REGULATION OF THE ATR SIGNALLING PATHWAY BY ADENOVIRUS

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A thesis submitted to the
University of Birmingham
for the degree of
DOCTOR OF PHILOSOPHY

School of Cancer Sciences
CRUK Institute for Cancer Studies
The Medical School
University of Birmingham

SEPTEMBER 2012

Dedicated to my parents,

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And

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ABSTRACT

Adenovirus has evolved to bypass, or inactivate host cell cycle checkpoints that would otherwise inactivate cell cycle arrest or apoptotic programmes in the infected cell. Adenovirus 5 (Ad5) inhibits both ATM and ATR activation, whereas Adenovirus 12 (Ad12) inhibits ATM activation and differentially regulates ATR activation. However, both Ad5 and Ad12 types inhibit ATR-dependent Chk1 phosphorylation. Ad5 E4orf3 promotes the relocalization of the MRN complex in order to inhibit Chk1 activation during infection, whereas Ad12 E4orf3 is unable to inactivate MRN by this method.

To determine how Ad12 inhibits the ATR-dependent Chk1 phosphorylation we investigated the relationship between Ad12 and known mediators of the ATR signalling pathway required for Chk1 phosphorylation and activation. Here we show that Ad12 has evolved to inhibit Chk1 phosphorylation by targeting the ATR/ATRIP activator, TopBP1 for degradation. We have determined that Ad12 E4orf6, independent of E1B-55K, associates with the cellular Cul2-containing ubiquitin ligase to promote TopBP1 degradation. Indeed, using RNA interference we have shown that Ad5 and Ad12 differentially activate Cullin-containing ubiquitin ligase complexes during infection such that Ad5 utilizes Cul5, and Ad12 utilizes Cul2. Furthermore, we have also determined that Ad12 E4orf3 promotes the degradation of two ATR signalling pathway mediator proteins, Timeless and Tipin, in an Ad12E1B-55k/E4orf6-independent, and Cul2-dependent fashion, during infection. Taken together, our data provides evidence to indicate that Ad12 inhibits ATR-dependent activation of Chk1 by targeting TopBP1, Timeless, and Tipin for Cul2-dependent degradation.

Given the ability of Ad oncoproteins to target cellular tumour suppressor gene products, many researchers have strived to identify novel, cellular Ad oncoprotein-interacting

proteins using a wide range of techniques. Previous research from our laboratory identified the centrosomal protein, WDR62, as possible E1B-55K interacting protein. The second aim of this study was to expand our current knowledge of this protein and determine its role during infection. Here we show that E1B-55K interacts with WDR62 *in vivo* and colocalizes with it at centrosomes. We also provide evidence to show that WDR62 functions in the cellular DNA damage response. Indeed, cells depleted of WDR62 by RNA interference resulted in a UV-sensitive phenotype, defects in ATR activation and G_2/M checkpoint control, as well as displaying supernumerary centrosome during mitosis. Furthermore, we identified by mass spectrometry, BRCA1, ATR, RPA70, and DNA-PK as possible interacting-proteins for WDR62. Taken together, our data provide evidence for novel functions of a cellular E1B-55K interacting protein, WDR62, in the ATR signalling pathway, and give further credence to the importance of studying the biological functions of adenovirus oncoproteins to further our understanding of fundamental cellular signalling pathways.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisors Andy Turnell and Grant Stewart, for all their support, advice and supervision that they have given me during my time I Birmingham, for which I am eternally grateful. In particular I would like to thank Andy for initially giving me the opportunity to do my PhD with him, as well as being my supervisor and my friend, having an open door policy for even the simplest of scientific queries, and constantly reminding me that Nottingham Forest have won back to back European cups, which Manchester united have yet failed to achieve. I would also like to thank Roger Grand for his advice, resolute encouragement, and support, in particular his help with mathematical calculations in tissue culture.

I am also grateful for the funding given to me by The University of Birmingham Medical School. Much of the work carried out in this thesis could not have been possible without the kind gifts of reagents from Thomas Dobner, David Cortez, Iain Morgan, Naoko Yoshizawa-Sugata, and members of the AT group. A special mention goes to Ashley Martin and Neil Shimwell for their help with mass spectrometry.

I would also like to thank members of the Turnell/Grand, AT, and Stankovic groups, past and present for their support and friendship. I would like to thank Andrew Blackford for his help and advice on data presented in chapter three of this study. Two deserved special mentions go to the ‘Ginger legend’ Garry Sedgwick and Natalie Forrester. Garry took me under his wing when I first arrived to Cancer Sciences and truly made me feel welcome and quickly became one of my good friends, as well as making my time in the lab fun with his dry humour and ‘interesting’ conversations. Natalie provided the female touch in the lab, as well as being ‘one of the lads’ with

great banter, accompanying me to football matches at Old Trafford, keeping me in check, and also just being truly great friend. I would also like to thank my former housemate, and good friend, David Da Costa, who has supported me during my thesis, in particular during late night thesis writing. A special mention also goes to my childhood friends Man Voong, Robert Horne, Martin Coxon and Jeffery Arnold, in particular my best friend Man who has always been there when I was frustrated and needed a break from writing, and has helped make my time at home writing my thesis endurable.

Finally I would like to thank my family for putting up with me during the PhD, especially during the write up. I would like to thank my amazing wife Krupa for her continued love and support and putting up with me in general, whom I love with all my heart. I would also like to show my appreciation to my two sisters Sonal and Nisha, for their love and encouragement. And finally, my parents Nalin and Vibhuti, to whom I would like to express my gratitude and never ending love for their guidance and always believing me, to them I dedicate this thesis.

Fear less, hope more, eat less, chew more, whine less, breathe more, talk less, say more, hate less, love more, and good things will be yours

Swedish proverb

Try not to become a man of success but a man of value.

Albert Einstein

Science is simply common sense at its best, that is, rigidly accurate in observation, and merciless to fallacy in logic.

Thomas Huxley

Science is wonderfully equipped to answer the question "How?" but it gets terribly confused when you ask the question "Why?"

Erwin Chargaf

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LIST OF ABBRIEVIATIONS

53BP1	p53-binding protein 1
9-1-1	Rad9-Hus1-Rad1
Ad	Adenovirus
Ad5	adenovirus type 5
Ad12	adenovirus type 12
AdPol	DNA polymerase
ASPM	abnormal spindle-like, microcephaly associated
A-T	Ataxia telangiectasia
APC/C	Anaphase promoting complex/cyclosome
APS	Ammonium persulphate
ATCC	American Type Culture Collection
ATLD	Ataxia telangiectasia-like disorder
ATM	Ataxia-Telangiectasia Mutated
ATR	ATM-Rad3-related
ATRIP	ATR-interacting protein
BAK	BCL2 antagonist killer
BAX	BCL2-associated X
BCL2	B-cell lymphoma-2
BLM	Bloom helicase
BP	Base pairs
BRCA1	Breast cancer susceptibility gene 1
BRCT	BRCA1 C-terminal
BSA	Bovine Serum Albumin
BUBR1	Budding uninhibited by benzimidazoles 1 homolog beta
C-terminal	Carboxy-terminal

CAR	Coxsackievirus-adenovirus receptor
CBP	CREB binding protein
CENPJ	Centromeric protein J
CDC	Cell division cycle
CDK	Cyclin-dependent kinase
CDK5RAP2	Cyclin-dependent kinase 5 regulatory associated protein 2
CK2	Casein kinase 2
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CMV	Cytomegalovirus
CR	Conserved regions
CRL	Cullin ring ligase
CtBP	C-terminal binding protein
CtIP	CtBP-interacting protein
Cul	Cullin
DAPI	4’,6-diamidino-2-phenylindole
Daxx	Death domain-associated protein
dCMP	Deoxycytidine monophosphate
DBP	DNA binding protein
DDR	DNA damage response
DMEM	Dulbecco’s modified Eagles medium
DMSO	Dimethyl-sulphoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DSBs	DNA double strand breaks
DTT	Dithiothrietol
DUB	De-ubiquitylating

E1A	early region 1A
E1B	early region 1B
E1B-AP5	E1B55K-associated protein 5
E4orf3	Early region 4 open reading frame 3
E4orf6	Early region 4 open reading frame 6
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
FA	Fanconi anaemia
FANCD2	Fanconi anaemia complementation group D2
FCS	Foetal calf serum
FHA	Forkhead-associated
GLI1	Glioma-associated oncogene homology
GST	Glutathione S-transferase
H2A	Histone 2A family member
H3K9me3	H3 at lysine residue 9
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HEK	Human embryonic kidney cells
HER	Human embryonic retinoblast cells
HIV-1	Human immunodeficiency virus type 1
HNRPU	Heterogeneous nuclear ribonucleoprotein U-like
HPV	Human papillomavirus
HRP	Horseradish peroxidase
HTLV-1	Human T-cell lymphotrophic virus 1
HU	Hydroxyurea
ICP0	Infected cell protein 0
IF	Immunofluorescence

IgG	Immunoglobulin G
IP	Immunprecipitaion
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IR	Ionising radiation
ITR	Inverted terminal repeats
JNK	c-Jun N-terminal kinase
KAP1	KRAB-associated protein 1
kb	Kilo base
kDa	Kilo dalton
KSHV	Kaposi's sarcoma-associated herpesvirus
LB	Luria broth
LiDS	DNA ligase IV deficiency syndrome
LTag	Large tumour antigen
MAD2	Mitotic arrest-deficient 2
MCL1	Myeloid cell leukaemia 1
MCPH	Autosomal recessive primary microcephaly
MDC1	Mediator of DNA damage checkpoint 1
MDM2	Murine double minute 2
MG132	N-(benzyloxycarbonyl)leucinylleucinylleucinal
MHC	Major histocompatibility complex
MLP	Major late promoter
MCM	Minichromosome maintenance
Mre11	Meiotic recombination 11
MRN	Mre11-Rad50-NBS1
mRNA	Messenger RNA
mRNP	mRNA ribonucleoprotein
N-terminal	Amino-terminal

NBS	Nijmegen breakage syndrome
NEDD8	Neural precursor cell-expressed developmentally down-regulated 8
NES	N-terminal nuclear export signal
NF1	Nuclear factor 1
NF2	Nuclear factor 2
NF-κB	Nuclear factor-kappa B
NHEJ	Non-homologous end joining
NLS	Nuclear localization signal
Non-sil	Non-silencing
NRS	Nuclear retention signal
Oct1	Octamer-binding protein 1
P	Phosphorylated
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PCC	Premature chromosome condensation
PCR	Polymerase chain reaction
PCNA	Proliferating cell nuclear antigen
p.f.u	Plaque forming unit
PIKK	Phosphatidylinositol 3-kinase-like kinase
PML	Promyelocytic leukemia
POD	PML oncogenic domains
PP2A	Protein phosphatase 2A
pTP	TP precursor
OFC	Occipitofrontal head circumference
ORF	Open reading frame
RAP80	Receptor protein 80

Rb	Retinoblastoma
RBX1	RING-box 1
RIDDLE	Radiosensitivity, immunodeficiency, dysmorphic features and learning difficulties
RING	Really interesting new gene
RNA	Ribonucleic acid
RNF168	RING-finger 168
RNF8	RING-finger 8
RNP	Ribonucleoprotein
RPA	Replication protein A
RSR	Rad17-RCF2-5
RSV	Rous sarcoma virus
RT-PCR	Reverse transcriptase PCR
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SDT	Ser-Asp-Thr
SMC1	Structural maintenance of chromosomes 1
SOC	Super optimal catabolite
SOCS	Suppressor of cytokine signalling
SSBs	Single-stranded DNA breaks
STIL	SCL/TAL1-interrupting locus
SUMO-1	Small ubiquitin-like modifier 1
SV40	Simian virus 40
TBS	Tris-buffered saline
TBST	TBS containing Tween 20
TEMED	N,N,N’,N’ -tetramethylethylenediamine-1,2-diamine

TFIID	Transcription factor IID
TIF1	Transcriptional intermediary factor 1
Tipin	Timeless-interacting protein
Tim	Timeless
Tim-2	Timeout
TopBP1	Topoisomerase (DNA) II binding protein 1
TP	Terminal protein
TRIM	Tripartite motif
γTuRC	Gamma-tubulin ring complex
Ub	Ubiquitin
Ubc13	Ubiquitin-conjugating enzyme 13
USP1	Ubiquitin-specific protease 1
UV	Ultraviolet
WB	Western blotting
WDR62	WD40 repeat-containg protein 62
wt	Wild-type
VHL	von Hippel-Lindau
VRC	viral replication centres
XRRC4	X-ray repair complementing defective repair in Chinese hamster cells 4
XP	Xeroderma pigmentosum

CHAPTER 1

INTRODUCTION

1.1. ADENOVIRUS

1.1.1. DNA tumour viruses

Cell growth and division is a very tightly controlled process that is governed by a complex balance of signalling pathways. Defects in these signalling pathways can lead to unrestrained cell proliferation leading to the formation of tumours, causing a disease called cancer. Cancer as a disease has been recognised for centuries, dating back as far as the ancient Egyptian era where the aetiology of the disease was attributed to the "will of the gods" (Diamandopoulos 1996). Over the centuries many theories on the cause of cancer have been discussed, however it was not until the beginning of the 20th century that an infectious aetiology was considered (Javier and Butel 2008). In 1911 Peyton Rous founded the scientific field of tumour virology by discovering an avian retrovirus, Rous sarcoma virus (RSV), which was found to induce tumours in chickens (Rous 1911). The significance of this ground breaking research was not fully appreciated for many years; however it did eventually pave way for the discovery of many cancer-causing viruses, which in turn has vastly contributed to the discovery of oncogenes and tumour suppressor genes. The study of cancer-causing viruses has been split into two general fields; RNA tumour viruses and DNA tumour viruses. A representative member from every group of DNA tumour viruses that have been studied which include adenoviruses, polyomaviruses, papillomaviruses, and herpesviruses, have been shown to cause tumours in animals or humans (Levine 1988). Furthermore, in 2002 it was estimated that 1.9 million new cases of cancer (17.8% of human cancers worldwide) were attributable to the infectious agents: human papillomavirus (HPV), human immunodeficiency virus type 1 (HIV-1), hepatitis B virus (HBV), hepatitis C virus

(HCV), Epstein-Barr virus (EBV), Kaposi's sarcoma-associated herpesvirus (KSHV) and the human T-cell lymphotrophic virus 1 (HTLV-1) (Parkin 2006).

Unlike RNA tumour viruses, DNA tumour virus viral oncogenes bear little or no resemblance to any cellular proto-oncogenes. Instead, the products of these viral oncoproteins serve to disrupt host cellular processes, many of which are down regulated in cancer cells, such as apoptosis and cell cycle checkpoint activation, and therefore increase the chances of the cell becoming tumorous. In fact, under the correct experimental conditions, primary cells can undergo transformation once transfected with viral oncogenes, causing them to bypass normal growth barriers and gain tumour-like characteristics. These transformed cells display characteristic morphological and growth changes such as reduced-serum requirements, decreased adhesion, disruption of the cytoskeleton, loss of contact inhibition, and immortalisation (Hanahan and Weinberg 2000).

With the exception of a few (EBV, KSHV, HPV), at present, most DNA tumour viruses are not thought to be causative agents of human cancers, albeit the fact that they can transform cells in culture and cause tumours in animal models under certain conditions. Instead, over the past 40 years the study of DNA tumour viruses and their oncogenes has revolutionised our understanding of many cellular processes which include cell cycle regulation, transcription and DNA replication (Javier and Butel 2008). One example of the impact that tumour virology has had in the scientific field is the discovery of the tumour suppressor, p53. Immunoprecipitation studies using antibodies against the simian virus 40 (SV40) large tumour antigen (LTag) in transformed cells led to the discovery of p53 (Lane and Crawford 1979; Linzer and Levine 1979), which later was also found to interact with adenovirus (Ad) early region 1B 55kDa (E1B-55K) oncoprotein in Ad-transformed cells (Sarnow et al. 1982), and also the HPV E6

oncoprotein (Werness et al. 1990). Furthermore, the retinoblastoma gene product, pRB, has also been shown to interact with SV40 LTag, Ad early region 1A (E1A), and HPV E7 oncoproteins; pRB interaction with E1A served to provide significant insights into pRB function (DeCaprio et al. 1988; Whyte et al. 1988; Dyson et al. 1989).

1.1.2. Adenovirus identification and classification

Adenoviruses are non-enveloped icosahedral DNA tumour viruses, with a linear double-stranded (ds) DNA genome that undergo a lytic cycle of replication. This family of viruses have been studied for the best part of 60 years, in which time they have been used as a model system to study viral entry, viral assembly, transcription and DNA replication, mRNA splicing, cell cycle control, cell transformation and tumourigenesis. Originally discovered in 1953 at the National Institutes of Health (Bethesda, Maryland) by researchers trying to identify the virus of the "common cold", viral agents were isolated from the adenoid and tonsil tissue grown in cell culture, and hence, subsequently named adenovirus (Rowe et al. 1953). The next significant discovery in the adenovirus field came in 1962 when Trentin and colleagues discovered that adenovirus type 12 (Ad12) was able to induce tumour formation in new born rodents in a laboratory setting (Trentin et al. 1962). This was not only a significant discovery in the adenovirus field, but in the tumour virus field as a whole, as it was the first time that a human virus was shown to cause cancer, albeit under experimental conditions (Javier and Butel 2008).

There are currently 5 accepted genera in the *Adenoviridae* family which have been determined by their common ancestor, and these are *Mastadenoviridae*, *Aviadenoviridae*, *Atadenovirus*, *Siadenoviridae*, and *Ichtadenovirus.* Avianadenoviruses and Ichatadenoviruses infect birds and fish respectively, whereas

Atadenoviruses and Siadenoviruses have a slightly broader range of host which include ruminants, reptilian, and avian hosts (Davison et al. 2003). Mastadenoviruses infect mammalian hosts, of which there are over 50 known human serotypes that have been subdivided into groups A-G according to their sequence homology, oncogenic potential in rodents, and their capacity to agglutinate erythrocytes of rat, monkey and human (see Table 1.1) (Russell 2009). Members of the group A adenoviruses which includes Ad12 are known to be highly oncogenic, which is in contrast to group C viruses that includes the widely studied adenovirus type 5 (Ad5) which has been shown to be non-oncogenic (Trentin et al. 1962; Trentin et al. 1968; Mackey et al. 1979). Our current understanding is that adenoviruses are not causative agents of cancer in humans; however DNA from both oncogenic and non-oncogenic viruses are able to transform human and rodent primary cells (McBride and Wiener 1964; Russell 2009). The non-oncogenic Ad5 E1 DNA is able to transform human embryonic kidney cells (HEK), whereas oncogenic Ad12 E1 DNA is able to transform human embryonic retinoblast cells (HER) (Graham et al. 1977; Byrd et al. 1982).

