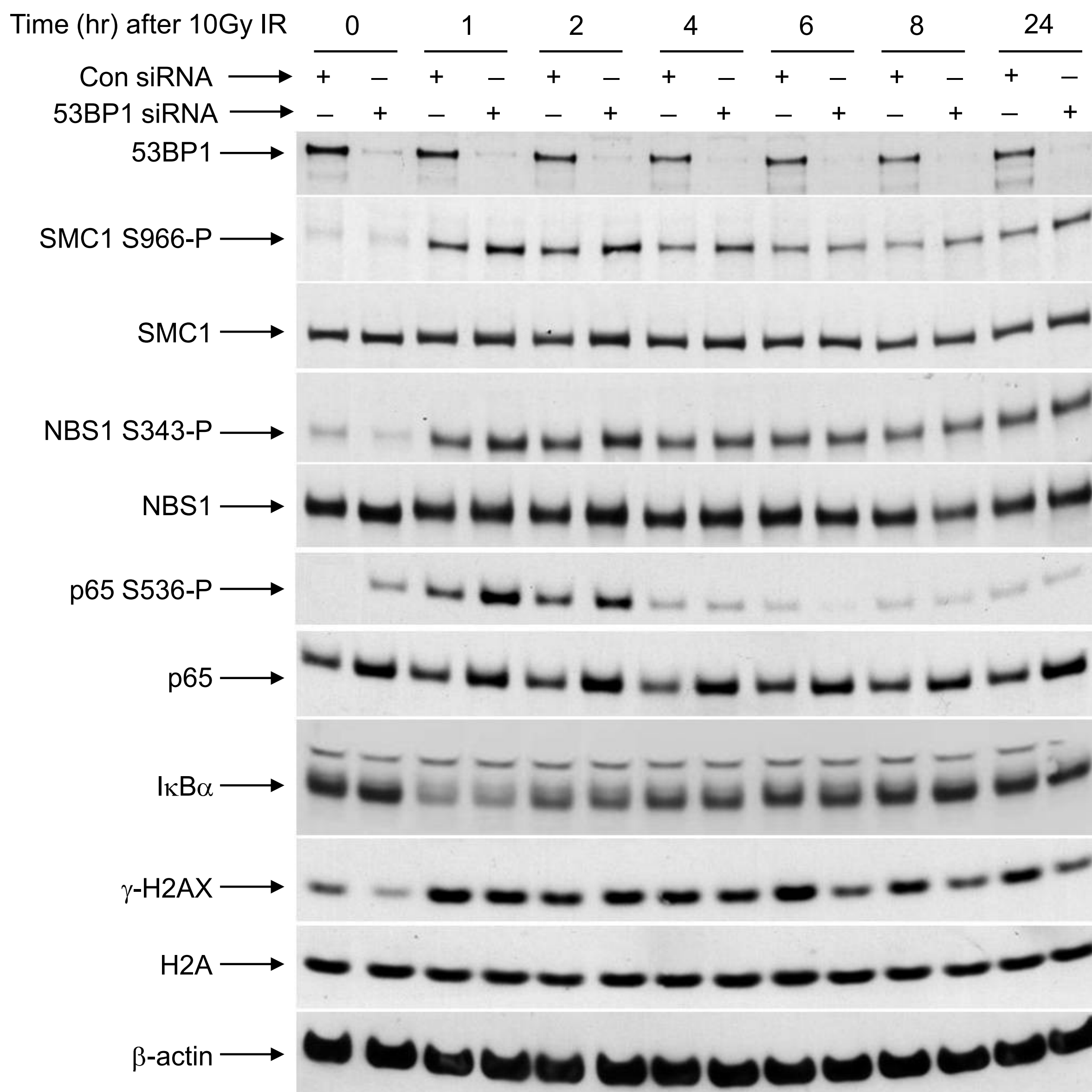


Figure 5.10 ***Depletion of 53BP1 induces hyperphosphorylation of p65 in response to IR****. U2OS cells were transfected with either control or 53BP1 siRNA, 72 hours after transfection, cells were either mock-treated or irradiated with a 10 Gray dose and harvested at the times indicated. Whole cell extracts were prepared, separated by SDS-PAGE and subjected to immunoblotting using the antibodies indicated. β-actin was used as a loading control.*

depleted cells compared to control cells indicating that 53BP1 is not required for efficient activation of the ATM-dependent DNA damage response. However, at late time points after IR, H2AX phosphorylation was reduced in cells lacking 53BP1 suggesting that 53BP1 may be required to sustain phosphorylation of H2AX or that the damage is repaired quicker than when compared to control cells treated with the same dose of IR. These data suggest that 53BP1 is impacting on p65 stability and is repressing NF-κB transcriptional activity in response to IR in a similar manner to when cells were treated with TNFα (Figure 5.2).

5.3 DISCUSSION

This study demonstrates a novel function for 53BP1 as a negative regulator of NF-κB transcriptional activity. Despite the fact that it is known that NF-κB activity plays crucial roles in numerous cellular processes, relatively little is known about how the NF-κB response is controlled.