Adenoviruses readily infect a broad range of species and tissues; however their ability to cause disease is relatively restricted as a result of the effective defences mounted by the infected host. Despite this, it is known that approximately a third of the human Ad serotypes can cause respiratory, gastrointestinal, and ocular diseases that occur in children, military recruits, and immunocompromised individuals (Echavarria 2008). Furthermore, it is known that some adenovirus serotypes can cause pneumonia, hepatitis, hemorrhagic cystitis, colitis, pancreatitis, meningoencephalitis, and disseminated disease in AIDS and other immunocompromised patients (Echavarria 2008).

Table 1.1. Classification of human adenoviruses.

Group	Serotypes	Oncogenicity in rodents	Transformation *in vitro*
A	12, 18, 31	High	Yes
B1	3, 7, 16, 21, 50	Moderate	Yes
B2	11, 14, 34, 35, 55	Moderate	Yes
C	1, 2, 5, 6	Low or none	Yes
D	8, 9, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-49, 51, 53, 54	Low or none	Yes
E	4	Low or none	Yes
F	40, 41	None reported	Yes
G	52	None reported	Unknown

1.1.3. Adenovirus structure and genome

Adenoviruses are relatively large viruses that weigh around 150 MDa and have a diameter of approximately 70 nm. A combination of electron microscopy and X-ray crystallography techniques have contributed towards elucidating the structure of adenovirus virions, whereby it has been determined that the virion is composed of two major structural elements, the outer capsid and the core. It was initially determined that adenoviruses have a nonenveloped icosahedral structure, with 240 surface features (homotrimeric hexons) and long fibres with terminal knobs extending from penton bases at the 12 vertices, which together form the outer caspid (Brenner and Horne 1959; Horne et al. 1959; Valentine and Pereira 1965). Furthermore, proteins IIIa, VI, VIII, and IX have all been shown to associate with the hexons and pentons, and have been implicated in helping to cement the virion capsid (Saban et al. 2006).

Adenoviruses have linear, dsDNA genomes, that are 26-45 kb in size, which ranks it as medium-sized when compared to other DNA viruses, and encode over 40 different proteins (Davison et al. 2003). The Ad genomes are characterized by inverted terminal repeats (ITR) approximately 100-140 bp in size, and are condensed in the core of the virion, where they are associated with the proteins V, VII, and X, as well as the terminal protein (TP) which binds covalently to the 5' termini of each ITR and acts as a primer for DNA replication (Smith et al. 2010; Rauschhuber et al. 2011). The Ad infectious cycle can be split in two phases, early and late, which are separated by the commencement of DNA replication (Russell 2000). Therefore the Ad genome by convention is organized into three sections; the early region which consists of 6 transcription units (E1A, E1B, E2A, E2B, E3, and E4), the intermediate region with 2 transcription units (IX and IVa2), and the late region which consist of a single transcription unit which is transcribed to produce five families of late mRNAs [L1-L5]

(Fig. 1.1;(Tauber and Dobner 2001). The E1, E2, and E4 regions are discussed below. The E3 region is required for immune evasion, whereas the intermediate and late genes products are essential for transcriptional activation of the major late promoter (MLP) to regulate the early to late switch, and synthesis of structural proteins respectively (Russell 2000).

1.1.4. Adenovirus DNA replication

The adenovirus fibre and penton base proteins play very distinct roles in the uptake of the adenovirus particle into the cell. The fibre protein promotes entry into the cell through its interactions with various cell receptors which include the major histocompatibility complex (MHC) class I molecule, and the coxsackievirus-adenovirus receptor (CAR), which tethers the virus to the cell surface (Wu and Nemerow 2004). Binding of the penton base protein to a coreceptor (which is an αv integrin for all adenovirus subgroups with the exception of subgroup F) triggers signals for virus internalization by clathrin-coated pits (Mathias et al. 1998; Meier and Greber 2003). Capsid dissociation and structural rearrangements that occurs as a result of endosome acidification allows the virus particle to be released into the cytoplasm, where it is then transported to the nuclear pore complex, allowing the viral DNA to be transported into the host cell nucleus, allowing it to use host cell transcription machinery for gene expression (Wu and Nemerow 2004).

Ad DNA replication occurs around 6-8 hours post-infection, and utilizes the three viral proteins transcribed from the E2 region. These are the TP precursor (pTP) and the DNA binding protein (DBP), and DNA polymerase (AdPol), encoded by the E2A and E2B genes, respectively (van der Vliet and Levine 1973; de Jong et al. 2003; Liu et al. 2003). Furthermore, *in vitro* studies have shown that there are at least three cellular

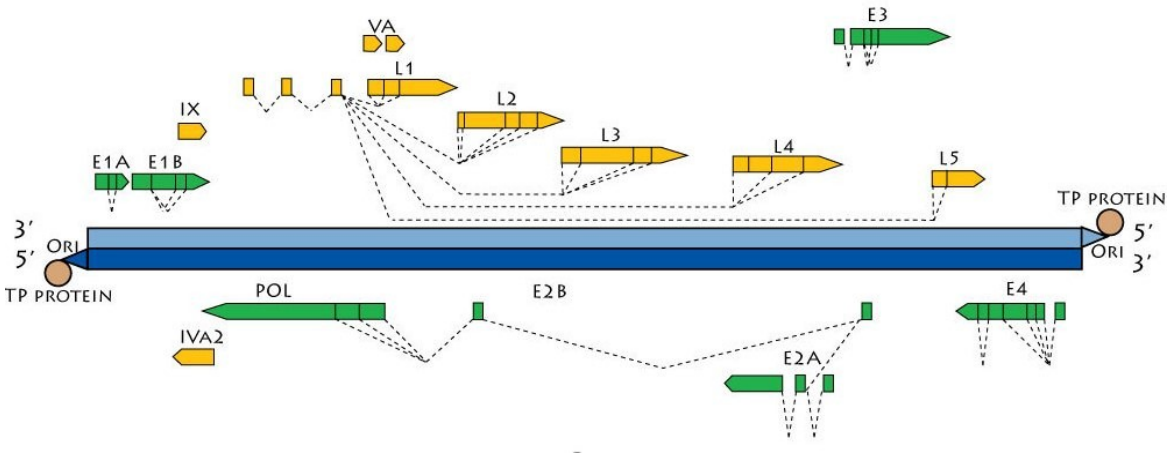

Fig. 1.1. Adenovirus genome organization. The relative positions and orientation of the major transcription factors are shown. The early genes are shown in green and the intermediate and late genes are shown in yellow. (Adapted from http://viralzone.expasy.org/all_by_species/183.html)

proteins that play a vital role in Ad DNA replication, which include the transcriptional factors nuclear factor 1 (NF1) and octamer-binding protein 1 (Oct1) (also known as nuclear factor 3), as well as nuclear factor 2 (NF2) which is a type 1 DNA topoisomerase (Nagata et al. 1982; Nagata et al. 1983; Pruijn et al. 1986).

Viral DNA replication is initiated by a novel protein-priming mechanism at either end of the linear genome, in which Ad pol catalyses the covalent linkage between the β-OH group of a serine residue in pTP and the α-phosphoryl group of the 5'-terminal residue deoxycytidine monophosphate (dCMP) to form the pTP/dCMP complex, which acts as a primer for synthesis of the nascent strand (Liu et al. 2003). DBP promotes the binding of NF1 to Ad pol, whereas Oct1 binds to pTP, which enhances their affinity for viral DNA sequences (van Leeuwen et al. 1997; de Jong et al. 2003). The combined actions of NF1 and Oct1 can increase DNA replication up to 200-fold, and these cellular proteins, together with the three viral proteins pTP, DBP, and Ad pol, form the stable pre-initiation complex (de Jong and van der Vliet 1999; de Jong et al. 2003). DBP also plays a major role in promoting elongation in an ATP-independent manner, hence bypassing the requirement for a helicase to unwind the DNA double helix, although complete replication of the viral genome requires the topoisomerase activity of NF2, given that elongation cannot continue beyond 30% of the template strand without it (Van der Vliet 1995; de Jong and van der Vliet 1999). After replication, pTP is cleaved by a virally encoded protease, to produce its mature, smaller form TP, which remains covalently attached to the 5' end of the genome. The role of the covalently attached pTP has yet to be fully elucidated, although it is thought to play a role in unwinding of the DNA duplex at the origin of replication, as well as protecting viral DNA from exonucleases, and allowing DNA-nuclear matrix association (Dunsworth-Browne et al. 1980; Stillman et al. 1981; Schaack et al. 1990).

Adenoviruses have evolved to bypass antiviral responses by controlling host cellular processes like cell cycle checkpoints and apoptosis, in order to facilitate viral DNA replication and keep the host cell alive long enough to produce viral progeny. The mechanisms employed by adenovirus to achieve this are outlined later in this chapter, with particular focus on how Ad negates the cellular DNA damage response (DDR).

1.1.5. E1A

E1A is one of the most extensively studied viral oncogenes, and is the first to be transcribed during adenovirus infection (approximately 1 hour post-infection), preceding the onset of viral DNA replication. Transcription of *E1A* produces two major mRNA products, 12S and 13S, as well as 3 minor mRNA products, 9S, 10S, and 11S, named after the sedimentation coefficients of their respective mRNAs (Boulanger and Blair 1991). The 12S and 13S E1A proteins are identical given that they are transcribed from the same mRNA, and both contain the three highly conserved regions CR1, CR2, and CR4, with the 13S form containing an extra conserved region CR3 in the C-terminal half due to alternative RNA splicing, which control Ad early gene expression (Gallimore and Turnell 2001). E1A is not a DNA-binding protein itself, yet it plays a role a major role in both the transcriptional activation and repression of many early viral and cellular genes. Instead, the conserved regions of E1A proteins have been shown to interact with a number of cellular proteins, most of which play a role in transcriptional regulation (some of which are illustrated in Fig. 1.2), which is crucial for adenovirus to manipulate normal cell cycle control in order to facilitate viral DNA replication (Gallimore and Turnell 2001; Berk 2005).

The first cellular protein that was found to bind to E1A was the product of the tumour suppressor gene *Rb-1*, pRB, which significantly, was the first link between a product of

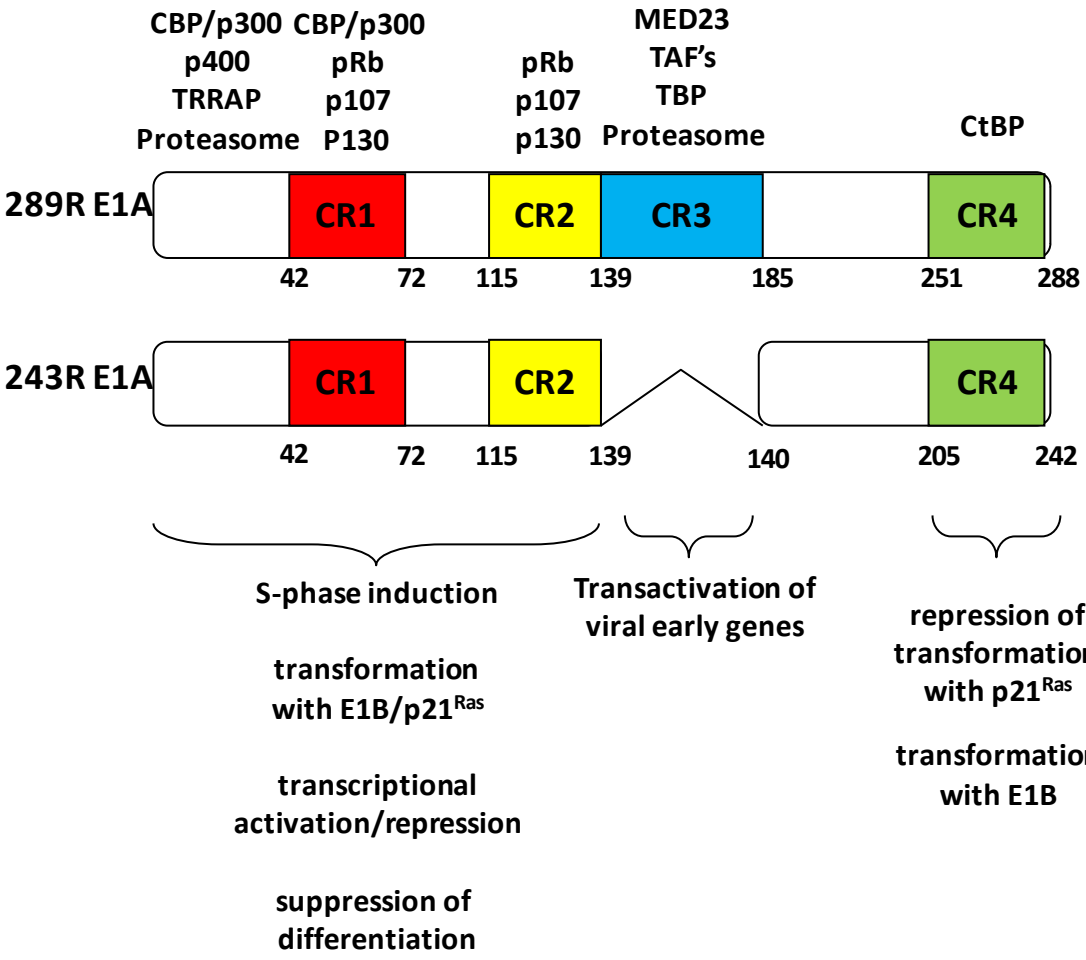

Fig. 1.2. Linear representation of Ad5 12S and 13S E1A and its biological functions. The conserved regions (CR) of the 12S and 13S E1A are represented by the coloured rectangles. E1A interacts with a number of host cellular factors listed above to carry out its functions which are also listed. (Zheng 2010)

a tumour suppressor and E1A (Whyte et al. 1988). Furthermore, E1A also binds to the two related pocket proteins p107 and p130, which are functionally and biochemically similar to pRB. The CR1 and CR2 regions are responsible for the E1A interaction with pRB, and consequently interfere with the transcriptional repression activity of pRB by causing it to be displaced from E2F-responsive promoters (Frisch and Mymryk 2002). The displacement of pRB from E2F complexes allows E2F-dependent transcription of cyclin-dependent kinase (CDK) 2, cyclin E, and cyclin A, resulting in the host cells being pushed into S-phase, bypassing normal cell growth restrictions, and priming the cell for viral DNA replication (Grand et al. 1998). Furthermore, the displacement of pRB from E2F complexes also allows for E2F to activate transcription of the E2 gene from the E2 early promoter, which is important for production of proteins that are vital for viral DNA replication (Bagchi et al. 1990).

Another way that E1A promotes S phase entry is through the interactions between CR1 and the transcriptional co-activators p300 and CREB-binding protein (CBP), which possess intrinsic histone acetyl transferase activity (Howe et al. 1990; Howe and Bayley 1992). Transcription of cellular genes, such as p53 and nuclear factor-kappa B (NF-κB), which are involved in cell cycle regulation are affected as a consequence of the E1A-p300/CBP interaction, further promoting host cell progression into S phase and viral replication (Ben-Israel and Kleinberger 2002).

The pRB- and CBP/p300-binding domains of E1A are required for E1A-dependent stabilization of p53 and stimulating p53-dependent apoptosis in primary cells. E1A also binds to proteasome subunits to inhibit p53 degradation (Querido et al. 1997; Samuelson and Lowe 1997; Turnell et al. 2000; Zhang et al. 2004). However, stabilization of a pro-apoptotic protein like p53 is quite likely to be deleterious for a replicating virus, thus these actions are counteracted by the expression of other viral

proteins, E1B and E4, although there is evidence to suggest that functional p53 might be able to promote viral replication and late virus protein expression (Rhodes et al. 2005).

1.1.6. E1B

The products of the Ad *E1B* gene are involved in a number of different mechanisms that are crucial for efficient viral replication and Ad-mediated cell transformation. There are five known gene products encoded from the Ad *E1B* gene, which are generated by alternative splicing of a common mRNA precursor. E1B-55K and E1B-19K are the two proteins which are the best characterized, and individually, both have been shown to function in concert with E1A to transform rodent cells in culture, albeit they have no transforming potential of their own (Sieber and Dobner 2007). These two proteins are two unrelated polypeptides whose primary functions are to inhibit apoptotic effects of p53 and to cooperate with E1A to promote Ad-mediated cell transformation (Berk 2005). Whilst much research has gone into characterizing the functions of E1B-55K and E1B-19K, very little is known about the other three proteins, E1B-84R, E1B-93R, and E1B-156R, which are designated as such in relation to the numbers of their amino acid residues (Fig. 1.3; (Sieber and Dobner 2007).

E1B-19K is a functional homologue of the cellular B-cell lymphoma-2 (BCL2) family of proteins (Cuconati and White 2002). Expression of E1A promotes degradation of the myeloid cell leukaemia 1 (MCL1) protein, also a BCL2 homologue, which results in the release of the pro-apoptotic BCL2 antagonist killer (BAK) protein, normally bound to MCL1 in uninfected cells (Cuconati et al. 2003). This then allows BAK to form oligomers with another pro-apoptotic protein, BCL2-associated X (BAX), resulting in pore formation in the outer mitochondrial membrane, leading to the release of pro-

apoptotic proteins, which include cytochrome c and Smac/DIABLO into the cytoplasm, and subsequent activation of the caspase-9 and caspase-3 mediated apoptotic programmes (Cuconati and White 2002; Cory et al. 2003; Cuconati et al. 2003). During infection, E1B-19K binds to, and sequesters BAX and BAK, and thus inhibits the caspase-mediated apoptotic programme, which could potentially cause premature cell death and restricted viral replication as a result of p53 activation (White 2001).

E1B-55K is a multifunctional protein that utilizes a number of different mechanisms to facilitate efficient viral replication during infection. A schematic representations of Ad5 E1B-55K protein is shown in Figure 1.4. E1B-55K also functions in concert with E1A to promote complete oncogenic transformation of mammalian cells (Barker and Berk 1987). The first interaction between an adenovirus E1-protein and a cellular protein to be identified is the one between E1B-55K and p53 in transformed cells, where they are found in a stable complex within subcellular structures in the cytoplasm near the nucleus (Sarnow et al. 1982; Zantema et al. 1985). These subcellular structures are now known as aggresomes, which are formed at the microtubule organizing centre in response to misfolded proteins that occur when their rate of synthesis exceeds their rate of degradation; it is believed that the sequestration of p53 into these aggresomes by E1B-55K inhibits the protein from performing its normal functions (Liu et al. 2005). The transforming ability of E1B-55K correlates with its role as a transcriptional repressor of p53, whereby it binds with high affinity to p53 at p53-responsive promoters, thus repressing its transcriptional activity (Yew and Berk 1992; Yew et al. 1994). In contrast to Ad5 E1B-55K, Ad12 E1B-55K only associates weakly to p53, and does not co-localize at cytoplasmic aggresomes, but instead has a diffuse nuclear localization (Zantema et al. 1985; Grand et al. 1994). Despite the difference in the binding affinities for p53, both Ad5 E1B-55K and Ad12 E1B-55K are able to inhibit

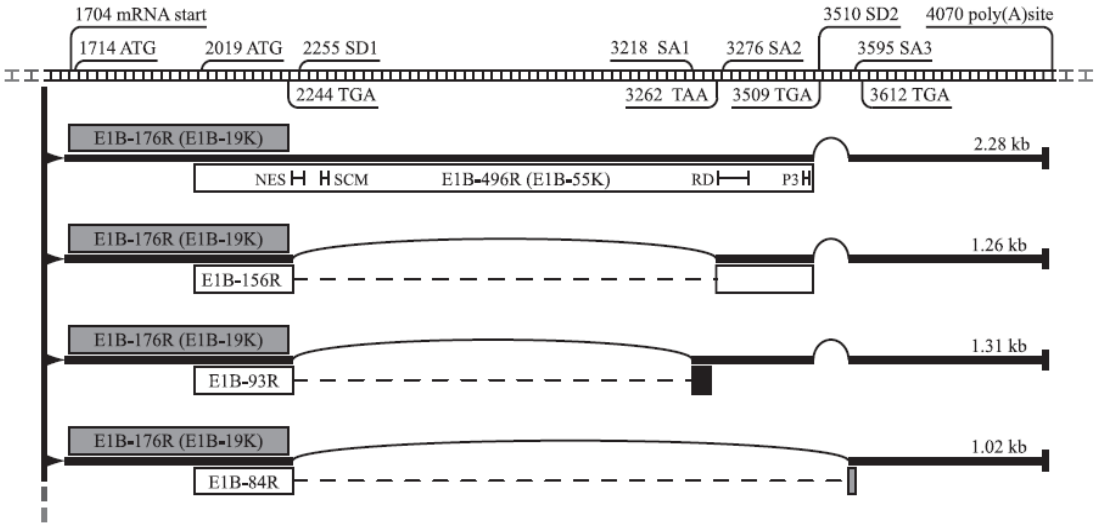

Fig. 1.3. Linear representation of Ad5 *E1B* gene products. The numbers at the top represent the nucleotides in the Ad5 sequence. The four alternatively spliced mRNAs are depicted by the thick black lines, whereby the arced and dashed lines that connect them represent their non-coding introns. The grey, black, and white shaded boxes next to the mRNAs represent their gene products, indicating the used reading frames 1-3 respectively. The open reading frames encoding E1B-156R, E1B-93R, and E1B-84R are generated through fusion of two different exons. (Sieber and Dobner 2007).

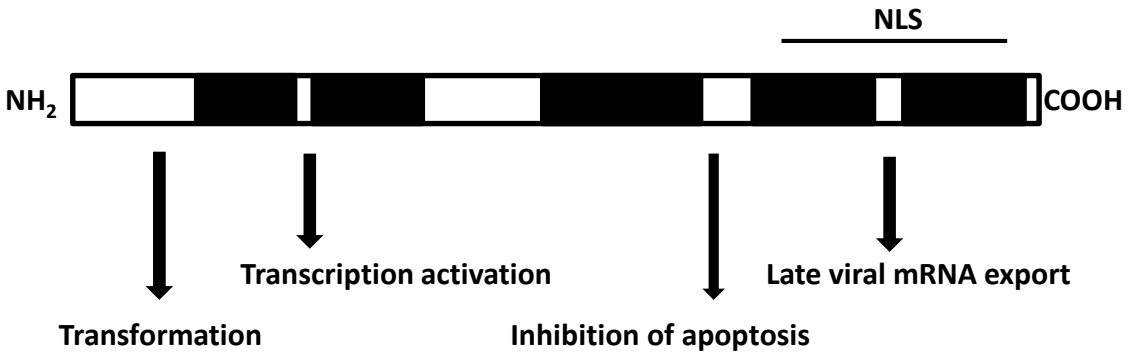

Fig. 1.4. Linear representation of Ad5 E1B-55K and its biological functions. The coloured rectangles represent the five different E1B-55K binding motifs. The biological functions of this protein are also listed. NES, nuclear export signal; SCM, SUMO1 conjugation motif; RNP, ribonucleoprotein motif; ZnF, putative C_2H_2 zinc finger; CKI/II, caesin kinase I/II phosphorylation sites; NLS, nuclear localization signal. (Zheng 2010).

p53 transcriptional activity (Yew and Berk 1992).

E1B-55K undergoes a post-translational modification by the small ubiquitin-like modifier 1 (SUMO-1) via the ΨKxE consensus motif required for SUMO-1 conjugation, which surrounds the lysine residue at amino acid position 104 (Endter et al. 2001). A single mutation (K104R) inhibits E1B-55K SUMOylation, which results in a significant reduction in the ability of E1B-55K to transform primary baby rodent cells in cooperation with E1A, as well as reducing the inhibition of p53-mediated transactivation (Endter et al. 2001). Overexpression of SUMO-1 results in relocalization of E1B-55K into the nucleus, whereas a mutation in the SUMO conjugation motif restores the proteins cytoplasmic location, and therefore suggests that SUMO-1 conjugation and deconjugation acts as a molecular switch to control E1B-55K distribution in the cell (Endter et al. 2001; Kindsmuller et al. 2007). In contrast to Ad5 E1B-55K, Ad12 E1B-55K has diffuse, nuclear distribution in transformed cells, and is not detectably SUMOylated (Zantema et al. 1985; Endter et al. 2001). Furthermore, E1B-55K also regulates p53 SUMOylation (Muller and Dobner 2008). E1B-55K-mediated SUMOylation of p53 is essential for maximum inhibition of p53 activity, whereby it tethers p53 into promyelocytic leukemia (PML) nuclear bodies to decrease its mobility, and then facilitates its nuclear export and subsequent degradation (Pennella et al. 2010).

The roles of the E1B-55K protein are generally better understood during infection than transformation, where it generally functions in concert with the early region 4 open reading frame 6 (E4orf6) protein to perform both early and late roles that are crucial for viral replication (Berk 2005). The roles of Ad12 E1B-55K during infection have not been as extensively characterized when compared to Ad5 E1B-55K, but it is thought to function in a similar fashion to the Ad5 protein (Grand et al. 1999; Stracker et al. 2005).

One such function that E1B-55K carries out together with E4orf6, is to promote the proteasomal degradation of p53, thus allowing the virus to evade cellular p53-dependent events like apoptosis and cell cycle arrest (Querido et al. 2001). E1B-55K and E4orf6 function in concert to recruit p53 to a E3 ubiquitin ligase complex containing the cellular proteins Cullin (Cul) 5, RING-box 1 (RBX1), and elongins B and C, where it is then ubiquitylated, and targeted to the 26S proteasome for proteasomal degradation (Querido et al. 2001; Harada et al. 2002). Although E4orf6 is able to bind to p53 independently, it has been suggested that E1B-55K acts as a substrate adapter for the complex, and E4orf6 recruits the Cullin ring ligase (CRL) as it has been found to contain motifs termed BC boxes, that bind to elongins B and C (Dobner et al. 1996; Blanchette et al. 2004; Cheng et al. 2007). Furthermore, a number of other cellular proteins that are involved in the cellular DNA damage response are also targeted by E1B-55K and E4orf6 for degradation, which include; DNA ligase IV, Mre11, and BLM, and will be discussed in more detail later in this chapter (Stracker et al. 2002; Liu et al. 2005; Baker et al. 2007; Orazio et al. 2011).

It has also been shown that through their ability to recruit the CRL5, E1B-55K and E4orf6 also cooperate to inhibit host cell nuclear mRNA export, and promote late nuclear viral mRNA export to the cytoplasm during the late phase of infection (Woo and Berk 2007; Blanchette et al. 2008). Cells expressing either mutant E1B-55K or E4orf6, or both, have been shown to have a defect in late nuclear viral mRNA export to the cytoplasm, and cannot inhibit host cell mRNA export from the nucleus to the cytoplasm, as well as a reduction in the synthesis of viral late proteins when compared to that of *wt* Ad5 (Babiss et al. 1985; Cutt et al. 1987; Gonzalez et al. 2006). These functions are likely to be associated with the fact that both E1B-55K and E4orf6 have an N-terminal nuclear export signal (NES), and a carboxy-terminal (C-terminal) nuclear

localization signal (NLS), which are required for shuttling between the nucleus and the cytoplasm (Goodrum et al. 1996; Weigel and Dobbelstein 2000; Dosch et al. 2001). E1B-55K also has a ribonucleoprotein (RNP) RNA-binding motif which is essential for functionality of the protein (Horridge and Leppard 1998). Together these observations suggest that a possible model for viral mRNA export is that an mRNA ribonucleoprotein (mRNP) whose degradation causes the inhibition of cellular mRNA export from the nucleus, yet promotes viral mRNA export, maybe ubiquitylated and targeted for proteasomal degradation; an E1B-55K substrate that may carry out these functions has yet to be identified (Berk 2005).

1.1.7. E4 region

The E4 transcription unit is located at the far right hand side of the adenovirus genome and is transcribed in the leftward direction which produces a primary transcript of around 2800 nucleotides in length (Tauber and Dobner 2001). Alternative splicing of this transcript generates at least 18 different mRNAs that encode for at least seven orfs: orf1, orf2, orf3, orf3/4, orf4, orf6 and orf6/7, which, with the exception of orf3/4, have all been shown to be present in infected cells (Cutt et al. 1987; Tauber and Dobner 2001; Thomas et al. 2001). E4 proteins collectively play a number or roles which are vital for efficient viral replication via a complex network of protein interactions with viral and cellular proteins that function in DNA replication, mRNA transport, virus particle assembly, transcriptional regulation, and host cell shutoff (Halbert et al. 1985; Weiden and Ginsberg 1994; Tauber and Dobner 2001). It has been shown that mutations in individual orfs result in only minimal effects on viral growth in cultured cells, rendering them dispensable for lytic growth, however a mutation in the E4orf6 coding region was found to be modestly defective, whilst mutations resulting in loss of both E4orf3 and E4orf6 resulted in significant defects in DNA replication,

accumulation of viral mRNAs, and host cell shutoff (Halbert et al. 1985). Furthermore it has been shown that E4orf3 and E4orf6 can compensate for each other's defects as they have been shown to be functionally redundant, as both proteins are involved in inhibiting adenovirus DNA concatamer formation, promoting late viral protein synthesis, augmenting viral DNA replication, and shut-off of host protein synthesis, although they carry out these functions via different mechanisms (Halbert et al. 1985; Huang and Hearing 1989; Weiden and Ginsberg 1994).

The *E4orf6* gene encodes for a 34 kDa protein that plays a role in regulating a number of host cell pathways in order to promote viral gene expression and viral replication. Most of the functions carried out by E4orf6 require another viral oncoprotein E1B-55K, although E4orf6 in some instances can act alone. The E4orf6 proteins has been shown to bind directly to p53 at the C-terminal regulatory domain of the protein, which inhibits the interaction between the N-terminal activation domain of p53 and TAFII31, a component of the transcription factor IID (TFIID), thus blocking p53-mediated transcriptional activation (Dobner et al. 1996).

E4orf6 contains a N-terminal NES, and C-terminal NLS, which are required for shuttling between the nucleus and the cytoplasm, as well as an amphipathic arginine-rich α-helical nuclear retention signal (NRS), which has been shown to be required for E1B-55K-E4orf6 complexes to localize in the nucleus (Orlando and Ornelles 1999). E4orf6 also contains a functional zinc-binding region, which has been shown to be required for its interaction with E1B-55K, and three functional BC boxes, which are required for recruitment of CRL complexes, as well as also mediating its indirect interaction with E1B-55K (Boyer and Ketner 2000; Blanchette et al. 2004; Cheng et al. 2007). As discussed above, E4orf6 functions in concert with E1B-55K to counteract the effects of E1A-induced p53 stabilization by promoting the proteasome-mediated

degradation of p53 (Querido et al. 2001; Blanchette et al. 2004). Viruses harbouring mutations that express E4orf6 proteins lacking BC boxes are defective in production of late viral proteins, export of late viral mRNAs, and viral growth (Blanchette et al. 2008). This shows that the interactions between E4orf6 and E3 ubiquitin ligase complexes are required for these late functions, suggesting that E3 ubiquitin ligase activity may play a part in mRNA transport and stability.

The *E4orf3* gene encodes for a highly conserved 11 kDa protein which was the first gene product to be identified from the E4 region in infected cells and found to associate with the nuclear matrix (Sarnow et al. 1982; Downey et al. 1983). Like E4orf6, E4orf3 also interacts with E1B-55K to carry out some of its functions, as well as functioning alone. E4orf3 has been shown to reorganize cellular and viral proteins in to nuclear track like structures that surround sites of viral replication, the most studied of which is promyelocytic leukemia (PML) protein; E4orf3 is able to reorganize PML oncogenic domains (PODs) into these elongated nuclear track structures (Carvalho et al. 1995; Doucas et al. 1996). Further research has shown that E4orf3 specifically targets the PMLII isoform to reorganize PODs into nuclear tracks, and the reorganization of PODs into these nuclear structures is important for viral replication, as overexpression of PML inhibits the E4orf3-mediated reorganization of the PODs, causing a severe delay in adenoviral replication (Doucas et al. 1996; Hoppe et al. 2006). Furthermore, like E4orf6, E4orf3 has been shown to interact with E1B-55K, and reorganize it into nuclear tracks (Leppard and Everett 1999).

More recent research has shown the E4orf3 also interacts with members of the transcriptional intermediary factor 1 (TIF1) family of proteins, TIF1α and TIF1γ, which like PML, are members of the tripartite motif (TRIM) family of proteins. E4orf3 recruits TIF1α to PML nuclear tracks during infection, however the functional

significance of this reorganization during infection remains to be elucidated (Yondola and Hearing 2007). Furthermore, E4orf3 not only reorganizes TIF1γ to PML nuclear tracks, but also promotes the proteasomal degradation of this protein, independent of E1B-55K, E4orf6, and CRLs (Forrester et al. 2012).

Like E1B-55K and E4orf6, E4orf3 is also able to inhibit p53 transcriptional activity via epigenetic silencing of p53 promoters, and occurs independent of E1B-55K/E4orf6 mediated degradation of p53 (Soria et al. 2010). E4orf3 forms a scaffold in the nucleus, which directs heterochromatin formation via trimethylation of histone H3 at lysine residue 9 (H3K9me3) at p53 target promoters, thus silencing p53-mediated transcription in response to DNA damage (Soria et al. 2010). The role of E4orf3 in reorganizing other DNA damage proteins will be discussed later.

The *E4orf4* gene encodes for a highly conserved 14 kDa protein which does not appear to be essential for Ad infection, and instead appears to have an inhibitory role in Ad replication by negatively regulating the E1A transactivation of both the E2 and E4 promoters (Bondesson et al. 1996; Mannervik et al. 1999). The primary cellular target for E4orf4 was found to be the Bα subunit of the protein phosphatase 2A (PP2A), which together have been shown to function in many processes which include regulation of viral and cellular gene expression, down regulation of virus-induced signal transduction, and induction of p53-independent apoptosis (Kleinberger and Shenk 1993; Kleinberger 2000). E4orf4 together with PP2A, in the absence of other viral proteins, is able to induce p53-independent apoptosis in human cancer cells, as well as inducing G_2/M arrest in mammalian cells prior to apoptosis (Marcellus et al. 2000; Kornitzer et al. 2001). These functions of E4orf4 make it an excellent potential target for cancer therapy seeing as a high incidence of human tumours lack functional

p53 and are thus susceptible to treatments which require p53-dependent apoptosis (Branton and Roopchand 2001).

E4orf6/7 is a 17 kDa protein that also appears to be non-essential for adenovirus infection, yet it is able to functionally compensate for E1A expression during viral infection (O'Connor and Hearing 2000). The expression of E4orf6/7 in the absence of E1A causes the displacement of Rb and p107 from E2F complexes, and allows E2F to bind to the E2a promoter region and promote expression of E2 (O'Connor and Hearing 2000). E4orf1 is also not essential for adenovirus infection, however it has been shown display oncogenic properties as it essential for Ad9-induced mammary tumours in rats (Javier 1994; Thomas et al. 2001)

1.1.8. Biological significance of studying adenoviruses

As discussed adenoviruses have often been used as a model system to study many fundamental cellular and molecular processes (Berk 2005; Weitzman and Ornelles 2005). Scientists studying Ad2 found that an Ad2 pre-mRNA moleule was reorganized in such a way that it resulted in a variety of mature mRNA molecules with an assorted arrangement of exons; this process which we now know as alternative splicing was first described in adenovirus, for which Phillip Sharp and Richard Roberts were awarded the Nobel Prize in Physiology or Medicine in 1993 (Berget et al. 1977; Chow et al. 1977). The study of adenovirus oncogenes have also played a major role in identification and/or characterization of many cellular proteins, in particular the E1A viral oncogene, which has been used to study a range of cellular proteins, in particular pRB, E2F, p300, and CtBP (Yee and Branton 1985; Kovesdi et al. 1986; Whyte et al. 1988; Boyd et al. 1993). Furthermore, adenovirus was found to directly interact with p53, initially via

E1B-55K, which lead to subsequent studies that were crucial for discovering the fundamental nature and function of the protein (Sarnow et al. 1982; Russell 2000).