When activation of the NF-κB pathway in response to TNFα was assessed in cells depleted of 53BP1 alterations in both the stability and phosphorylation of p65 were observed (Figure 5.2 and 5.3). Basal p65 levels were elevated in the absence of 53BP1 suggesting that 53BP1 could be affecting the protein stability of p65 and/or the expression of p65. To investigate this further, the mRNA levels of the p65 NF-κB subunit would have to be determined using q RT-PCR. A functional knock on effect of stabilising p65 could account for the increased NF-κB-dependent transcription and phosphorylation of p65 on serine 536 and serine 468 observed in 53BP1 deficient cells. However, the level of increased and sustained phosphorylation of p65 following TNFα stimulation in cells with compromised expression of 53BP1 far exceeds the modest increase in basal levels of p65 observed in these cells suggesting that 53BP1 may be modulating the NF-κB response by numerous mechanisms. Moreover, despite the well established biochemical links between p53 and NF-κB, this role of 53BP1 in suppressing the phosphorylation of p65 occurs independently of p53 since similar effects of 53BP1 loss on p65 stability and phosphorylation were also observed in a p53 null cell line (Figures 5.2, 5.3, 5.5 and 5.6). Interestingly, the increase in NF-κB activity observed in 53BP1 depleted cells is not specific to TNFα because exposing these cells to 10 Grays of IR elicited a similar response (Figure 5.2 and 5.10). This is consistent with findings demonstrating that IR, like TNFα, can activate NF-κB (Criswell *et al* 2003). Together these data suggest that 53BP1 is

repressing NF-κB transcriptional activity, independently of p53. However, this is likely to be via an indirect mechanism given that co-immunoprecipitation studies failed to detect an interaction between 53BP1 and p65 (Figure 5.8). It is conceivable that one possible way in which 53BP1 could be mediating repression of NF-κB activity is that it is negatively regulating the interaction between p65 and its co-activator, CBP/p300. As a consequence, loss of 53BP1 allows CBP/p300 to acetylate p65 resulting in augmentation of NF-κB transcriptional activity. This has been demonstrated to be the case with other negative regulators of NF-κB such as the ARF tumour suppressor. ARF has been demonstrated to repress NF-κB activity by enhancing the interaction of p65 with its known regulators, HDAC1 and TCEAL7. HDAC1 has been shown to directly deacetylate p65 whereas TCEAL7 inhibits p300-mediated NF-κB activity by interfering with the binding of p300 to p65 (Rattan *et al* 2010, Rocha *et al* 2003). To support this hypothesis, immunoprecipitation studies from cells with and without 53BP1 will have to be conducted to ascertain if 53BP1 is modulating the acetylation status of p65 and whether this is mediated by affecting its ability to interact with p300 and HDAC1. Furthermore, depletion of p300 in cells lacking 53BP1 will help to determine if the hyperphosphorylation of p65 that occurs in these cells is p300-dependent.

Co-immunoprecipitation studies presented in this study have shown that 53BP1 can interact directly with both p300 and CBP (Figure 3.2). It is therefore possible that the presence of 53BP1 may compete with p65 for its association with CBP/p300. However, it is also known that the acetylation of p65 is not always associated with stimulation of its ability to function as a transcription factor. p300-mediated acetylation of p65 on lysine 122 and lysine 123 have been shown to have an inhibitory effect, thus the function of 53BP1 to repress the activity of NF-κB may be via its ability to promote acetylation on these regulatory lysine residues (Kiernan *et al* 2003). The derivation of antibodies specific for individual acetylated lysine residues of p65 will be required to directly determine whether 53BP1 suppresses the function of NF-κB by preventing activatory acetylation or promoting inhibitory acetylation.

The activation of NF-κB in response to different stimuli has both cytoplasmic and nuclear aspects to its regulation. The findings presented here demonstrating that a loss of 53BP1 does not affect degradation of IκBα and the nuclear translocation of p65 as well as 53BP1 only being present in the nucleus indicates that it is unlikely that the cytoplasmic component of this signalling cascade is subjected to regulation by 53BP1 (Schultz *et al* 2000) (Figures 5.2, 5.7

and 5.10). However, cellular fractionation would have to be carried out in unstimulated and stimulated cells with and without 53BP1 to determine whether hyper-phosphorylated form of p65 observed in cells lacking 53BP1 was present in the nuclear or cytoplasmic compartment of the cell.

Due to the involvement of 53BP1 in the DNA damage response and that NF-κB is activated in response to DNA damage, it is possible that the increase in p65 basal levels and NF-κB transcriptional activity observed in TNFα stimulated 53BP1 deficient cells was due to elevated levels of DNA damage in these cells. However, the fact that inhibiting the activity of ATM and ATR by the incubation of cells with caffeine failed to return the phosphorylation of p65 on serine 536 in cells depleted of 53BP1 and stimulated TNFα back to normal levels indicates that this abnormal regulation of NF-κB is unlikely to arise due to an activated DNA damage response (Figure 5.9). Furthermore, absence of detectable increases in the levels of γ-H2AX, phospho-NBS1 or phospho-SMC1 in unstimulated cells lacking 53BP1 compared with control siRNA treated cells supports this notion.

The sustained NF-κB response observed in 53BP1 depleted cells in response to IR and TNFα indicates that the NF-κB response is not being terminated correctly in cells lacking 53BP1 (Figure 5.2 and 5.10). Therefore, one could hypothesise that perhaps 53BP1 is involved in regulating the termination of the NF-κB response. However, in a similar manner to its activation, termination of the NF-κB response is a complex process involving many post-translational mechanisms as well as both a cytoplasmic and nuclear component. One of the best studied mechanisms is the degradation and resynthesis of IκB proteins, which control the translocation of NF-κB in and out of the nucleus. Since, 53BP1 did not affect IκBα degradation and resynthesis following TNFα stimulation nor did it inhibit translocation of NF-κB in and out the nucleus, this suggests that 53BP1 is regulating NF-κB via an IκB-independent mechanism (Figure 5.2, 5.3, 5.4 and 5.7). Recently, it has been shown that ubiquitylation and degradation of promoter bound p65 by the proteasome is required for efficient termination of nuclear NF-κB activity (Saccani *et al* 2004) Ubiquitylation of p65 is controlled by a variety of E3 ubiquitin ligases including SOCS1, which is a component of the ECS-ubiquitin ligase complex and COMMD1, which interacts with this complex. Interestingly, the elevated levels of basal p65 and increase in NF-κB activity in 53BP1 depleted cells mirror those observed when either SOCS1 or COMMD1 expression is

compromised indicating that 53BP1 may functionally regulate the turnover of NF-κB through the activity of this ubiquitin ligase complex (Maine *et al* 2007, Ryo *et al* 2003). However, only a small fraction of p65 is turned over using this mechanism indicating defects in this pathway are unlikely to significantly contribute to the sustained NF-κB response observed in cells depleted of 53BP1.