Adenoviruses were the first family of human viruses to be described as capable of causing cancer, where they have been shown to promote tumour formation in rodents (Trentin et al. 1962). The Ad early gene products, E1A, E1B, E4orf3 and E4orf6 can function together to mediate cellular transformation by utilizing what has been described as a "hit and run" mechanism seeing as there are no detectable viral DNA sequences, or viral proteins, in the resulting tumour cells (Trentin et al. 1962; Nevels et al. 2001). Adenoviruses have been associated with a number of human conditions in both healthy and immunocompromised individuals, yet despite this, they are not thought to be associated with human cancers (Sarantis et al. 2004; Echavarria 2008). Although a recent study which utilized real-time quantitative PCR assay managed to detect Ad DNA from serotypes that belong to groups B and D, in paediatric brain tumours, and this was further confirmed by *in situ* hybridization assays, suggesting that some Ad serotypes might have tumourigenic capabilities in humans (Kosulin et al. 2007).

The Ad5 *E1B-55K* mutant virus, *dl*1520 (also known as ONYX-015), is an oncolytic virus which has been of particular interest with regards its potential use as a therapeutic tool in cancer treatment. In theory, adenoviruses that do not express E1B-55K will be unable to replicate efficiently in healthy human cells that express p53, but instead will be able to replicate in tumour cells that express mutant p53 (Bischoff et al. 1996). Ad5 *dl*1520 has been shown to preferentially infect and lyse cancer cells, although there are conflicting reports of its efficacy in clinical trials of cancer patients (Heise et al. 1999; Turnell et al. 1999; Khuri et al. 2000; Cherubini et al. 2006). Adenoviruses are able to infect a broad range of cells, can be genetically altered with relative ease, and are able

to remain fairly stable *in vitro*, which is why they have been used as vehicle for gene delivery since the early 1990's, and are currently the vector of choice for gene therapy, representing a fourth of all vectors utilized in gene therapy clinical trials (Robinson et al. 2011). It is likely that adenoviruses will continue to be used, in the future, to explore fundamental cellular processes, and be central to potential therapeutic strategies.

1.2 THE DNA DAMAGE RESPONSE

1.2.1. DNA damage and cancer

The cell's genome is under constant attack from agents that can directly damage the DNA, such as ultraviolet (UV) or ionising radiation (IR) or indirectly as a consequence of normal cellular metabolic processes that result in by-products such as oxygen free radicals. Approximately 10,000 DNA lesions are repaired by each cell every day. These can be in the form of many different types of lesion including oxidative base damage, double-stranded, single-stranded breaks or mismatches (Abraham 2001; Lilley et al. 2007). Complex molecular pathways have evolved to recognize and correct cellular DNA damage, which are collectively termed the DNA damage response (DDR) and is thought to be the key early anti-cancer barrier that cells must negotiate before becoming tumorous (Bartkova et al. 2005). The DDR utilizes the cell-cycle machinery, which is overlaid with a series of surveillance pathways called cell-cycle checkpoints. These checkpoints detect damaged or abnormally structured DNA and then co-ordinate cell-cycle progression with DNA repair. Cell-cycle checkpoint activation slows or arrests cell-cycle progression which allows the appropriate DNA repair mechanisms to correct genetic lesions so that they are not passed onto the next generation of daughter cells (Abraham 2001; Lilley et al. 2007). These processes are not perfect and consequently

mutations in oncogenes and tumour suppressor genes, as well as other cell regulatory genes, can occur despite a functional cellular DNA damage response (Lobrich and Jeggo 2007). Defects in these processes can lead to errantly or unrepaired DNA, which can result in genomic instability and subsequent cancer development. This is supported by the fact that individuals who harbour inherited mutations in their DNA damage response genes are predisposed to developing tumours (O'Driscoll et al. 2006).

Interestingly, DNA damaging agents such as IR and alkylating agents are used in a controlled manner in current cancer treatments. This is because most tumour cells have defects in the DNA damage response machinery, and are therefore more susceptible to the cytotoxic effects of DNA damage agents than the surrounding healthy cells. These treatments however have many adverse side effects as a result of DNA damage caused in the normal cells, which include the gastrointestinal problems, hair loss, immune suppression, and possible secondary tumour development (Kastan and Bartek 2004). Given these side effects, therapies that utilize specific DNA damage pathway inhibitors are being developed and are currently being administered in clinical trials in combination with traditional therapies. An example of such inhibitors are ones that target the poly (ADP-ribose) polymerase (PARP) family of enzymes, which play a role in base excision DNA repair pathway. Inhibitors target the PARP1 and PARP2 isoforms, and have been shown to enhance the antineoplastic potential of chemotherapeutic agents, as well as showing preferential killing of neoplastic cells when treating patients with breast or ovarian cancers that are caused by mutations in either the *BRCA1* or *2* genes (Kummar et al. 2012).

Mutations in DDR genes have been attributed to a number of rare human genetic conditions, which include ataxia telangiectasia (A-T), ataxia telangiectasia-like disorder (ATLD), Nijmegen breakage syndrome (NBS), NBS-like syndrome, RIDDLE

(radiosensitivity, immunodeficiency, dysmorphic features and learning difficulties) syndrome, Fanconi anaemia (FA), DNA ligase IV deficiency syndrome (LiDS), Li-Fraumeni syndrome, Seckel syndrome and xeroderma pigmentosum (XP; (Jackson and Bartek 2009). These diseases present with many different symptoms, of which the most common are sensitivity to agents that cause DNA damage, as well as a predisposition to the development of tumours.

1.2.2. p53

The tumour suppressor protein p53 was first discovered in 1979 in a complex with SV40 LTag using immunoprecipitation assays (Lane and Crawford 1979; Linzer and Levine 1979). Originally, p53 was described as an oncogene as it was shown to have transforming activity, although this was heavily disputed as the original gene sequence for the *wt* TP53 was questioned, and it was later found to actually be a suppressor of transformation (Eliyahu et al. 1984; Parada et al. 1984; Finlay et al. 1989). Since its discovery, p53 has been the one of the most extensively studied proteins and has been described as the 'guardian of the genome' as it has been shown to regulate a number of cellular processes in response to cellular stress, and has been shown to be mutated in over 50% of human cancers (Lane 1992; Whibley et al. 2009).

The major role that p53 plays is that of transcription factor that binds to a very loose DNA sequence found in the regulatory regions of p53-responsive genes (Meek 2004). This tumour suppressor is able to regulate various genes that function in cellular processes like cell cycle control, DNA repair, senescence and apoptosis, in response to different cellular stresses as illustrated in Figure 1.5 (Whibley et al. 2009). In response to DNA damage, p53 acts as a molecular switch between DNA repair and apoptosis.

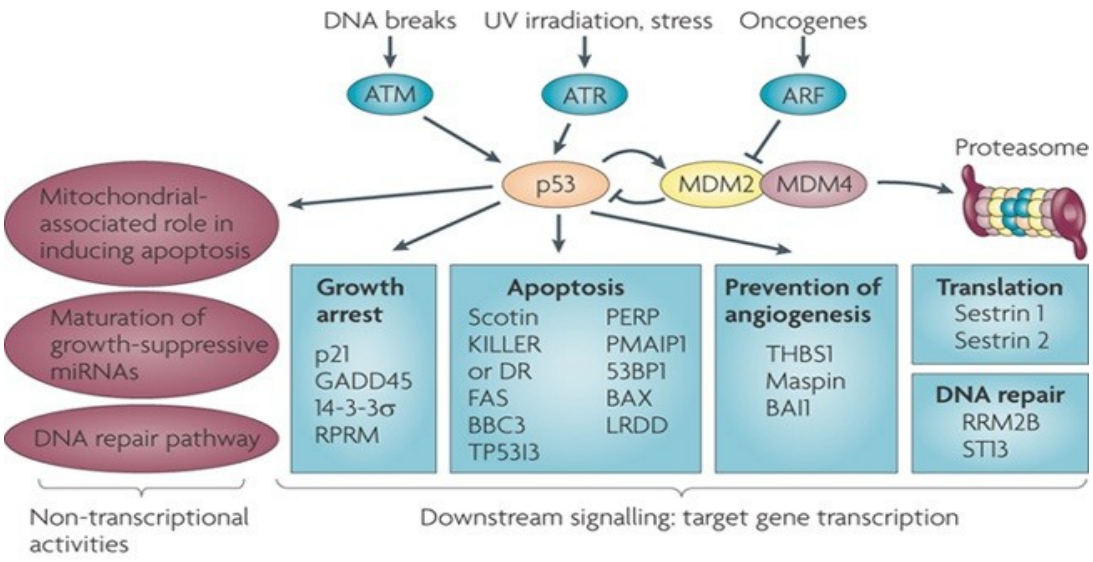

Fig. 1.5. The p53 pathway. This illustration highlights the complexity of p53 regulation and its importance in maintaining genome integrity. Upstream signalling to p53 increases its level and activates its function as a transcription factor in response to a wide variety of stresses, whereas downstream components execute the appropriate cellular response. p53 function is regulated by at least 50 known enzymes which effect its interaction with the MDM2-MDM4 complex. In unperturbed cells, MDM2-MDM4 bind to p53 and ubquitylate it, targetting it for proteasome-mediated degradation. The functions of MDM2-MDM4 are blocked under stressed conditions by protein-binding events, phosphorylation, and degradation. This then allows p53 to transcriptionally activate or repress genes involved in cell cycle arrest, DNA repair, angiogenesis, apoptosis and senescence (Brown et al. 2009).

When DNA damage occurs, p53 acts to induce cell cycle arrest to allow the repair of the damage and then subsequent entry into the normal cell cycle. However, when damage is too severe and irreparable, p53 activates the apoptotic pathways to cause cell death in order to prevent the damaged DNA being passed on to daughter cells (Latonen and Laiho 2005).

In unperturbed cells the expression levels of p53 remain quite low via mechanisms that promote ubquitylation and subsequent proteasome-mediated degradation of the protein. These processes are mainly regulated by the murine double minute 2 (MDM2) E3 ubquitin ligase, which binds to the N-terminal of p53 and ubquitylates multiple lysine residues located in its C-terminal region (Rodriguez et al. 2000). A second mechaninsm by which MDM2 acts as a negative regulator of p53 function is by binding directly to the N-terminal transactivation domain of p53 thus inhibiting its transcriptional activity (Momand et al. 1992; Oliner et al. 1993). MDM2 also promotes the relocalization of p53 from the nucleus into the cytoplasm, thus removing it from its site of action (Roth et al. 1998). Furthermore, MDM2 itself is also transcriptionally regulated by p53 via a p53 response element in the MDM2 gene, hence creating an auto-regulatory negative feedback loop through which both the expression levels of p53 and MDM2 can be regulated (Wu et al. 1993). MDM2 has also been referred to as an oncongene as it has been observed to be overexpressed in over forty various forms of malignancies, though a mechanism for its involvement remains to be identified; it has been postulated that overexpression of MDM2 results in aberrant p53 degradation (Leach et al. 1993; Rayburn et al. 2005). In addition to MDM2 there are a large profile of proteins that can also play a role in p53 regulation, most of which act via post-translational modifications of the protein, such as phosphorylation, methylation and acetylation (Whibley et al. 2009). Many of these modifications occur withihin the N-terminal region of p53, which

prevents MDM2 interaction and results in the stabilization of p53 (Brooks and Gu 2003).

1.2.3. Cell cycle checkpoints

The cellular response to DNA damage initiates an entire ensemble of processes that include cell cycle arrest, activation of DNA repair genes, and if needed, activation of apoptotic pathways. The cell cycle checkpoints are an integral component of the DDR, and are active surveillance mechanisms that are required to maintain DNA integrity (Sancar et al. 2004). In response to DNA damage, these checkpoints allow the cell cycle to arrest, allowing time for the cell to repair the lesion, and if repair is successful the cell cycle is allowed to progress error-free. However if the damage is irreparable, the cell will undergo apoptosis.

G1, S, G2, and M are the four phases of the cell cycle that occur in all eukaryotic cells, and transitions between these phases are tightly controlled. Checkpoints are activated in response to DNA damage. There are three main DNA-damage regulated checkpoints, the G_1/S checkpoint, which can block S phase entry, the intra-S phase checkpoint, which can delay S phase progression, and the G_2/M phase checkpoint, which can inhibit mitotic entry (Fig. 1.6). There are a large plethora of proteins involved in the DDR that form an integrated network to signal DNA lesions and activate the checkpoints, which can be simply grouped into sensors, mediators, signal transducers, and effectors, although some of these proteins can act as both signal transducers and sensors, and some mediators also have been shown to function in more than one step (Fig. 1.7) (Sancar et al. 2004).

Ataxia-Telangiectasia Mutated (ATM) and ATM-Rad3-related (ATR) are members of the phosphatidylinositol 3-kinase-like kinase (PIKK) family that function as key

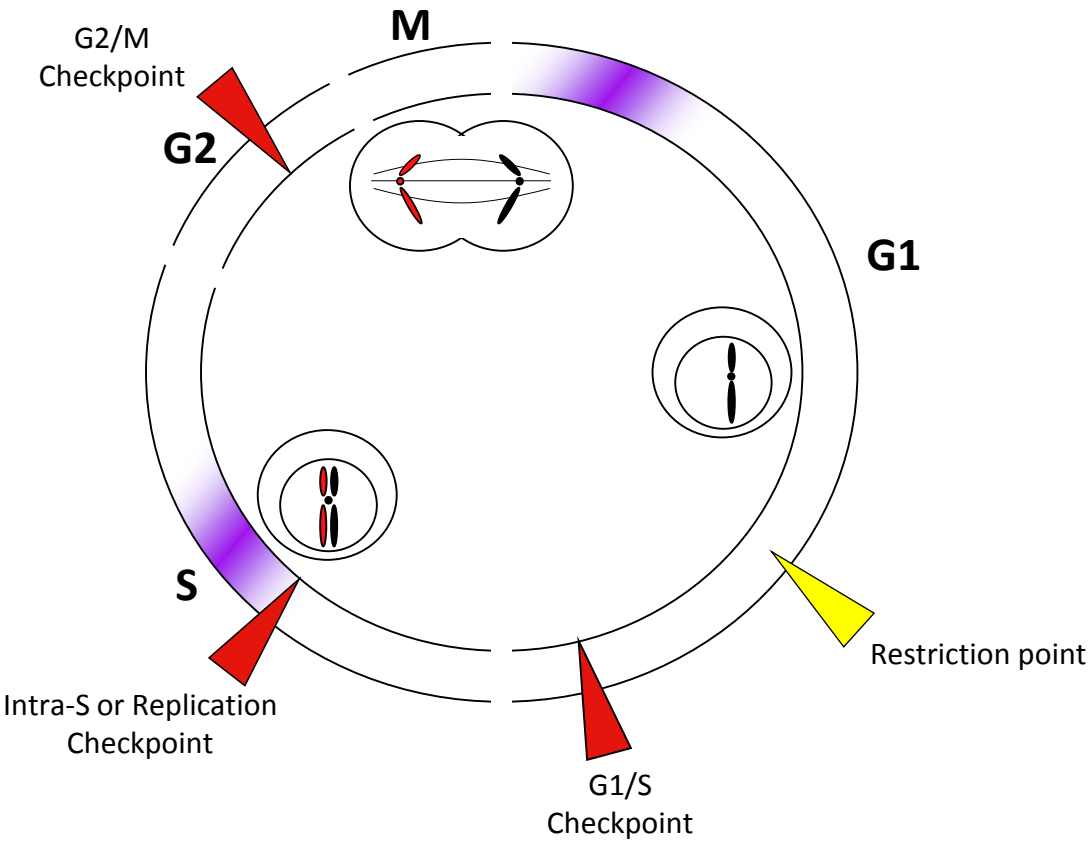

Fig. 1.6. Schematic representation of the cell cycle. DNA damage activates cell cycle checkpoint. The yellow triangle depicts the restriction point, which is the point at which cells are committed to S-phase entry. The Red triangles depict the checkpoints, whereby the G_1/S checkpoint block S phase entry, the intra-S phase checkpoint delays S phase progression, and the G_2/M phase checkpoint inhibits mitotic entry. Cell cycle arrest at these checkpoints allows time to repair damaged DNA.

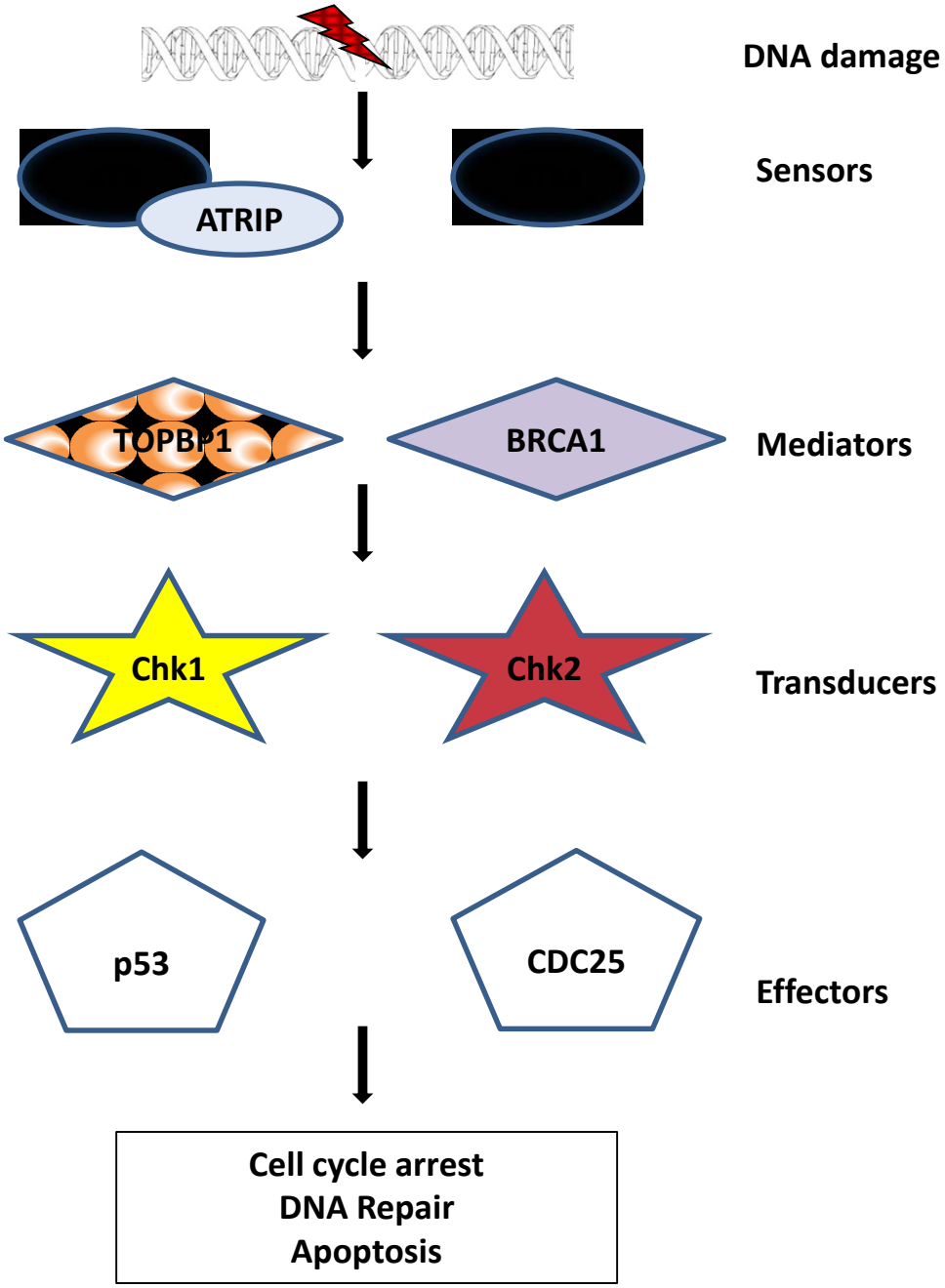

Fig. 1.7. Transduction of the DNA damage signal. DNA damage is detected by sensor proteins, which in turn activates the signalling cascade. This recruits mediators and transducers which amplify the signal and pass it on to effector proteins, which results in cell cycle arrest to allow DNA repair, or apoptosis if damage is too severe. (Sancar et al. 2004)

transducers of signals initiated in response to DNA damage. These proteins phosphorylate and subsequently activate a number of proteins involved in the checkpoint pathways, a number of which are common substrates for both proteins. These kinases respond to different types of DNA damage, as ATM responds primarily to DSBs, whilst ATR responds to single-stranded DNA breaks SSBs and stalled DNA replication forks (Bakkenist and Kastan 2004). However, cross talk between the two pathways has been shown to occur as there is evidence for an ATR response to DSBs, as well ATM activation via ATR signalling in response UV and hydroxyurea (HU) treatment (Adams et al. 2006; Jazayeri et al. 2006; Stiff et al. 2006).

Mutations in the *ATM* gene have been shown to result in the rare genetic condition called Ataxia telangiectasia, where patients lack functional ATM proteins and display symptoms such as cerebellar degeneration, immunodeficiency, hypersensitivity to ionizing radiation-IR (as they are unable to repair DSBs), and a predisposition to certain cancers (Savitsky et al. 1995). However, evidence indicates that the *ATM* gene is not essential for cell survival, as *ATM*-null mice are still viable (Barlow et al. 1996). In contrast, *ATR*-null mice display early embryonic lethality, showing that *ATR* is essential for cell survival (Brown and Baltimore 2000). Hypomorphic mutations in *ATR* cause a rare condition called Seckel syndrome, where patients still have low levels of functional ATR and display growth and mental retardation (O'Driscoll et al. 2003).

1.2.4. ATM kinase activation

In normal unperturbed cells, ATM activity is minimal as it exists as a homodimer in the nucleus, whereby the kinase domain of the protein is blocked by its tight binding to an internal domain in the adjacent ATM protein which surrounds the serine 1981 (Kastan and Bartek 2004). The formation of DSBs leads to ATM undergoing conformational

change, which results in autophosphorylation of the protein at serine 1981 and subsequent dissociation of ATM dimers, thus releasing the activated ATM monomers (Bakkenist and Kastan 2003). The recruitment of proteins to sites of DSBs occurs rapidly, although the autophosphorylation of ATM does not appear to be dependent on its recruitment to these lesions, rather it results from a change in the higher-order structure of chromatin that occurs at some distance away from the DNA damage sites (Bakkenist and Kastan 2003). Despite this, ATM-mediated activation of cell cycle checkpoints requires ATM to be localized at sites of DSBs that occurs through its interactions with the C-terminal region of Nijmegen breakage syndrome 1 (NBS1) protein (Falck et al. 2005; You et al. 2005). The meiotic recombination 11 (Mre11) and Rad50 proteins are both binding partners for NBS1, and together form the Mre11-Rad50-NBS1 (MRN) complex, which are amongst the first proteins to be recruited to DNA damage foci and have been shown to be required for efficient ATM kinase activation (Trujillo et al. 1998; Uziel et al. 2003).

There are many other proteins that are required for efficient activation of ATM, which include mediator of DNA damage checkpoint 1 (MDC1), breast cancer susceptibility gene 1 (BRCA1), and p53-binding protein 1 (53BP1), which like NBS1, contain BRCA1 C-terminal (BRCT) domains. MDC1 is phosphorylated by casein kinase 2 (CK2) at the N-terminal region that is enriched with Ser-Asp-Thr (SDT) repeats, which facilitates its interaction with the forkhead-associated (FHA) domain of NBS1, and this association is required for the MDC1-MRN-ATM complex to be tethered to chromatin-flanking sites of unrepaired DSBs (Chapman and Jackson 2008; Melander et al. 2008; Spycher et al. 2008).

Upon recruitment to sites of DSBs, ATM phosphorylates a number or transducer and effector proteins which activate signalling cascades that lead to checkpoint activation and DNA repair (Fig. 1.8). The major transducer protein in this cascade is the checkpoint kinase 2 (Chk2) protein, which is phosphorylated in the ATM pathway, causing the stabilization of p53 by phosphorylating it at sites which interfere with MDM2 binding, and thus resulting in subsequent activation of cell cycle checkpoints or apoptosis (Hirao et al. 2000). Thus, ATM activation, and ATM-mediated phosphorylation of its downstream targets can be summarised as occurring in two steps, the first being autophosphorylation of itself to dissociate it from its homodimer form in to monomers, and secondly localization of these activated monomers to sites of DNA damage where its substrates are located (Kastan and Bartek 2004).

1.2.5. ATR kinase activation

In contrast to ATM, ATR exists as in a heterodimeric complex with the ATR-interacting protein (ATRIP), which has been deemed its essential binding partner, as loss of either gene results in the destabilization of both proteins (Cortez et al. 2001). Until recently it was believed that ATR-ATRIP complexes were not subject to post-translational modification in response to DNA damage, neither was there an increase in ATR kinase activity, however, recent studies have shown that autophosphorylation of ATR occurs on T1989 in response to DNA damage (Bartek and Mailand 2006; Liu et al. 2011; Nam et al. 2011). ATR-ATRIP is recruited to sites of DNA damage through an interaction between ATRIP and replication protein A (RPA) (Zou et al. 2003). RPA is a heterotrimeric ssDNA-binding complex which consists of 14, 32, and 70 kDa subunits, that coats ssDNA, and recruits the ATR-ATRIP complex to DNA via interactions between RPA70 and ATRIP (Zou and Elledge 2003; Ball et al. 2007). The sole recruitment of ATR to sites of DNA damage is not sufficient for an ATR-

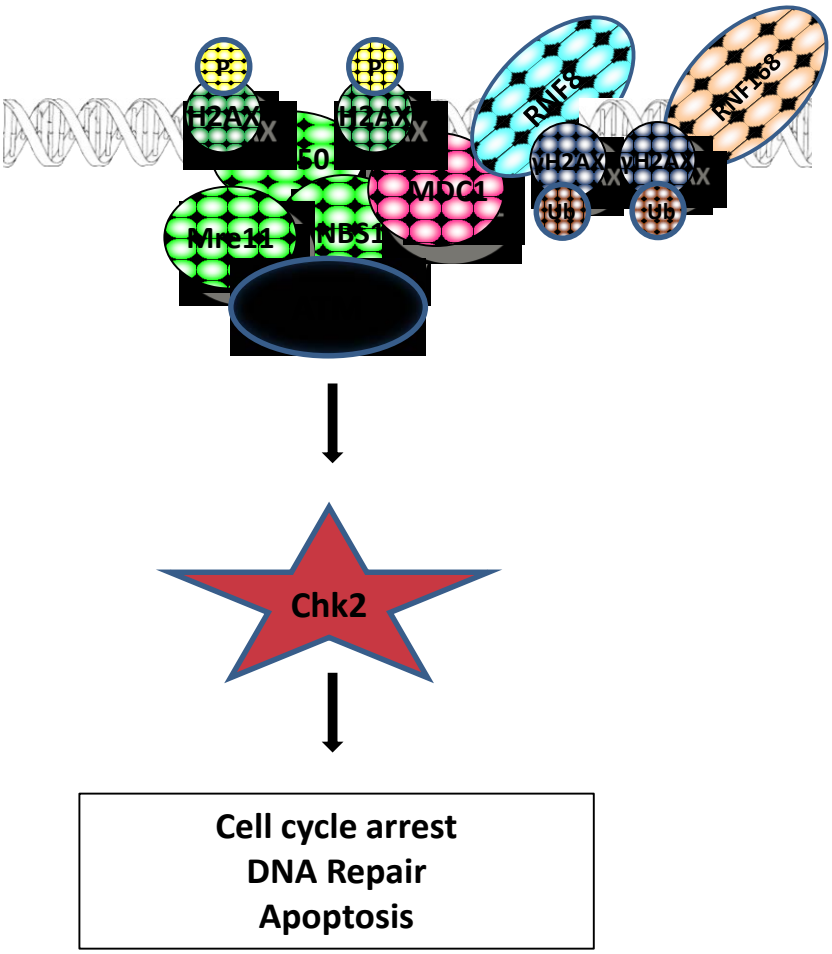

Fig. 1.8. ATM signalling pathway. DSBs are sensed by MRN complex which recruits the ATM kinase to these lesions. ATM phosphorylates H2AX which then recruits a series of mediator proteins such as MDC1 and transducer proteins such as Chk2. RNF8 and RNF168 ubiquitylate γH2AX to further amplify the signal and allow further recruitment of mediator proteins. Activation of ATM kinase activity by MRN allows phosphorylation of downstream transducer proteins such as Chk2, which promotes cell cycle arrest, DNA repair, and apoptosis (Weitzman et al. 2010).

dependent damage response. RPA also recruits the Rad17-RCF2-5 (RSR) complex known as the 'clamp loader', and the Rad9-Hus1-Rad1 (9-1-1) complex which functions as the sliding checkpoint clamp (Zou et al. 2002; Zou et al. 2003). Rad17 is required to load the 9-1-1 complex which requires ATP, and occurs independently of ATR localization or function (Zou et al. 2002). Until recently the functional relevance of loading of the 9-1-1 complex was unclear, but now it is known to be required to recruit topoisomerase (DNA) II binding protein 1 (TopBP1) to sites of DNA damage through the proteins interactions with the C-terminal of Rad9 (Delacroix et al. 2007; Lee et al. 2007). TopBP1 functions to directly activate ATR by stimulating its kinase activity via interactions with both ATR and ATRIP (Kumagai et al. 2006; Mordes and Cortez 2008). Therefore the recruitment of TopBP1 to DNA lesions requires 9-1-1, which links it to ATR-ATRIP, resulting in checkpoint signalling. In support of this, TopBP1 has been shown to be essential for certain ATR-dependent signalling events, including checkpoint kinase 1 (Chk1) and NBS1 phosphorylation (Kumagai et al. 2006). A model for ATR activation in response to ssDNA damage is illustrated in Figure 1.9.

The major transducer protein in the ATR signalling pathway is the Chk1 kinase, which is phosphorylated by ATR on serine residues 317 and 345 (Liu et al. 2000; Zhao and Piwnica-Worms 2001). Chk1 phosphorylation also requires the combined actions of Claspin, Timeless (Tim), and Timeless-interacting protein (Tipin) to mediate its recruitment to ATR (Chini and Chen 2003; Unsal-Kacmaz et al. 2005; Chou and Elledge 2006).

1.2.6. TopBP1

TopBP1 is a multifunctional protein, and is involved in transcriptional regulation, DNA

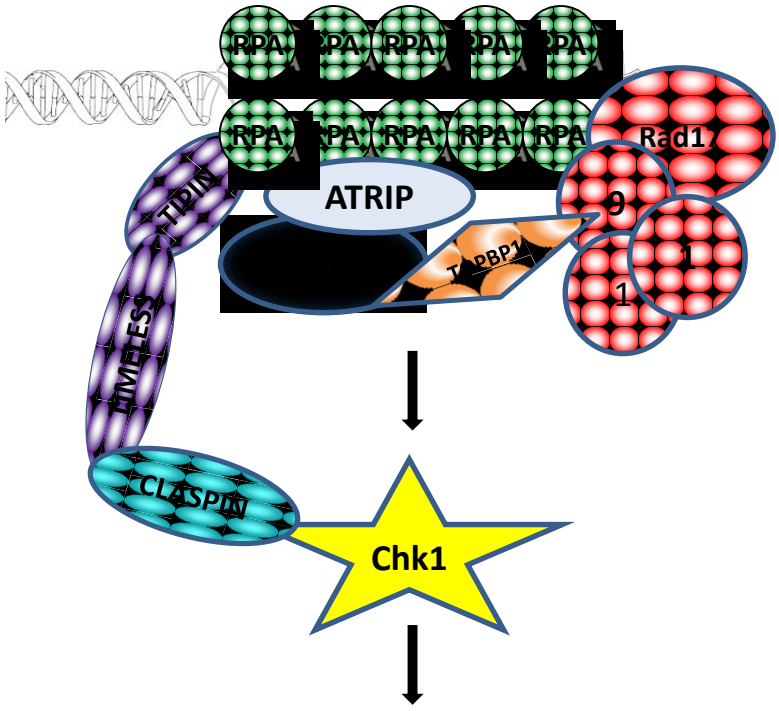

Fig. 1.9. ATR signalling pathway. The generation of ssDNA as a result of replication stress or single-stranded breaks becomes coated in RPA. RPA then recruits ATR-ATRIP, Rad17, Rad9-Hus1-Rad1 complex to ssDNA. Rad17 loads the 9-1-1 complex, which in-turn recruits TopBP1 to bind and activate ATR. Tipin binds to RPA, which stabilizes Tim-Tipin complexes and Claspin onto RPA-coated ssDNA, which allows recruitment of Chk1 to ATR. Activation of ATR kinase activity phosphorylates downstream transducer proteins such as Chk1, which promotes cell cycle arrest, DNA repair, and apoptosis (Weitzman et al. 2010).

replication, and checkpoint signalling (Garcia et al. 2005). TopBP1 is a structurally and functionally conserved protein amongst eukaryotes, and was originally identified as a topoisomerase IIβ interacting partner; it contains 8 BRCT domains that are often found in proteins that function in cell cycle checkpoint regulation and cellular response to DNA damage (Yamane et al. 1997; Garcia et al. 2005).

The best characterized function of TopBP1 is its role as an ATR activator. TopBP1 has been shown to contain an ATR-activating domain in the C-terminal region of the protein which lies between the 6^{th} and 7^{th} BRCT domain, where it binds to ATR to activate its kinase activity; activation of ATR is required for both the phosphorylation of its downstream targets, and the regulation of the G_2/M checkpoint (Yamane et al. 2003; Kumagai et al. 2006). However, the interaction between TopBP1 and ATR described above is not regulated by DNA damage, and until recently it was unclear how activation of ATR by TopBP1 in response to DNA damage occurred (Mordes et al. 2008). Recent research has shown that autophosphorylation of ATR at T1989 in response to DNA damage is directly recognised by the TopBP1 BRCT domains 7 and 8, and allows TopBP1 to interact with ATR-ATRIP resulting in subsequent activation of the ATR kinase activity and substrate recognition (Liu et al. 2011). As described above, TopBP1 is recruited to ssDNA by the 9-1-1 complex via its interaction with the C-terminal region of Rad9 (Delacroix et al. 2007; Lee et al. 2007). Furthermore, depletion of TopBP1 results in decreased Chk1 phosphorylation, and the partial abrogation of the G_2/M checkpoint, further suggesting that TopBP1 plays a role in Chk1 activation (Yamane et al. 2003). Also, like ATR-null mice, *TopBP1*-null mice display early embryonic lethality, showing that *TopBP1* is essential for cell survival (Brown and Baltimore 2000; Jeon et al. 2011).

TopBP1 function has also been attributed to cellular response to DSBs. It is known that there is crosstalk between the ATM and ATR signalling pathways that are activated in response to DNA damage (Adams et al. 2006; Jazayeri et al. 2006; Stiff et al. 2006). In response to DSBs, ATM has been shown to regulate TopBP1 by phosphorylating it at Ser-1311, and strongly enhancing its association with ATR (Yoo et al. 2007). Cells that express a mutant TopBP1 that prevents this phosphorylation from occurring are defective in ATR-mediated Chk1 phosphorylation in response to DSBs, demonstrating that TopBP1 is essential for the ATM-mediated activation of ATR in response to DNA damage that causes DSBs (Yoo et al. 2007). The interaction between ATM and TopBP1 requires MRN, in particular the NBS1 subunit, as depletion of NBS1 inhibits ATM from interacting with TopBP1 (Yoo et al. 2009). TopBP1 binds directly to NBS1 via its BRCT 1 and 2 domains and the two tandem BRCT repeats on NBS1 (Yoo et al. 2009). Furthermore, TopBP1 also interacts with 53BP1 through its BRCT domains 4 and 5 to mediate the DNA damage checkpoint function of 53BP1 in G_1, where TopBP1 is recruited to sites of DNA DSBs by 53BP1, specifically in the G_1 phase of the cell cycle (Cescutti et al. 2010).