Whilst regulating the turnover of NF-κB represents a major aspect of its regulation, post-translational modifications of components of this pathway also play a critical role in its activation and termination. In this respect it is important that NF-κB is de-modified in order for the IκBα negative feedback loop to work because modified NF-κB does not bind to IκBα very efficiently (Chen and Greene 2003). The observation that p65 is not being dephosphorylated efficiently in 53BP1 deficient cells following TNFα stimulation indicates that 53BP1 may also be regulating the phosphatase, WIP1, which has been recently demonstrated to target serine 536 (Chew *et al* 2009). Whilst the levels of WIP1 were unaffected by loss of 53BP1 (data not shown) it is possible that loss of 53BP1 affects the ability of WIP1 to bind to and dephosphorylate p65. To implicate abnormal regulation of WIP1 as a contributory factor in the elevated and sustained levels of phosphorylated p65 in 53BP1 depleted cells, assessment of whether forced over-expression of WIP1 in these cells can correct the p65 hyperphosphorylation phenotype may support a role of 53BP1 in regulating NF-κB activity by affecting the function of a known phosphate.

Given that the phosphorylation status of p65 is intimately linked with its ability to bind p300 and therefore its level of acetylation, it is quite possible that inability of p65 to be dephosphorylated in cells lacking 53BP1 may be linked to abnormalities in its ability to be acetylated/deacetylated properly. Again, assessment of the levels and kinetics of p65 acetylation in these cells may shed some light on this.

One important question that needs to be addressed is what is the physiological consequence of 53BP1-mediated inhibition of NF-κB activity? NF-κB plays a crucial role in cell survival, so it would be interesting to ascertain whether like ARF and TCEAL7, 53BP1 is promoting apoptosis by repressing anti-apoptotic NF-κB target genes and consequently, loss of 53BP1 is causing an increase in cell survival by inducing the expression of anti-apoptotic genes. Interestingly, the microarray data does indicate that this may be occurring because in 53BP1 depleted cells, the expression of FADD, which is involved in apoptosis, was decreased

whereas the anti-apoptotic protein XIAP and components of the PI3K pathway, another important cell survival pathway, were increased (Figure 4.5 and 4.6).

Collectively, these data suggest an additional biological role for 53BP1 as a negative transcriptional regulator of NF-κB activity. However, the mechanism and biological consequence remains unclear.

CHAPTER 6

CHAPTER 6 FINAL DISCUSSION

6.1 A ROLE FOR 53BP1 AS A TRANSCRIPTIONAL REGULATOR

The role of 53BP1 as a regulator of gene transcription has been a subject of debate due to contradictory studies. Data presented here supports a role for 53BP1 in transcriptional regulation.

Data in chapter 3 shows that 53BP1 can interact with the transcriptional co-activators CBP and p300 and enhance their transcriptional activities indicating that 53BP1 acts as a cofactor for CBP/p300. Moreover, the capability of 53BP1 to function as a CBP/p300 cofactor was dependent on its BRCT domains since depletion of these domains dramatically reduced the interaction between CBP/p300 and 53BP1, as well as the ability of 53BP1 to potentiate the transactivation activities of CBP/p300. In addition, 53BP1 was shown to modulate p53 transcriptional activity as illustrated by the fact that 53BP1 can differentially regulate a subset of p53 target genes including p21 and PUMA. These data are consistent with several reports indicating that 53BP1 can regulate the transcriptional activity of p53. However, unlike these reports, these data suggest that 53BP1 does not function as a strict co-activator or co-repressor of p53, but rather acts as a modulator of p53 transcriptional activity.

Microarray analysis revealed that 53BP1 could differentially regulate a diverse array of genes both before and after DNA damage. These genes were involved in a wide range of cellular processes such as transcription, metabolism, cell cycle regulation and DNA repair. Intriguingly, despite the observations in chapter 3 implying that 53BP1 modulated the expression of a subset of p53 responsive genes, no p53 responsive genes were identified as being differentially regulated in response to DNA damage. However, microarrays are designed to take a snapshot of gene expression at the specific time point at which the RNA is isolated. Furthermore, it has been reported that in response to DNA damage p53 regulates the expression of its target genes in a temporal manner, with cell cycle arrest genes being expressed first and pro-apoptotic genes being expressed at later stages of the p53 response (Zhao *et al* 2000). As a result, it is possible that major differences in the expression of some IR-inducible p53 target genes may have been missed due to the time point chosen. Alternatively, the fold differences in the expression of some p53 target genes may have been

small and/or statistically insignificant. Therefore, these genes would have been removed by the statistical analysis and the filtration process. In addition, MTC is very stringent in order to ensure the rate of false positives is low. However, as a consequence, the rate of false negatives is high, therefore some p53 target genes may have been classed as false negatives. Interestingly, both the univariate and multivariate analyses implied that 53BP1 was involved in regulating the TNFR1 signalling pathways before IR, in particular the NF-κB pathway. Several genes encoding proteins involved in TNFR1 signalling, as well as pathways linked to TNFR1 were found to be differentially regulated by 53BP1. Further investigation of a possible role for 53BP1 in TNFR1 signalling revealed that 53BP1 regulated NF-κB transcriptional activity. Depletion of 53BP1 resulted in an increase in NF-κB transcriptional activity in response to both TNFα and IR indicating that 53BP1 is repressing NF-κB transcriptional activity. However, the underlying mechanism remains unclear since it was observed that 53BP1 and p65 do not associate with each other directly.