In unperturbed cells, TopBP1 plays a role in DNA replication by functioning as a modulator of G_1/S transition (Kim et al. 2005). TopBP1 is able to transcriptionally regulate E2F1 by repressing it in a pRB-independent fashion by recruiting a component of the SWI/SNF chromatin-remodelling complex, Brg/Brm, to E2F1- responsive promoters, which represses E2F1 activity and inhibits E2F1-dependent apoptosis at G_1/S (Liu et al. 2004). Subsequent studies have revealed that the phosphorylation of TopBP1 at Ser-1159 by Akt results in the oligomerization of the protein, which is essential for its interaction with, and the repression of, E2F1 (Liu et al. 2006). Furthermore, TopBP1 depletion results in cells being unable to enter S phase, as it

causes the defective chromatin-loading of replication factors, and the up-regulation of p21 and p27 cdk inhibitors and the down-regulation of cyclin E/CDK2 (Jeon et al. 2007). TopBP1 is also required for the formation of the pre-initiation complex in mammalian cells and *Xenopus* egg extracts, and is required to load cell division cycle 45 (CDC45) onto replication origins; TopBP1 association with Treslin promotes CDC45 loading (Fig. 1.10) (Schmidt et al. 2008; Kumagai et al. 2010).

TopBP1 has been shown to be functionally similar to BRCA1 as well as sharing sequence conservation within their respective BRCT domains. As discussed above both proteins are required for Chk1 activation and G_2-M regulation, as well as being able to partially compensate for each other's function (Yamane et al. 2003). Furthermore, like *BRCA1* and *BRCA2*, *TopBP1* has been shown to a possible susceptibly gene for breast and ovarian cancers, as there is commonly occurring TopBP1 Arg309Cys alteration that has been linked with an increased risk to hereditary breast and ovarian cancer, although there are conflicting reports as to whether this is the case (Karppinen et al. 2006; Blaut et al. 2010). Furthermore, TopBP1 has been shown to bind to p53 and inhibit its transcriptional activity via an interaction between its BRCT 7 and 8 domains and p53's DNA-binding domain (Liu et al. 2009). This function of TopBP1 is essential in normal cells for G_1/S transition, however, in breast cancer tissues TopBP1 has been found to be overexpressed and linked with high tumour grade and short patient survival time; it has been postulated that the oncogenic potential of overexpressed TopBP1 resides in its ability to inhibit p53 (Liu et al. 2009).

1.2.7. Timeless and Tipin

Timeless (Tim) is a protein that is essential for the regulation of circadian rhythms and was first identified in *Drosophila melanogaster* in 1994. The mammalian homologues

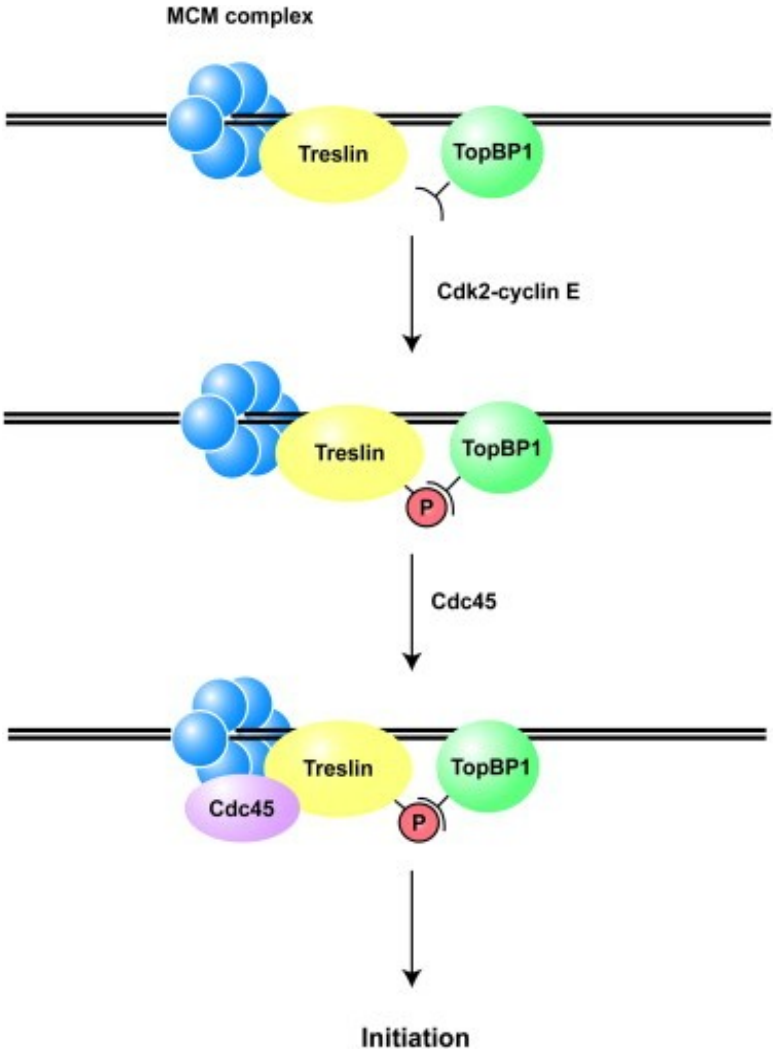

Fig. 1.10. TopBP1 promotes DNA replication. TopBP1 binds to chromatin along with Treslin. Cdk2 promotes phosphorylation of Treslin which is required for it form a complex with TopBP1. The TopBP1-Treslin complex is required for the recruitment and loading of CDC45 onto replcation origins (Kumagai et al. 2010).

were discovered later in 1998, and have been since shown to function in cell cycle progression, DNA replication, cellular responses to DNA damage (Sehgal et al. 1994; Koike et al. 1998; Kondratov and Antoch 2007). Unlike its *Drosophila* homologue, the role of Tim in the mammalian clock is debatable, and it also appears that it is not actually a true homologue of *drosophila* Tim, but instead shares a greater homology with another *Drosophila* protein, Timeout (Tim-2) (Benna et al. 2000; Gotter et al. 2007).

Tim has functions in the ATR pathway as a mediator protein and interacts with Chk1, ATR, and ATRIP in response to DNA damage that occurs as a consequence of exposure to the genotoxic agents UV and HU (Unsal-Kacmaz et al. 2005). The importance of its role in the ATR signalling pathway is highlighted by the fact that depletion of Tim reduces Chk1 phosphorylation in cells treated with HU (Unsal-Kacmaz et al. 2005). The mechanism behind which Tim promotes Chk1 activation is relatively unclear, however, seeing as it has been shown to bind to Claspin it has been suggested that it functions to recruit Chk1 to ATR, whereupon Chk1 is then phosphorylated (Gotter et al. 2007).

Tim exists in the nucleus of cells with its binding partner Tipin, an interaction that occurs through the N-terminal region of both proteins, and has been shown to be important for the stability for both proteins, as it has been shown that down-regulation of either protein by small interfering (si)RNA leads to reduced protein levels and relocalization to the cytoplasm of the other protein (Gotter 2003; Chou and Elledge 2006; Yoshizawa-Sugata and Masai 2007). Like Tim, depletion of Tipin inhibits Chk1 phosphorylation in response to DNA damage caused by genotoxic agents, thus showing that Tipin is also required for an efficient DDR (Unsal-Kacmaz et al. 2007). Furthermore, Tipin binds to RPA2 in response to DNA damage, which stabilizes Tim-

Tipin complexes and Claspin onto RPA-coated ssDNA, and promotes the Claspin-mediated phosphorylation of Chk1 by ATR (Unsal-Kacmaz et al. 2007; Kemp et al. 2010).

Tim/Tipin also function to stabilize replication forks and promote sister-chromatid cohesion (Leman et al. 2010). Tim/Tipin co-localize with RPA at nuclear replication sites and interact with MCM2 (Tim), MCM6, MCM7 (Tipin), and Polδ and Polε DNA polymerases, where they are then thought to coordinate helicase-induced DNA unwinding and polymerase-mediated DNA synthesis (Chou and Elledge 2006; Gotter et al. 2007). Replication fork stabilization by Tim/Tipin is essential, as depletion of these proteins result in defective damage repair in response to fork collapse and chromosome fragmentation, as well as impaired sister-chromatid cohesion and defective mitotic progression (Leman et al. 2010). Further evidence for the role of Tim in maintaining genome integrity comes from a study that shows that like TopBP1 and ATR, *Timeless*-null mice also display embryonic lethality (Gotter et al. 2000).

1.2.8. Mediators of the DNA damage checkpoint

In addition to TopBP1, there are a number of proteins that have been identified as DDR mediators, many of which, like TopBP1, possess BRCT domains. Well known examples include MDC1, 53BP1, and BRCA1 (Schultz et al. 2000; Stewart et al. 2003). In response to DNA damage, the principle function of these proteins is to provide a base, or scaffold, for DNA damage signal amplification, whilst simultaneously providing signal transduction specificity (Sancar et al. 2004). The recruitment of mediator proteins to sites of DNA damage require the ATM-mediated phosphorylation of histone 2A family member X (H2AX), which then marks the chromatin regions flanking the DSBs (Rogakou et al. 1999; Niida and Nakanishi 2006).

Mediator proteins are phosphorylated by either ATM or ATR, and carry out their functions through interactions with sensors, transducers, effectors and other mediators in a cell cycle-dependent manner (Houtgraaf et al. 2006).

Like TopBP1, other mediator proteins have multiple roles in the DDR. For example, MDC1 localizes the MRN complex to sites of DSBs as well as recruiting the RING-finger 8 (RNF8) ubiquitin ligase to these lesions; RNF8 ubiquitylates H2A and H2AX and consequently recruits 53BP1 and BRCA1 to DSB sites (Huen et al. 2007; Mailand et al. 2007). Claspin is another mediator protein with multiple functions. It is required principally to recruit Chk1 to ATR to allow for the ATR-dependent phosphorylation of Chk1. Claspin also associates with, and stabilizes, replication forks during unwinding and DNA replication (Chini and Chen 2003; Yoo et al. 2006). Depletion of mediator proteins such as TopBP1, BRCA1, and 53BP1 cause defects in DNA damage checkpoint activation and sensitize cells to genotoxic stress agents, demonstrating that these proteins are required for rapid and efficient cell cycle arrest in response to DNA damage (Stewart et al. 2003; Yamane et al. 2003).

1.2.9. Role of Transducer and effector proteins in checkpoint activation

Chk1 and Chk2 are two key, specific transducers of signals from DNA damage sensors to cell cycle checkpoints. Signals from ssDNA are primarily transduced to Chk1 by ATR-mediated phosphorylation, which activates Chk1 kinase activity, whilst Chk2 kinase activity is stimulated in response to ATM-mediated phosphorylation and primarily transduces signals from DSBs, although there is some overlap between the functions of the two proteins (Matsuoka et al. 2000; Zhao and Piwnica-Worms 2001; Gatei et al. 2003). Like ATR, *Chk1*-null mice display embryonic lethality, whereas like ATM, *Chk2*-null mice are still viable (Takai et al. 2000; Hirao et al. 2002). Once

activated, Chk1 and Chk2 dissociate from chromatin and activate the appropriate effectors proteins depending on the cell cycle phase; effectors include the essential regulators of cell cycle progression, p53, and the phoshotyrosine phosphatase, CDC25A (Chen et al. 2003; Li and Stern 2005; Smits et al. 2006). The effector proteins are what give DNA damage checkpoint signalling pathways their unique identities, as the sensor and signal transducer proteins are common in all three pathways (Sancar et al. 2004).

The G_1/S checkpoint is activated in response to DNA damage to prevent cells from entering S phase and commencing DNA replication in the presence of DNA aberrations. Cells that have entered the G_1 phase of the cell cycle typically become committed to enter S phase once they have passed the restriction point. However, if DNA becomes damaged during G_1, entry into S phase becomes inhibited even if cells have passed the restriction point (Sancar et al. 2004). The two main effectors in the G_1/S checkpoint are p53 and CDC25A. Initial G_1/S arrest occurs when activated Chk1 and Chk2 phosphorylate CDC25A; phosphorylation inactivates CDC25A by promoting its nuclear exclusion and proteolytic degradation (Bartek and Lukas 2001). CDC25A degradation inhibits the initiation of DNA replication, as CDC25A is normally required to activate CDK2 and promote the CDK2-dependent phosphorylation, and loading of CDC45 onto replication origins (Costanzo et al. 2000). To maintain G_1/S arrest, ATM and ATR either phosphorylate p53 directly at Ser15, or indirectly via Chk1 and Chk2 at Ser20, which activates and stabilizes the p53 protein, allowing it to stimulate the transcription of its target genes, one of which is the CDK inhibitor, p21 (Bartek and Lukas 2001; Sancar et al. 2004). Both cyclin E-CDK2 and cyclin D-CDK4 are inhibited by p21; inactivation of CDKs by p21 inhibits pRB phosphorylation, and prevents pRB release from E2F and the transcription of genes required for S phase progression (Harper et al. 1993; Lin et al. 2001).

The intra-S phase checkpoint is activated to block DNA replication in response to either DNA damage that occurs during S phase, or by unrepaired damaged DNA that has managed to escape the G_1/S checkpoint (Sancar et al. 2004). There are two commonly known pathways that exist to activate the intra-S phase checkpoint, both of which are p53-independent. The first pathway involves the phosphorylation of CDC25A by checkpoint kinases, much like in the G_1/S checkpoint, to prevent CDC45 loading onto replication origins (Falck et al. 2001). The second mechanism involves a Chk1/2-independent pathway, and is dependent upon the ATM-dependent phosphorylation of structural maintenance of chromosomes 1 (SMC1), Fanconi anaemia complementation group D2 (FANCD2), BRCA1 and the MRN complex; phosphorylation of these proteins results in replication inhibition, although the exact mechanism of how this branch of the intra-S phase functions remains unclear (Kim et al. 2002; Nakanishi et al. 2002; Yazdi et al. 2002). As mentioned above, the intra-S phase is generally thought to be p53-independent, however, recent studies have shown a possible role for p53 in this checkpoint where it acts with Chk1 to prevent DNA synthesis (Ahmed et al. 2011).

The G_2/M checkpoint is activated in response to cells that have undergone DNA damage in the G_2 phase to block them from entering mitosis (Houtgraaf et al. 2006). The initiation and maintenance of this checkpoint requires both ATM and ATR kinase activity. Chk1 and Chk2 phosphorylate and inactivate CDC25A, which in turn inhibits CDC2/cyclin B1 and prevents cells from entering mitosis (Sanchez et al. 1997; Mailand et al. 2002). Furthermore, both Chk1 and Chk2 phosphorylate and activate the WEE1 kinase, which also leads to CDC2/cyclin B1 inhibition (Parker and Piwnica-Worms 1992; McGowan and Russell 1993). The maintenance of G_2/M arrest is again mediated by p53 via its transcriptional activation of CDK inhibitors such as p21, although unlike

the G_1/S checkpoint, this pathway is non-essential (Lakin and Jackson 1999; Taylor and Stark 2001).

1.2.10. The role of ubiquitin in the DNA damage response

The Nobel prize in Chemistry was awarded in 2004 to Avram Hershko, Aaron Ciechanover, and Irwin Rose for their discovery of the function of ubiquitin (Welchman et al. 2005). Ubiquitin is 76 amino acid polypeptide that can be covalently attached to substrate proteins; poly-ubiquitylation is widely recognised as a post-translational modification that signals proteins for degradation by ATP-dependent protease, the 26S proteasome (Thrower et al. 2000; Xie 2010). However, it is becoming increasingly apparent that conjugation of ubiquitin to proteins, does not necessarily promote degradation, and that ubiquitin is required for many cellular functions, such as cellular differentiation, cell cycle progression, DNA repair, and apoptosis (Welchman et al. 2005). Furthermore, there is increasing evidence to show that DNA damage response proteins are continuously ubiquitylated and deubquitylated to regulate their function (Zhang et al. 2006).

Ubquitylation is the process by which ubiquitin is covalently attached to its target protein, and occurs in three steps, with each step requiring a separate type of enzyme: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-protein ligase (E3) (Hershko et al. 1983; Welchman et al. 2005). The E1 enzyme binds to free ubiquitin which is adenylated in an ATP-dependent manner to activate it, whereupon it is then modified by a *trans*-thiolation reaction allowing it to conjugate to the E2 enzyme, which associates with the E3 enzyme, that acts as the bridge between the substrate and the E2 conjugating enzyme, and allows for ubiquitin to be transferred onto the amino group of the substrate (Fig. 1.11)

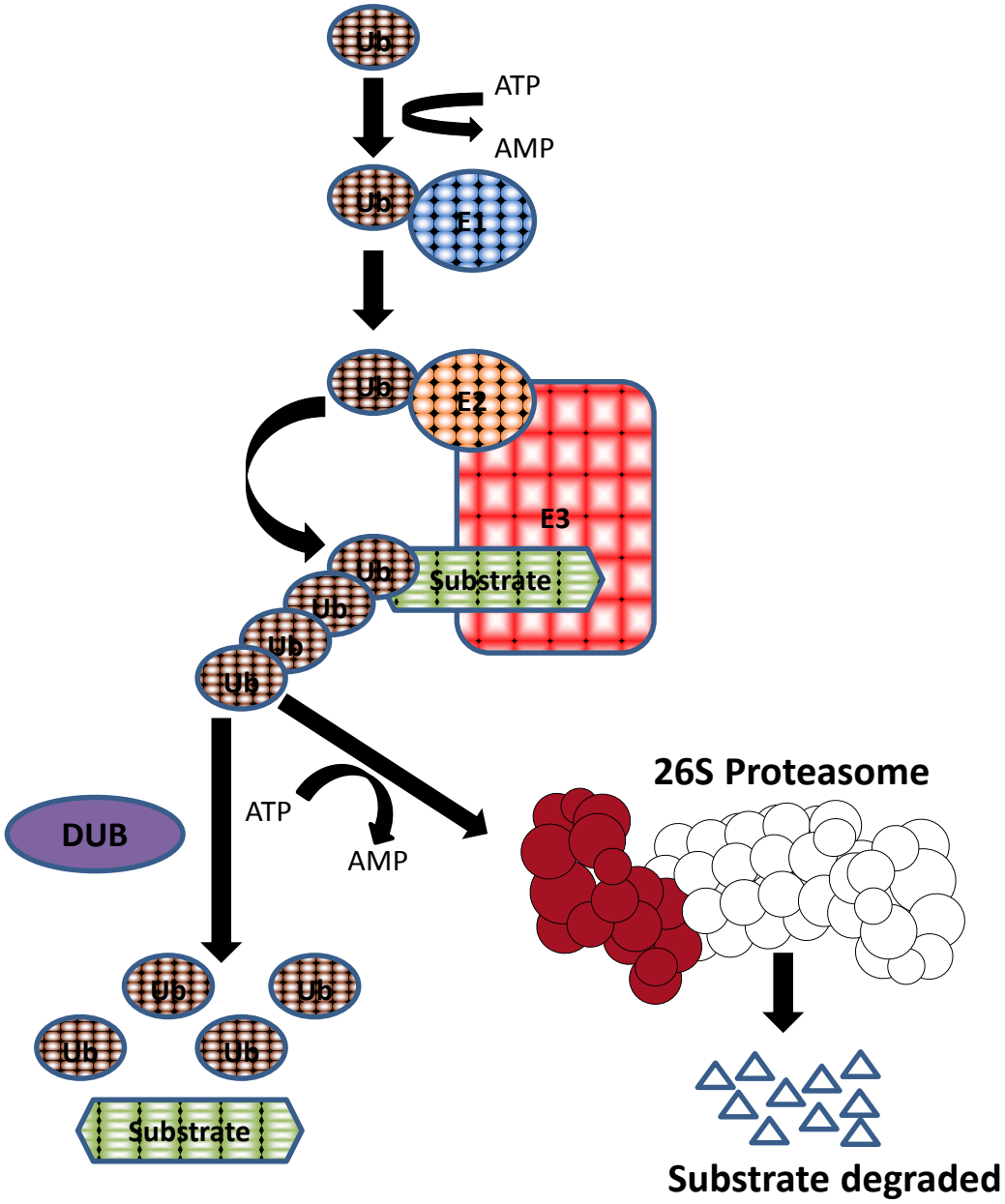

Fig. 1.11. Protein ubiquitylation and 26S proteasome-mediated degradation. The covalent attachment of ubiquitin (Ub) to its substrate is dependent on ATP hydrolysis and occurs in three steps, which requires three different enzymes (E1, E2, and E3). E1 binds and activates Ub and then transfers it to E2 Ub-conjugating enzyme. The E3 Ub-protein ligase then mediates the attachment of Ub to a lysine residue on it substrate, which are then recognized by the 26S proteasome and subsequently degraded. Deubiquitylating enzyme (DUB) is able to reverse ubiquitylation. (Hoeller et al. 2006)

(Weissman 2001; Welchman et al. 2005). There are also many de-ubiquitylating (DUB) enzymes that exist in the cell which act to reverse ubiquitylation by catalysing the hydrolysis of ubiquitin-substrate bonds (Wilkinson 2000).