6.2 53BP1 MAY LINK TRANSCRIPTION FACTOR COMPLEXES TO THE CHROMATIN

It is possible that the ability of 53BP1 to function as a transcriptional regulator is due to its ability to interact with chromatin and proteins that are involved in chromatin remodelling. 53BP1 can interact with chromatin through its Tudor domain, which has been shown to be a methyl-histone binding domain (Kim *et al* 2006). In particular, it has been demonstrated that the Tudor domain of 53BP1 interacts with di-methylated histones including H4K20 and possibly H3K79 (Botuyan *et al* 2006, Huyen *et al* 2004). However, in undamaged chromatin these histone marks are buried within the nucleosome, therefore conformational changes in the chromatin are required to expose these marks and enable 53BP1 to access the chromatin. In response to DNA damage, it has been proposed that exposure of these histone marks is facilitated by post-translational modifications of histones surrounding the DSB, which promote relaxation of the chromatin, thereby allowing 53BP1 to be recruited to sites of DSBs (van Attikum and Gasser 2009). Interestingly, it has been shown that 53BP1 can interact transiently with chromatin in the absence of DNA damage (Bekker-Jensen *et al* 2005, Santos *et al* 2010). This is likely to be a result of the dynamic conformational changes that occur in

nucleosomes, which allow proteins access, even to buried regions of chromatin (Li and Widom 2004, Li *et al* 2005).

To facilitate cellular processes that involve DNA such as transcription, DNA replication and DNA repair, the structure of the chromatin needs to be altered to allow the transcription machinery and DNA repair proteins access to the DNA. This is achieved by both modifications of the tails of histones as well as by chromatin remodelling via proteins that alter the interaction between histones and the DNA. In addition to chromatin, 53BP1 interacts with several chromatin remodelling proteins. In response to DNA damage, 53BP1 has recently been shown to interact and recruit the chromatin-associated protein EXPAND1 to sites of DSBs. EXPAND1 binds to chromatin through its histone binding PWWP domain and triggers chromatin relaxation, thereby facilitating DNA repair and cell survival by increasing the accessibility of the chromatin to DNA repair proteins (Huen *et al* 2010a). Currently it is unclear whether the chromatin remodelling activities of EXPAND1 and/or the interaction with 53BP1 are required for transcriptional activation, as well as DNA repair. However, EXPAND1 inactivation resulted in chromatin condensation in unperturbed cells indicating that it is likely that it functions in other cellular processes besides the DDR. As demonstrated in this thesis, 53BP1 also interacts with the transcriptional co-activators CBP/p300 via its C-terminal region (see chapter 3). These proteins possess histone acetyltransferase activities enabling them to acetylate histones, thereby promoting chromatin decondensation. Relaxation of the chromatin results in transcriptional activation since it allows gene promoters/enhancers to become accessible to transcription factors and the basal transcription machinery. Furthermore, 53BP1 has been shown to interact with HDACs, specifically HDAC4 following DNA damage (Kao *et al* 2003). In contrast to HATs, HDACs induce chromatin compaction by deacetylating histones, which in turn results in transcriptional repression. In response to IR, HDAC4 has been shown to be important for maintaining the G2/M checkpoint since this checkpoint was abrogated in HDAC4-deficient cells (Kao *et al* 2003). It has been suggested that HDAC4 functions to repress the promoters of genes involved in the G2/M transition of the cell cycle including *CDC25C*, *CDK1* and *Cyclin B2* (Basile *et al* 2006).

These observations indicate that 53BP1 functions in transcriptional regulation at the chromatin level. Furthermore, through its ability to bind to chromatin as well as chromatin modifying proteins, it is likely that 53BP1 regulates transcription by acting as a molecular bridge to connect transcription factor complexes with the chromatin (Figure 6.1).

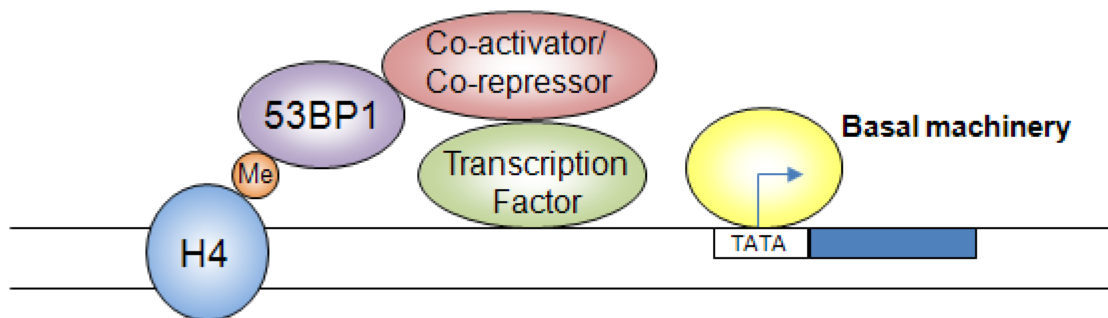


Figure 6.1 ***A model for the role of 53BP1 in gene transcription.*** *53BP1 connects transcription factor complexes with the chromatin by acting as a molecular bridge.*

Interestingly, a similar model has been suggested for the ability of BRCA1 to function as a transcriptional co-regulator (discussed below). This model provides a plausible explanation as to how 53BP1 is able to regulate NF-κB transcriptional activity without directly interacting with p65. Moreover, it also indicates how 53BP1 is able to regulate the expression of the diverse array of genes that were identified by the microarray analysis since CBP/p300 can interact with a wide variety of transcription factors. Due to 53BP1 serving as a scaffold protein, it is possible that 53BP1 may interact with other transcriptional co-activators such as PCAF and hGCN5 and co-repressors such as HDAC1 and HDAC2, as well as other chromatin remodelling proteins/complexes such as the SWI/SNF complex, and facilitate their recruitment to the chromatin.