As mentioned above, ubiquitylation and deubiquitylation regulates the function of a number of proteins involved in the DDR pathways, and there are an increasing number of E3 ubiquitn ligases that are being identified that function in these pathways, which include RNF8, RNF168, and HERC2 (Huen et al. 2007; Mailand et al. 2007; Stewart et al. 2009; Bekker-Jensen et al. 2010). The E3 ubiquitin ligase RNF8 and the E2 Ub-conjugating enzyme UBC13 ubiquitylate γ-H2AX in response to IR, which is required for receptor protein 80 (RAP80), BRCA1, and 53BP1 accumulation at sites of DSBs (Huen et al. 2007; Mailand et al. 2007). RNF168 is an E3 ubiquitin ligase (mutated in RIDDLE syndrome) which binds to ubiquitylated H2A and along with UBC13 operates downstream of RNF8 to amplify the RNF8-dependent ubiquitylation of histones (Stewart et al. 2009). Recent data has shown that another E3 ubiquitin ligase, HERC2, interacts with both RNF8 and RNF168, and is required to facilitate assembly of UBC13 with RNF8, and maintain levels of RNF168 (Bekker-Jensen et al. 2010). Furthermore BRCA1 has been shown to display E3 ubiquitin ligase activity, as there is evidence to suggest that it ubiquitylates CtBP-interacting protein (CtIP) and Claspin; BRCA1-mediated ubiquitylation of Claspin selectively triggers Chk1 activation (Yu et al. 2006; Sato et al. 2012). Another DDR protein that is ubiquitylated is proliferating cell nuclear antigen (PCNA), which is mono-ubiquitylated by the E3-ubiquitin ligase activity of Rad18 in response to DNA damage (Hoege et al. 2002). The ubiquitin is removed in undamaged cells by the DUB, ubiquitin-specific protease 1 (USP1), which also de-ubiquitylates FANCD2 after DNA repair (Nijman et al. 2005; Huang et al. 2006). In DNA damaged cells, USP1 undergoes autocleavage, thus inactivating it and allowing

both PCNA and FANCD2 to remain ubiquitylated and function in DNA repair pathways (Huang and D'Andrea 2006). Another DUB that has been shown to play a role in the DDR is USP28, which has been shown to inhibit the degradation of a number of proteins in response to IR, including ATRIP, TopBP1, NBS1, MDC1, 53BP1, and Chk2 (Zhang et al. 2006).

1.3. REGULATION OF THE CELLULAR DNA DAMAGE RESPONSE BY ADENOVIRUS DURING INFECTION

1.3.1. Concatenation of adenovirus genomes

During its replication life cycle, the terminal portions of the Ad linear ds DNA genome has the potential to mimic a DNA DSB in the host cell, whilst viral DNA replication generates ssDNA intermediates that are also capable of eliciting a cellular DNA damage response (Weitzman et al. 2004). As described above, the DDR is a rapid and efficient process which would prove to be detrimental to Ad DNA replication in the host cell. Therefore Ad has evolved a number or mechanisms to inactivate these pathways in order to facilitate efficient replication of its genome in the host cell.

The first indication that Ad DNA was recognised by the host cell as damaged DNA came from observations of Ad5 mutant viruses lacking the E4 region. Infections with these viruses resulted in production of large concatemers of viral DNA, which are covalently linked monomers of DNA joined by no specific orientation (Weiden and Ginsberg 1994). Concatenation of the Ad genome results in loss of ITRs which contain the origins of Ad DNA replication, thus rendering them as dead-end molecules. Subsequent studies using the mutant Ad5 virus *dl*1004, which lacks the entire E4

region, showed that this virus was defective for DNA replication and late protein synthesis due to the formation of large viral concatamers (Stracker et al. 2002). Furthermore it was found that *dl*1004 infection of mutant cell lines that did not express Mre11, DNA ligase IV, and DNA-PKcs rescued the concatemer phenotype, suggesting that core components of the ATM and NHEJ pathways are likely to be inactivated during infection with *wt* Ad (Stracker et al. 2002). Using the *dl*1004 virus and *wt* Ad it was later shown that the MRN complex is required for ATM and ATR activation in responses to DNA damage, as infection with the mutant virus yielded a DDR, whilst the infection with the *wt* Ad virus did not (Carson et al. 2003). A more recent study questioned whether the DDR response and concatemer formation was indeed the cause of defective DNA replication and late protein synthesis seen in E4 mutant viruses (Lakdawala et al. 2008). This study revealed that inhibition of viral DNA replication was a direct consequence of MRN activity, rather than activation of DDR pathways and concatemer formation (Lakdawala et al. 2008).

1.3.2. Adenovirus-mediated degradation of cellular proteins

There are an ever-increasing number of cellular proteins that are being recognised as targets for Ad-mediated degradation. The Ad oncoproteins have been shown to hijack E3 ubiquitin ligase complexes to facilitate degradation of their cellular substrates in an ubiquitin-dependent proteasome-mediated pathway. Ad predominantly target CRLs during infection. CRLs are the largest family of multi-subunit E3 ligases in eukaryotes with eight members (Cul1, 2, 3, 4A, 4B, 5, 7, and PARC/Cul9) (Sarikas et al. 2011). Adenoviruses have thus far only been shown to utilize Cul1, Cul2, and Cul5 (Querido et al. 2001; Isobe et al. 2009; Cheng et al. 2011; Forrester et al. 2011).

The work presented in this thesis focuses primarily on the relationship between Ads and Cul2 and Cul5. The CRL complexes that contain these scaffold proteins also have Elongin BC adaptor proteins, which link the substrate recognition von Hippel-Lindau (VHL) receptor boxes to Cul2, and suppressor of cytokine signalling (SOCS) to Cul5 (Kamura et al. 2004). The RBX1 E3 ligase component of the CRL is bound to the highly conserved C-terminal Cullin domains (Petroski and Deshaies 2005). The E3 ubiquitin ligase activity of CRLs are activated by a process known as neddylation, in which a ubiquitin-like protein, neural precursor cell-expressed developmentally down-regulated 8 (NEDD8) becomes covalently attached to a conserved lysine residue in the Cullin-homology domain (Petroski and Deshaies 2005). The conjugation of NEDD8 is essential for the ligase activity of CRLs, but it is reversible by deneddylation which subsequently inactivates it (Duda et al. 2008).

As described in section 1.1.6, Ad5 E1B-55K and E4orf6 function in concert to mediate the proteasome-mediated degradation of a number of proteins that function in the DDR pathway. The first to be described was p53, where E1B-55K replaces the SOCS-box as the substrate adaptor to bring p53 to CRL5, which is in turn recruited to the E1B-55K/E4orf6 complex by E4orf6 via one of its three BC box motifs that binds to the Elongin BC unit (Fig. 1.12) (Querido et al. 2001; Blanchette et al. 2004; Cheng et al. 2007). The inactivation of DNA ligase IV and Mre11 is believed to occur via the same mechanism as the E1B-55K-E4orf6-mediated proteasomal degradation of p53, although there are now conflicting reports in the literature with regards to the specific E3 ubiquitin ligases utilized by Ad to facilitate degradation of these proteins (Stracker et al. 2002; Baker et al. 2007; Cheng et al. 2011; Forrester et al. 2011). Furthermore E4orf6 functions independently of E1B-55K to dissociate DNA ligase IV from the XRCC4 complex prior to its degradation in order to prevent NHEJ of viral genomes

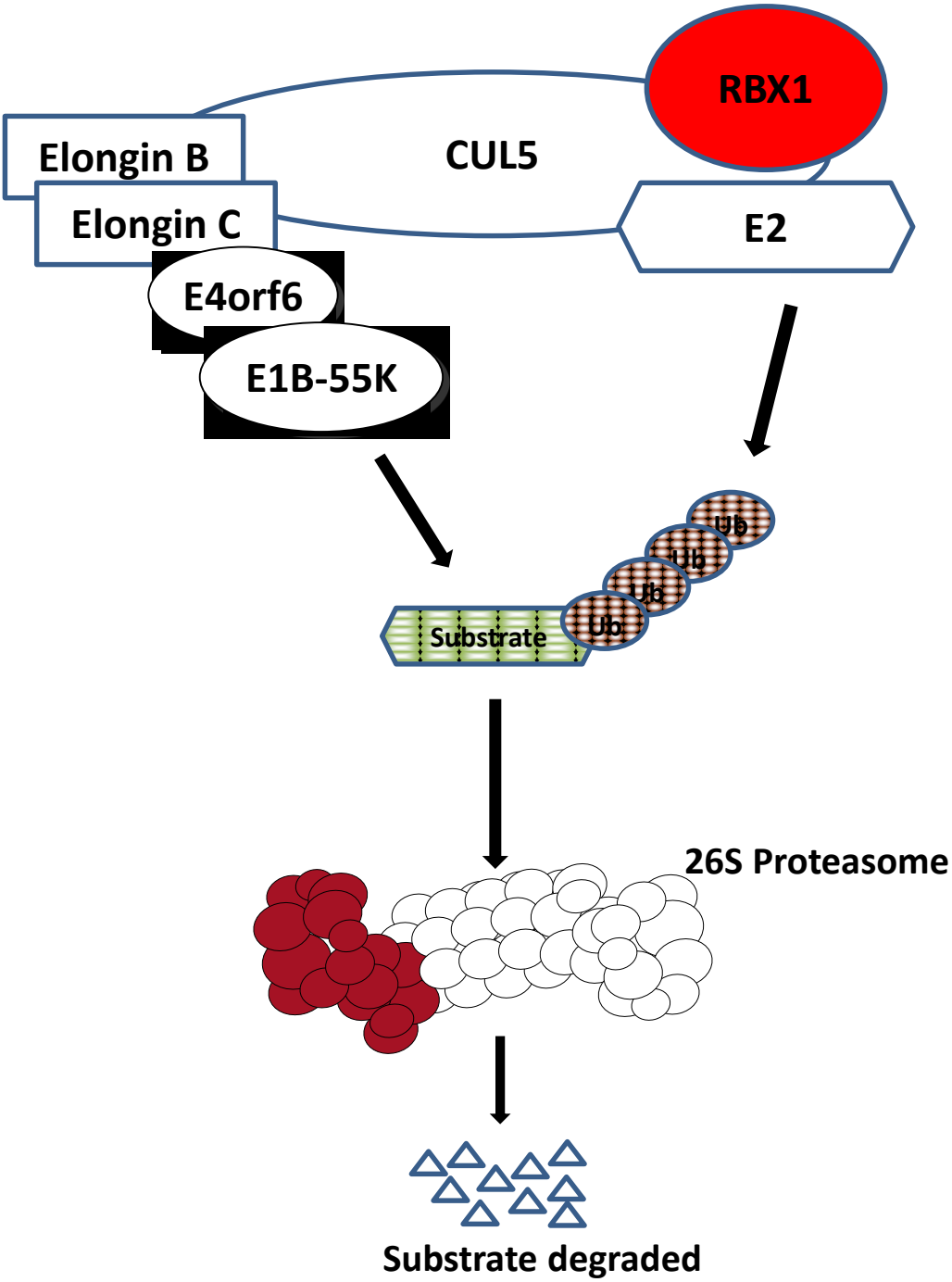

Fig. 1.12. Ad5-CRL mediated degradation of cellular proteins. Ad5 hijacks E3 ubiquitin ligase complexes containing the cellular CUL5, Elongins B and C, and RBX1. Ad5 E1B-55K acts as the substrate adaptor, and Ad5 E4orf6 recruits the CRL via interactions with the Elongins. The substrate is then polyubiquitylated and targeted for 26S proteasome-mediated degradation.

(Jayaram et al. 2008). The Bloom helicase (BLM) is a RecQ DNA helicase, which functions in DNA end resection, is also degraded by Ad5 utilising the same mechanism as for p53, however the functional relevance of this is yet unknown as BLM was shown not to promote concatemer formation (Gravel et al. 2008; Orazio et al. 2011).

Other cellular proteins that are targeted by adenovirus for proteasome-mediated degradation include the multifunctional death domain-associated protein (Daxx), which is degraded at late time points by mechanism that requires Ad5E1B-55K to recruit CRL5 via a highly conserved BC box motif in an E4orf6-independent manner (Schreiner et al. 2010). It is suggested that Daxx might act as a transcriptional repressor during early stages of infection, as Ad5 infection of cell lines that do not express Daxx result in increased early viral protein synthesis and viral progeny (Schreiner et al. 2010). In addition to Daxx, TIF1γ is also degraded via novel mechanism which involves E4orf3 and is E1B-55K/E4orf6 and CRL-independent (Forrester et al. 2012). Ad infection of cells that are depleted of TIF1γ display an increase in early and late viral proteins, suggesting that TIF1γ may act as a transcriptional repressor during both early and late stages of viral infection (Forrester et al. 2012). Ad infection also results in the degradation of the cell surface protein integrin alpha 3, which occurs in the classic E1B-55K/E4orf6 and CRL-dependent manner, and is thought to most likely to promote release and distribution of viral progeny, or to prevent re-infection (Dallaire et al. 2009).

The DDR proteins that are targeted by Ad for degradation vary between the different serotypes, for example, BLM, Mre11 and p53 are not degraded by group B and D viruses (Cheng et al. 2011; Forrester et al. 2011). As outlined above there are also different requirements for viral proteins that are needed to degrade the cellular targets. Degradation of Mrell, DNA ligase IV, and p53 require the combined actions of E1B-

55K and E4orf6, whereas Daxx only requires E1B-55K, and TIF1γ only E4orf3. The role of CRLs in Ad-mediated degradation will be considered in more detail in the results Chapters 3 and 4 presented in this thesis.

1.3.3. Relocalization of cellular proteins during Ad infection

In addition to degradation of components of the DDR pathways, Ad is also able to affect the localization of a number of DDR proteins (Turnell and Grand 2012). RPA32 is one such example, which is localized at viral replication centres (VRC) during infection with both *wt* and mutant viruses, presumably binding to the ssDNA that accumulates at these sites, and is hence used as a marker for VRCs (Stracker et al. 2005; Turnell and Grand 2012). TopBP1, ATR, ATRIP, E1B-AP5 (hnRNPUL1), Rad17 and Rad9 are further examples of DDR proteins that localize at VRCs during infection with *wt* adenoviruses (Carson et al. 2003; Blackford et al. 2008; Carson et al. 2009). The beneficial consequence of the relocalization of DDR proteins to VRCs is unclear, although it has been postulated that some components of the DDR maybe required for viral replication, however, this is awaits confirmation (Turnell and Grand 2012).

Ads can also relocalize proteins to other sites within the cell, one of which is the nuclear 'track-like' structures which surround sites of VRCs and is dependent on the E4orf3 protein (Carvalho et al. 1995). A number of DDR proteins localize to PML bodies, which also has a role in DNA damage sensing (Lombard and Guarente 2000). These PML bodies are disrupted during Ad infection and E4orf3 is able to reorganize PML oncogenic domains (POD) into these elongated nuclear track-like structures (Carvalho et al. 1995; Doucas et al. 1996). As discussed in section 1.1.7, E4orf3 also forms a scaffold in the nucleus, which directs heterochromatin formation via

trimethylation of histone H3 at lysine residue 9 (H3K9me3) at p53 target promoters, thus silencing p53-mediated transcription in response to DNA damage (Soria et al. 2010). E4orf3 is also able to inactivate the DDR independently of E1B-55K and E4orf6 by relocalizing the MRN complex into nuclear tracks, and this action by E4orf3 appears to be conserved between group C, D, and E serotypes, whilst group A and B Ads, Ad12 and Ad4, respectively, both lack a key isoleucine residue in their E4orf3 proteins that is required to relocalize MRN (Stracker et al. 2005; Forrester et al. 2011). The ATR pathway is selectively and differentially activated during adenovirus infection, as there is evidence to show that both RPA32 and Rad9 are phosphorylated in Ad12-infected cells, but not Ad5-infected cells; Chk1 is inactivated by both viruses (Blackford et al. 2008). Our laboratory has shown that the E1B-55K associated protein (E1B-AP5) is required for ATR activation during infection (Blackford et al. 2008). More recently E1B-AP5 has been shown to interact with both the MRN complex and BLM to facilitate 3' end resection (Blackford et al. 2008; Polo et al. 2012). It has been proposed that the Ad5-mediated inactivation of Chk1 during infection is a consequence of the MRN relocalization into nuclear tracks (Carson et al. 2009), however the mechanism by which Ad12 achieves this was unclear until recently, and is described in detail in the study presented in Chapter 3.