6.3 MEDIATOR PROTEINS FUNCTION IN DNA REPAIR AND TRANSCRIPTION

Together the data presented in this thesis indicates that 53BP1 can function both in transcription and DSB repair. 53BP1 is a member of a small family of mediator proteins that also includes MDC1, PTIP, BRCA1, TOPBP1, Claspin and MCPH1. Interestingly, there is evidence that many of these mediator proteins also play a role in transcriptional regulation, as well as DNA repair.

The C-terminal region of BRCA1, which contains the BRCT domains (residues 1560-1863) was shown to activate transcription when fused to the DNA binding domain of the yeast transcription factor, Gal4. This provided the first evidence that BRCA1 was involved in transcription (Monteiro *et al* 1996) and since then a substantial amount of data has been generated supporting a role for BRCA1 in transcriptional regulation. BRCA1 has been shown to associate with RNA polymerase II holoenzyme, a component of the core transcriptional machinery (Scully *et al* 1997a). Furthermore, it can bind to several DNA binding transcription factors and regulate their transactivation activities by acting as a transcriptional co-activator or co-repressor. BRCA1 has been shown to stimulate the transcriptional activities of p53 (Zhang *et al* 1998), STAT1 (Ouchi *et al* 2000), ATF1 (Houvras *et al* 2000), NF-κB (Benezra *et al* 2003), Oct-1 (Fan *et al* 2002, Saha *et al* 2010) and the androgen receptor (Park *et al* 2000, Yeh *et al* 2000). Conversely, BRCA1 represses the transactivation activities of the c-Myc oncoprotein (Wang *et al* 1998) and the estrogen receptor (Fan *et al* 2001). Several genes

have been identified that are regulated by BRCA1 including genes involved in DNA repair such as DDB2 and XPC and cell cycle arrest such as p21, GADD45 and p27 (Harkin *et al* 1999, Hartman and Ford 2002, MacLachlan *et al* 2002, Somasundaram *et al* 1997, Williamson *et al* 2002). BRCA1 has also been shown to associate with several proteins that modify the chromatin structure including the HATs CBP/p300 and hGCN5/TRRAP (Oishi *et al* 2006, Pao *et al* 2000), the histone deacetylases HDAC1 and HDAC2 (Yarden and Brody 1999) and components of the SWI/SNF chromatin remodelling complexes including BRG1 and BRD7 (Bochar *et al* 2000, Harte *et al* 2010). Since BRCA1 can interact with RNA polymerase II and various chromatin modifying proteins, it has been proposed that BRCA1 regulates transcription by linking transcription factor complexes with the basal transcription machinery.

TOPBP1 has also been shown to function as a transcriptional regulator and this is important for its role in promoting cell growth and survival. Through interacting with the SWI/SNF complex via BRG1, TOPBP1 has been shown to repress the pro-apoptotic activity of the E2F1 transcription factor both during the G1/S transition of the cell cycle and in response to DNA damage (Liu *et al* 2004). Also, in complex with the transcription factor Miz-1, TOPBP1 inhibits the ability of Miz-1 to activate its target genes *p21* and *p15INK4B* (Herold *et al* 2002). TOPBP1 also represses the expression of the proto-oncogene c-Abl by recruiting HDAC1 to the *c-Abl* promoter (Zeng *et al* 2005). More recently, it has been demonstrated that TOPBP1 can interact with p53 and repress its transcriptional activity, as illustrated by the fact that depletion of TOPBP1 resulted in an increase in the expression of several p53 target genes involved in cell cycle arrest and apoptosis including p21, NOXA, GADD45 and BAX (Liu *et al* 2009). As well as functioning as a transcriptional co-repressor, TOPBP1 can enhance Ets-1 transcriptional activity by associating with the ePHD protein, SPBP (Sjottem *et al* 2007).

MCPH1 was initially identified as a transcriptional repressor of hTERT (human telomerase reverse transcriptase) (Lin and Elledge 2003). Subsequently, MCPH1 was shown to positively regulate the expression of CHK1 and BRCA1 through interacting with E2F1 (Lin *et al* 2005, Yang *et al* 2008b). Furthermore, MCPH1 also induces the expression of several other E2F1 responsive genes involved in cell cycle checkpoint activation, DNA repair and apoptosis including RAD51, DDB2, TOPBP1 and caspase 3 (Yang *et al* 2008b). MCPH1 has also been shown to interact with the SWI/SNF complex via its core subunits BAF170 and BAF155 (Peng *et al* 2009). Recruitment of the SWI/SNF complex to the chromatin by MCPH1 results

in relaxation of the chromatin. Loss of MCPH1 caused impaired chromatin relaxation as a result of the decreased association of SWI/SNF with the chromatin. Since MCPH1-mediated chromatin remodelling was shown to be important for recruitment of DNA repair proteins to the sites of DNA damage and therefore efficient DNA repair, it is possible that this is how MCPH1 regulates E2F1 transcriptional activity.

PTIP was identified in a yeast-two hybrid screen looking for novel interacting proteins that could potentially modulate PAX2 transcriptional activity (Lechner *et al* 2000). PAX2 is a member of the PAX family of transcription factors that are essential for organ and tissue development (Lang *et al* 2007). In *Xenopus* development, the PTIP homolog was shown to function as a transcriptional co-activator of SMAD2 in a TGFβ-dependent manner (Shimizu *et al* 2001). PTIP functions as a transcriptional regulator by stably associating with the MLL3/4 methyltransferase complex (Cho *et al* 2007, Patel *et al* 2007). By recruiting this complex to transcriptions factors including PAX2 and PAX5, PTIP promotes methylation of histone H3 on lysine 4 (H3K4) and subsequently transcriptional activation (Patel *et al* 2007, Schwab *et al* 2011). PTIP-mediated H3K4me has been shown to be important for immune system development since loss of PTIP significantly attenuated CSR in B cells (Daniel *et al* 2010, Schwab *et al* 2011). This defect in switching is due to transcriptional repression of the IgH locus caused by impaired H3K4me.

Interestingly, 53BP1, BRCA1, MCPH1, PTIP and TOPBP1 have all been shown to interact with chromatin modifying proteins suggesting that the mechanism by which mediator proteins function in transcriptional regulation is likely to be through modulating the chromatin structure. This would allow the DNA to become accessible to transcription factors and the core transcriptional machinery. Currently, it is unknown if MDC1 and Claspin also play a role in transcription. However, considering the majority of mediator proteins function in both DNA repair and transcription, it is conceivable that these proteins may also function in transcriptional regulation. Indeed this does appear to be the case for MDC1 since the mass spectrometric analysis identified both 53BP1 and MDC1 as a CBP/p300 interacting proteins (Figure 3.1).

6.4 MEDIATOR PROTEINS AND CANCER

The DDR has been suggested to act as a barrier to tumour progression by promoting cellular senescence or apoptosis of tumour cells and thereby delaying or preventing tumourigenesis (Bartek *et al* 2007). Consequently, inactivating mutations in many DDR proteins are selected for during the development of cancer including the mediator proteins BRCA1, 53BP1 and MCPH1. This allows tumour cells to overcome this barrier and progress towards malignancy.

BRCA1 plays a crucial role in suppressing tumourigenesis as demonstrated by the fact that heterozygous BRCA1 inactivating mutations are associated with an increased risk to develop breast and ovarian cancer. Typically women carrying germline mutations in BRCA1 have a 50-85% lifetime risk of developing breast cancer and a 20-40% lifetime risk of developing ovarian cancer (Fackenthal and Olopade 2007, King *et al* 2003). Breast cancers that arise in *BRCA1* mutation carriers are mostly early onset, high grade and invasive cancers that do not express the estrogen receptor, the progesterone receptor and do not have amplification of HER2, a phenotype that is termed as the 'triple negative' phenotype (Johannsson *et al* 1997). BRCA1 plays a key role in DNA repair and activation of cell cycle checkpoints including the G2/M checkpoint in response to DNA damage. Consistent with these functional roles, cells and tumours deficient for *BRCA1* exhibit severe genomic instability, characterised by aneuploidy, centrosomal amplification and chromosome aberrations such as translocations, deletions and chromosome breaks (Venkitaraman 2002). However, the genomic instability caused by BRCA1 deficiency triggers the DDR, which inhibits proliferation and induces apoptosis. Therefore, cells must acquire additional mutations to allow proliferation and tumourigenesis. Accordingly, BRCA1-mutated breast tumours have a high frequency of mutations in p53 and PTEN (Holstege *et al* 2009, Manie *et al* 2009, Saal *et al* 2008). In addition to its DDR functions, the ability of BRCA1 to regulate transcription has been observed to contribute towards its tumour suppressor activity. Cancer-associated mutations of the C-terminal region of BRCA1 abrogated the transactivation activity of BRCA1. Furthermore, cancer-associated transactivation deficient mutants of BRCA1 failed to induce p21 expression and inhibit cell cycle progression indicating that BRCA1-dependent induction of p21 contributes to the growth suppressive effects of BRCA1 (Somasundaram *et al* 1997). The link between BRCA1 in transcription and tumour suppression was demonstrated further by the fact that cancer predisposing mutations of BRCA1 were found to disrupt the interaction

between RNA polymerase II and the hGCN5/TRRAP HAT complex (Oishi *et al* 2006, Scully *et al* 1997a). Furthermore, the oncoprotein c-MYC is frequently over-expressed in BRCA1-linked breast cancer, which may be due to the ability of BRCA1 to suppress the oncogenic potential of c-Myc by repressing its transcriptional activity (Grushko *et al* 2004, Wang *et al* 1998). Moreover, BRCA1 mutations confer an increased risk for several types of steroid hormone-responsive cancers such as breast, endometrial, cervical and prostate. The latter is androgen responsive, whereas the other tumour types are estrogen responsive (Rosen *et al* 2005). This epidemiologic data correlates with the role of BRCA1 in regulating estrogen and androgen receptor activity.

MCPH1 has also been shown to be important for protecting against genomic instability and tumourigenesis. Loss of MCPH1 has been associated with breast, ovarian and prostate cancers (Rai *et al* 2006). Similar to BRCA1, loss of MCPH1 resulted in an increase in chromosomal aberrations and centrosomal abnormalities (Rai *et al* 2006). The genomic instability caused by loss of MCPH1 is due to the involvement of MCPH1 in DNA repair and cell cycle checkpoint control. Furthermore, the transcriptional function of MCPH1 is also likely to contribute to genomic instability since MCPH1 activates genes involved in genome maintenance by functioning as a transcriptional co-activator of E2F1 (Yang *et al* 2008b). In addition to genomic instability, another hallmark of cancer is the ability to proliferate indefinitely (Negrini *et al* 2010). The majority of tumour cells achieve this by enhancing the activity of telomerase by up-regulating the expression of the catalytic subunit hTERT (Shay and Bacchetti 1997). Therefore, this may be another way loss of MCPH1 causes tumourigenesis since MCPH1 acts as a repressor of hTERT transcriptional activity (Lin and Elledge 2003).

Loss of 53BP1 has been observed in a wide range of carcinomas including lung, gastric, laryngeal and renal as well as melanoma (Gorgoulis *et al* 2005, Nuciforo *et al* 2007). Furthermore, progression from precancerous lesions to carcinomas was associated with loss of 53BP1 in some tumours (Gorgoulis *et al* 2005). More recently, loss of 53BP1 has been associated with triple-negative breast cancer and familial breast cancer caused by *BRCA1/2* mutations (Bouwman *et al* 2010). Furthermore, loss of 53BP1 in triple-negative breast tumours correlated with a greater likelihood of metastasis and decreased survival. This suggests that mutations in *53BP1* might confer a survival advantage in the absence of BRCA1 and BRCA2. This is probably due to partial restoration of HR in BRCA1-deficient cells (Bunting *et al* 2010). However, constitutive activation of NF-κB is frequently observed in

breast cancer, in particular triple-negative breast cancer (Yamaguchi *et al* 2009). Therefore, since loss of 53BP1 resulted in an increase in NF-κB activity, it is possible that this may also be a mechanism by which loss of 53BP1 contributes to the development of breast cancer. Furthermore, constitutive NF-κB activation is also associated with many other cancers including those that were found to have lost 53BP1 indicating that inactivation of 53BP1 may be selected for during tumourigenesis as it contributes to dysregulation of NF-κB activity, thereby allowing tumour cells to survive (Prasad *et al* 2010).

In contrast to BRCA1, MCPH1 and 53BP1, TOPBP1 was found to be frequently over-expressed in breast cancer and this correlated with poor survival (Liu *et al* 2009). It has been suggested that this is likely to be due to the ability of TOPBP1 to promote cell survival by inhibiting p53-dependent and E2F1-dependent apoptosis, as well as G1/S cell cycle arrest.

Taken together these studies demonstrate that both the DNA repair and transcription functions of the mediator proteins are important for preventing tumourigenesis. Currently, there have been no reports linking MDC1, PTIP and Claspin to tumourigenesis. However, given that these proteins have been shown to be important for maintaining the integrity of the genome, it is likely that mutations in these genes do exist in tumours.

In conclusion, the data presented in this thesis suggest that 53BP1 functions in transcriptional regulation, as well as DNA repair by acting as a scaffold protein to provide structural support for DNA repair and transcription in the context of chromatin.

CHAPTER 7

CHAPTER 7 FUTURE WORK

7.1 VALIDATION OF 53BP1 SIRNA PHENOTYPE

siRNA induces specific gene silencing through RNA interference and is widely used to study the functions of genes. Initially, siRNA was thought to be very specific and only targeted the gene of interest. However, it is now recognised that siRNA can induce off-target effects, which occur when the siRNA down-regulates other genes besides the gene of interest (Jackson and Linsley 2010). Data presented in chapter 5 demonstrated that siRNA-mediated depletion of 53BP1 caused an increase in NF-κB activity in response to TNFα and IR. Therefore, to prove that this phenotype is due to reduced 53BP1 expression and not due to off-target effects, a rescue experiment needs to be performed to determine if this phenotype can be reversed through expression of a siRNA-resistant 53BP1 plasmid. Initial complementation experiments using transient transfection of a siRNA-resistant 53BP1 expression construct into 53BP1 siRNA depleted cells failed due to low transfection efficiency of the 53BP1 plasmid. The reason for this is likely to be due to the large size of the expression construct, which is known to reduce transfection efficiency. To counteract this problem, a siRNA resistant retroviral 53BP1 expression construct will have to be constructed and cell lines stably expressing endogenous levels of 53BP1 generated. The use of a retrovirus will prevent loss of 53BP1 expression that occurs in stable cell lines over time of prolonged in *vitro* culturing, which still retain the antibiotic selection marker (GS Stewart, unpublished).

7.2 DOES LOSS OF 53BP1 PROMOTE NF-κB-MEDIATED CELL SURVIVAL?

NF-κB plays a crucial role in cell survival as illustrated by the fact that it is constitutively activated in a variety of diseases including several types of cancer (Courtois and Gilmore 2006, Karin 2006). Moreover, loss of 53BP1 has been shown to be associated with cancer such as BRCA1-deficient breast cancer (Bouwman *et al* 2010). Since an increase in NF-κB activity was observed in 53BP1 deficient cells following treatment with TNFα and IR, it would be of interest to ascertain if loss of 53BP1 is promoting cell survival in an NF-κB-dependent manner. To establish if this is the case, cell viability, survival and apoptosis assays

such as an MTS/trypan blue dye exclusion assay, clonogenic survival and Annexin V staining measured by FACS or analysing caspase 3 cleavage respectively would be performed in cells transfected with control and 53BP1 siRNA. Furthermore, these experiments could also be conducted in the presence and absence of NF-κB inhibitors to determine if any effects observed are dependent on NF-κB activity. In addition, the mRNA expression levels of anti- and pro-apoptotic NF-κB responsive genes could be measured by qRT-PCR in control and 53BP1 depleted cells to determine if 53BP1 is differentially regulating these genes and therefore influencing the final outcome of the NF-κB response. Together these studies would help to elucidate the physiological consequence of 53BP1-mediated repression of NF-κB activity and may indicate why *53BP1* is inactivated in tumours.

7.3 MECHANISM OF 53BP1-MEDIATED REGULATION OF p53 AND NF-κB

In most instances HATs and HDACs function as transcriptional co-activators and co-repressors respectively through regulating the acetylation status of both histones and transcription factors such as NF-κB and p53. The findings presented in chapter 5 indicate that 53BP1 is negatively regulating NF-κB transcriptional activity and that this is likely to be via an indirect mechanism given that no interaction was observed between 53BP1 and p65. Therefore, since 53BP1 can interact with the HATs CBP/p300, as demonstrated in chapter 3, it is possible that 53BP1 may be modulating the acetylation status of NF-κB through regulating the interaction between p65 and CBP/p300. Therefore, a co-immunoprecipitation could be performed in the presence and absence of 53BP1 to ascertain if the interaction between CBP/p300 and p65 is enhanced in cells lacking 53BP1. The acetylation status of p65 could also be assessed by immunoprecipitation to determine if loss of 53BP1 affects the CBP/p300-mediated acetylation of p65. Furthermore, to establish if the increase in NF-κB transcriptional activity is CBP/p300-dependent, cells could be depleted of both 53BP1 and CBP/p300 and the transcriptional activity of NF-κB analysed by luciferase assay. In addition, since 53BP1 can interact with HDAC4 (Kao *et al* 2003), it would be of interest to investigate whether, in a manner similar to ARF, 53BP1 is repressing the transactivation potential of NF-κB through promoting the association of p65 with HDACs (Rocha *et al* 2003). To address this, control and 53BP1 depleted cells could be treated with trichostatin A to determine if

inhibiting HDAC activity abolishes 53BP1-mediated repression of NF-κB activity. In addition, a co-immunoprecipitation could be conducted to establish if the interaction between p65 and HDACs is enhanced in the presence of 53BP1.

It is well established that 53BP1 can associate with chromatin, therefore to determine if 53BP1 is modulating NF-κB transcriptional activity through regulating the recruitment of CBP/p300 and HDACs to the promoters of NF-κB responsive genes, chromatin immunoprecipitation (ChIP) assays could be utilised. However, initially NF-κB responsive genes whose expression is regulated by 53BP1 would need to be identified using either qRT-PCR or a microarray approach. Once identified, ChIP could be performed in wild type and 53BP1 deficient cells to determine whether 53BP1 binds to the promoters of these genes. Subsequently, ChIP could be utilised to establish whether CBP/p300 or HDACs are present on the promoters of these genes and whether depletion of 53BP1 affects the recruitment of these co-activators and co-repressors to the gene promoters. In addition, cells could be depleted of 53BP1 and either CBP/p300 or HDACs to ascertain if the expression of these 53BP1-regulated genes is altered by loss of CBP/p300 or HDACs.

As well as an increase in NF-κB transcriptional activity, depletion of 53BP1 in response to IR and TNFα resulted in a sustained NF-κB response suggesting a role for 53BP1 in regulating the termination of the NF-κB response. NF-κB transcriptional activity can be terminated through post-translational mechanisms such as deacetylation and dephosphorylation. Recently, the phosphatase WIP1 has been demonstrated to dephosphorylate p65 (Chew *et al* 2009). Since phosphorylation of p65 is sustained in the absence of 53BP1, it is possible that loss of 53BP1 affects the ability of WIP1 to dephosphorylate p65. To address this, WIP1 could be over-expressed in control and 53BP1 siRNA treated cells to see if this corrected the phenotype. Alternatively, the inability of p65 to be dephosphorylated in cells lacking 53BP1 may be due to p65 not being efficiently deacetylated. The studies highlighted above may shed some light on whether the acetylation status of p65 is contributing to the sustained phosphorylation of NF-κB.

The ubiquitin-mediated proteasomal degradation of p65 has been described as a termination mechanism. Interestingly, depletion of 53BP1 resulted in elevated p65 basal levels, as well as an increase in NF-κB transcriptional activity. To ascertain if 53BP1 is affecting the expression of p65 at the transcriptional or post-transcriptional level, the mRNA level of p65

could be assessed by qRT-PCR in control and 53BP1 deficient cells. In addition, these cells could be treated with either transcription inhibitors such as actinomycin D or protein synthesis inhibitors such as cycloheximide and the half-life of p65 assessed to determine if 53BP1 is regulating p65 mRNA or protein stability respectively. Furthermore, to establish if 53BP1 is promoting the ubiquitin-mediated proteasomal degradation of p65, control and 53BP1 deficient cells would be treated with the proteasome inhibitor, MG132 and the protein levels of p65 assessed by Western blot analysis. However, the substantial increases in p65 phosphorylation observed in 53BP1 depleted cells are unlikely to be solely caused by the modest increase in basal p65 levels indicating that 53BP1 may regulate NF-κB transcriptional activity by several mechanisms.

Data presented in chapter 3 indicates that 53BP1 can differentially regulate p53 target genes through acting as a modulator of p53 transcriptional activity. However, this work only focused on p21, PUMA and HDM2, therefore to understand precisely the role of 53BP1 in regulating p53 function, other p53 target genes would need to be identified whose expression was regulated by 53BP1. Since p53 target genes are differentially expressed in response to IR (Zhao *et al* 2000) and no p53 target genes were identified by the microarray study following 4 hours of IR, a time course could be performed over a 24 hour period in cells treated with control and 53BP1 siRNA to identify 53BP1-regulated p53 responsive genes. Once these genes have been identified either by using qRT-PCR or a microarray approach, ChIP experiments similar to those suggested for NF-κB could be conducted to try and establish if 53BP1 is modulating p53 transcriptional activity at the promoter level through regulating the recruitment of co-activators and co-repressors to the promoters of those genes that were identified as being differentially expressed by 53BP1. In addition, acetylation of p53 by CBP/p300 enhances the transactivation potential of p53, therefore it would also be interesting to address if 53BP1 is affecting the acetylation status of p53 through influencing the interaction of p53 with CBP/p300.

Taken together these studies could potentially elucidate the mechanism(s) by which 53BP1 regulates the transcriptional activities of p53 and NF-κB.

CHAPTER 8

CHAPTER 8 REFERENCES

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