In addition to VRCs and nuclear tracks, Ad infection and Ad5E1-mediated transformation result in the formation of subcellular structures known as aggresomes, which are formed at the microtubule organizing centre in response to misfolded proteins that occur when their rate of synthesis exceeds their rate of degradation, and are enriched in components of the ubiquitin-proteasome pathway (Sarnow et al. 1982; Zantema et al. 1985; Garcia-Mata et al. 2002; Liu et al. 2005). As described in section 1.1.6, E1B-55K is able to from a stable complex with p53 and sequesters it into

aggresomes (Liu et al. 2005). Furthermore, the MRN complex, Cul5, E1B-55K, E4orf3, and E4orf6 have all also been shown to localize at these cytoplasmic structures (Araujo et al. 2005; Liu et al. 2005). During Ad5 infection the MRN complex is localized to nuclear tracks by E4orf3, 8 hours post-infection, where it binds E1B-55K, and is then transported to the aggresomes, where Mre11 then undergoes degradation (Araujo et al. 2005; Liu et al. 2005). Ad also relocalizes p53 to aggresomes to promote its degradation, suggesting that cellular aggresomes are exploited by Ads to negate the restrictive effects that the DDR machinery has on viral growth (Liu et al. 2005).

1.3.4. Regulation of the cellular DNA damage response by other viruses

Over recent years there has been growing interest in the virology community with regards to the relationship between the DDR pathways and viral infection. Indeed, there are now a plethora of viral proteins from many different viruses that have been shown to interact with cellular proteins that function in the DDR pathway.

Some viruses have been shown to deregulate DDR pathways, whilst some have been shown to utilize or exploit them to aid viral growth, and others can do both. ATM has been shown to be required for efficient SV40 viral DNA replication, whereby ATM is phosphorylated and activated in SV40-infected cells to promote phosphorylation of the LTag viral protein on S120 (Shi et al. 2005). The MRN complex is also recruited to the VRCs during SV40 infection, where it is thought to play an active role in viral DNA replication (Boichuk et al. 2010). As with Ad infection, Mrell is degraded along with NBS1 in SV40-infected cells, albeit at late times by LTag utilising a Cul7-containing CRL, which is also required for viral replication (Zhao et al. 2008).

HPV is another example of a virus that degrades DDR proteins to facilitate viral replication. The tumour suppressor proteins p53 and pRb are targeted by E6 and E7

HPV viral proteins for proteasome-mediated degradation to promote S-phase by bypassing the G_1/S checkpoint (Moody and Laimins 2009). However, HPV also utilizes the DDR machinery to promote viral genome amplification and formation of viral replication foci by activating ATM (Moody and Laimins 2009).

HSV-1 is another virus that differentially targets the DDR machinery. It had initially been suggested that HSV-1 infection disrupts the ATR pathway in the infected cell by spatially uncoupling ATRIP from ATR and sequestering ATRIP and endogenous hyperphosphorylated RPA within virus-induced nuclear domains containing molecular chaperones and components of the ubiquitin proteasome (Wilkinson and Weller 2006). However, a more detailed investigation conducted by the same lab has now reported that the ATR-ATRIP interaction remains intact during infection, and they can be co-immunoprecipated from infected cells, and may actually function to promote viral gene expression and virus production (Mohni et al. 2010). ICP0 also recruits ATM to sites of HSV DNA replication to promote viral growth by promoting ATM-dependent Chk2 phosphorylation and G_2/M checkpoint activation (Lilley et al. 2005; Li et al. 2008). However, ICP0 promotes the degradation of RNF8 and RNF168, thus preventing full activation of the ATM pathway (Lilley et al. 2010).

These are just a few examples of the many mechanisms that viruses have evolved in order to negate and/or selectively activate the DDR pathways in order to facilitate the replication of their genomes efficiently in host cells. Thus, viruses prove to be powerful tools for increasing our understanding of the complex nature of the regulatory pathways required for maintaining genome integrity.

1.4. MICROCEPHALY

1.4.1. Autosomal recessive primary microcephaly

Microcephaly by definition means 'small head'. Microcephaly is a neurodevelopmental disorder whereby the occipitofrontal head circumference (OFC) is more than 2 standard deviations (SD) below the mean for the person's age, sex, and ethnicity (Kaindl et al. 2010). Microcephaly can be either inherited, or caused by various environmental factors such as alcohol consumption, viral infections, and radiation exposure during pregnancy or early infancy (Abuelo 2007). Microcephaly that arises congenitally is known as primary microcephaly, whereas post-natal development of microcephaly is termed as secondary microcephaly. There are conflicting reports in the literature which suggest that primary microcephaly occurs solely as a result of genetic influences, and secondary microcephaly occurs solely as a result of environmental factors (Opitz and Holt 1990). However, some genetic conditions such as Rett and Angelman syndromes develop microcephaly during late infancy or childhood, and are considered to have secondary microcephaly (Opitz and Holt 1990). Furthermore, environmental factors such as viral infections and other disruptive influences to foetal brain development have been shown to cause microcephaly that is apparent at birth, and is therefore termed primary microcephaly (Hughes and Miskin 1986; Corona-Rivera et al. 2001). It is difficult to estimate the incidence of microcephaly as there have been a variety of estimates published in the literature, but it is believed to be around 1.3-150 per 100,000 births, depending on the applied SD threshold to define microcephaly as well as the population (Kaindl et al. 2010).

Autosomal recessive primary microcephaly (MCPH), historically referred to as microcephaly vera ('true microcephaly'), is a rare heterogeneous genetic disorder that

is defined by an OFC of more than 3 SD below the average at birth, reduced brain volume, and mental retardation, but surprisingly no other neurological symptoms aside from mild seizures in some instances (Woods et al. 2005). It has long been known that MCPH is inherited as an autosomal recessive trait, but the genes involved have only become apparent in the 10-15 years, whereby it has been shown that at least 7 loci are associated with this condition, MCPH1-7 (Mochida 2009). Currently, mutations in 6 genes have been identified at these loci which are associated with inheriting MCPH, and these include *MCPH1* in MCPH1, *WDR62* (WD40 repeat-containg protein 62) in MCPH2, *CDK5RAP2* (cyclin-dependent kinase 5 regulatory associated protein 2) in MCPH3, *ASPM* (abnormal spindle-like, microcephaly associated) in MCPH5, *CENPJ* (centromeric protein J) in MCPH6, and *STIL* (SCL/TAL1-interrupting locus) in MCPH7 (Bond et al. 2002; Jackson et al. 2002; Bond et al. 2005; Shen et al. 2005; Kumar et al. 2009; Nicholas et al. 2010). These genes encode proteins that predominantly localize to the centrosome, and are involved in a range of cellular pathways which include cell cycle checkpoint control, DNA damage repair, cell cycle regulation, and apoptosis, as well as other functions involved in spindle formation and attachment (Kaindl et al. 2010). The functions of MCPH1 and WDR62 will be discussed in more detail in sections 1.4.2 and 1.4.3, respectively.

The *CDK5RAP2* gene product CDK5RAP2 protein localizes at the centrosome and plays a role in centrosome cohesion and spindle checkpoint regulation. CDK5RAP2 has an N-terminal interaction site with gamma-tubulin ring complex (γTuRC), which is required for docking of γTuRC to the centrosome (Fong et al. 2008). CDK5RAP2 is shown to localize at the centrosome for the entire cell cycle where it interacts with pericentrin and is required for centrosome cohesion (Graser et al. 2007). Furthermore CDK5RAP1 is involved in spindle checkpoint regulation by interacting with the

promoters of both BUBR1 (budding uninhibited by benzimidazoles 1 homolog beta) and MAD2 (mitotic arrest-deficient 2) to transcriptionally regulate them (Zhang et al. 2009).

The *ASPM* gene product ASPM plays a role in mitotic spindle function, and localizes at the centrosome during interphase and at the spindle poles during prophase through to telophase (Fish et al. 2006; Kaindl et al. 2010). ASPM functions in the pathway that defines the orientation of the cleavage plane during mitosis, which determines if their will symmetric or asymmetric division (Fish et al. 2006). ASPM also functions to accumulate LIN5 at meiotic and mitotic spindle poles to promote spindle organization (van der Voet et al. 2009).

The *CENPJ* gene product CENPJ is localised at the centrosome during the entire cell cycle by associating with γTuRC, and has been shown to play a role in centrosome and spindle function (Hung et al. 2004; Kaindl et al. 2010). CENPJ regulates microtubule assembly and disassembly at kinetochores and centrosomes, which is important during mitosis for chromosome segregation and spindle structure (Hung et al. 2004).

The *STIL* gene product STIL is 150 kDa cytosolic protein, which functions in mitotic entry, centrosome function, and control of apoptosis, and has been detected at mitotic spindles in metaphase (Campaner et al. 2005; Erez et al. 2007; Erez et al. 2008). Increased expression of STIL in some cancers correlates with increased metastatic potential and expression of mitotic spindle checkpoint genes (Ramaswamy et al. 2003; Erez et al. 2004). STIL is required for mitotic entry and survival of cancer cells, and is transcriptionally regulated by E2F (Erez et al. 2007; Erez et al. 2008). Furthermore, STIL binds the C-terminal of SUFU to suppress glioma-associated oncogene homology (GLI1) expression and promote cancer cell proliferation (Kasai et al. 2008).

1.4.2. MCPH1

The *MCPH1* gene which encodes the protein MCPH1 (also known as BRIT1 and microcephalin) is located at 8p23 and was the first gene in which mutations were identified in patients with MCPH (Jackson et al. 1998; Jackson et al. 2002). MCPH1 contains three BRCT domains, one in the C-terminal region and two located in the N-terminal region (Jackson et al. 2002). This protein has been shown to play a role in cell cycle checkpoint regulation, DNA repair, and chromosome condensation, and centrosome function.

MCPH1 has been implicated as a possible tumour suppressor, and functions in both ATM and ATR signalling pathways in response DNA damage (Rai et al. 2006). During IR-induced DDR, MCPH1 is recruited to DSBs through an interaction with its C-terminal BRCT domains and phosphorylated H2AX, where it functions as a mediator and colocalizes with other mediators such as 53BP1, NBS1, MDC1, and ATM (Rai et al. 2006; Wood et al. 2007). MCPH1 also colocalizes with ATR, RPA and Rad17 in response to UV-induced DDR, and is essential for ATR-mediated phosphorylation of RPA and Rad17 (Rai et al. 2006). Furthermore, MCPH1 has been shown act as a transcriptional regulator of BRCA1 and Chk1, and is thus required for the regulation of the intra-S and G_2/M DNA damage checkpoints (Xu et al. 2004; Alderton et al. 2006). It also functions downstream of Chk1 to promote the degradation of CDC25A to prevent premature entry into mitosis (Alderton et al. 2006). A more recent study has shown the MCPH1 interacts with E2F1 at the promoters of both Chk1 and BRCA1 to regulate their transcription, and via the same mechanism it also regulates other genes involved in DNA repair and apoptosis such as TopBP1, Rad51, DDB2, and p73 (Yang et al. 2008). Furthermore, MCPH1-mutant cell lines that are exposed to UV display

nuclear fragmentation, supernumerary centrosomes, and a defective G_2/M checkpoint, much the same as the ATR-mutant phenotype (Alderton et al. 2006).

MCPH1 also regulates chromosome condensation, which is apparent in patients with premature chromosome condensation syndrome (PCC), a condition that is also a result of mutation in the *MCPH1* gene and is characterized by misregulated chromosome condensation, microcephaly, and short stature (Trimborn et al. 2004). This phenotype can be seen in patients with MCPH1 microcephaly as well as in cells depleted of MCPH1 via siRNA, where a significant number of prophase-like cells are observed as a result of premature chromosome condensation in the early G_2-phase and delayed decondensation post-mitosis (Trimborn et al. 2004; Trimborn et al. 2006). Furthermore, MCPH1 has also been shown to be essential for centrosome function where it has been shown to localize throughout the cell cycle via its N-terminal BRCT domain, and siRNA-mediated depletion of MCHP1 resulted in impaired centrosomal function (Jeffers et al. 2008; Rai et al. 2008).

MCPH1 provides a link between microcephaly and DDR mechanisms, where there are also DDR disorders which present with microcephaly, which include NBS, Seckel syndrome, LIG4, FA, and XPA.

1.4.3. WDR62

Until recently the gene mutated at the MCPH2 locus was unknown, but has now been shown to be WDR62 (Nicholas et al. 2010; Yu et al. 2010). MCPH2 is the second most common MCPH, where genetic linkage to chromosome 19q12 was discovered 13 years ago. This means that WDR62 eluded discovery for over 10 years, whereafter it was eventually identified by classical and neo-classical reverse genetics, and has been shown to be comprised of 1523 amino acids, possessing at least 15 WD40 repeats

(Nicholas et al. 2010). MCPH2 is caused by missense and frame-shifting mutations in the WDR62 gene, which is an unusual mutation spectrum for MCPH genes as the majority normally harbour null mutations (Wollnik 2010).

Very little is known about the functions of the WDR62 protein. It has been shown to be a ubiquitously expressed scaffold protein that binds specifically to c-Jun N-terminal kinase (JNK), and potentiates its activity, as well as relocalizing JNK to a non-nuclear compartment, which results in inhibition of AP-1 transcription (Wasserman et al. 2010). Furthermore, WDR62 was found to localize at spindle poles during mitosis in neural precursor cells in a similar fashion to ASPM, but mutated WDR62 as seen in MCPH2 patients is unable to localize at the spindle poles suggesting that WDR62 may play a role in centrosome function akin to other MCPH proteins described above (Nicholas et al. 2010). Indeed, it was recently shown that WDR62 plays a role in mitotic spindle regulation. WDR62 was shown to localize at spindle poles during mitotic entry and remained there until the metaphase-anaphase transition; JNK-mediated phosphorylation of WDR62 was required to maintain metaphase spindle organization during mitosis (Bogoyevitch et al. 2012). The siRNA-mediated depletion of WDR62 from neuroprogenitor cells led to defects in spindle orientation, caused the centrosomes to be displaced from the spindle poles, and delayed mitotic progression, as well as causing the cell to exit the cell cycle and reducing their proliferative capability (Bogoyevitch et al. 2012).

WDR62 is a relatively poorly understood protein, whose functions still remain a focus for much research. Work presented in this thesis investigates the relationship between Ad and WDR62 and investigates a role for this protein in the DDR (Chapter 5).

1.4.4. Environmental causative factors of microcephaly

Microcephaly can be caused by both genetic influences and environmental factors, of which the main genetic causes of microcephaly have been described above. There are many described environmental factors that can cause either primary microcephaly which occurs during foetal development, or secondary microcephaly which occurs post-natally, and these include alcohol consumption, exposure to radiation, maternal diabetes, vascular accidents, death of a monozygous twin, and intrauterine infection (Abuelo 2007).

The most common teratogen that the foetus is exposed to is alcohol, and in the United States alone there is an incidence of foetal alcohol spectrum disorders in 1-2 per 1000 live births (Abuelo 2007). Infants display both pre- and postnatal growth retardation and are at risk for developing microcephaly and mental retardation with an average IQ of 65 (Abuelo 2007).

Cytomegalovirus (CMV) was first isolated from human cultures in the early 1970s; one of the first patients samples that contained CMV was that of a 3 month old infant that had microcephaly (Dudgeon 1971). Congenital CMV infection is well known cause of microcephaly. Another virus that has been known to cause microcephaly is HIV-1. HIV-1 infection in infants can cause secondary microcephaly, whereby onset of the disorder occurs between 2-4 months of age. In congenital HIV-1 infection babies are born normocephalic and show deceleration of head growth between 2 and 4 months, caused by aberrant excitatory amino acid neurotransmitter expression during early postnatal development, which is normally required for dendritic differentiation, synaptogenesis, and activity-dependent plasticity (Epstein and Gelbard 1999).

1.5 AIMS AND OBJECTIVES

As described in section 1.3.3, Ad5 and Ad12 differentially activate ATR during infection, however both inhibit Chk1 phosphorylation. Ad5 inhibits Chk1 activation by relocalizing the MRN complex into nuclear tracks through the action of Ad5 E4orf3, however this function is not conserved between certain serotypes. Ad12 E4orf3 lacks a key isoleucine residue required to carry out this function.

E1B-55K has been shown to interact with a number of proteins involved in the DDR making it a useful tool to identify novel DDR proteins. A proteomic study in our laboratory utilizing a mass spectrometry approach identified WDR62 as a possible interacting protein for E1B-55K (Forrester, N.A. PhD thesis (2011), The University of Birmingham).

Therefore the specific aims and objectives for this study were as follows:

1. To investigate the molecular mechanism by which Ad12 inhibits Chk1 activation.

2. Investigate the relationship between E1B-55K and WDR62.

It was hoped that the investigations outlined above would not only further our understanding of Ad infection, but also provide new insights into DDR function in both non-infected, and Ad-infected cells. Results arising from these investigations are presented in this thesis.

CHAPTER 2

MATERIALS AND METHODS

2.1. TISSUE CULTURE TECHNIQUES

2.1.1. Cell lines

All of the cell lines that were used throughout this study are summarized in the table below (Table 2.1).

Table 2.1. Human cell lines used in this study

Cell Line	Cell Type	Source	Origin	Culture Medium	ATCC® Number
A549	epithelial	small cell lung carcinoma	human	DMEM	CCL-185
HEK293	epithelial	embryonic kidney	human	DMEM	CRL-1573
HER911	epithelial	embryonic retinoblast	human	DMEM	(Fallaux et al. 1996)
HER2	epithelial	embryonic retinoblast	human	DMEM	(Byrd et al. 1982)
HER3	epithelial	embryonic retinoblast	human	DMEM	(Byrd et al. 1982)
HER10	epithelial	embryonic retinoblast	human	DMEM	(Byrd et al. 1982)
HeLa	epithelial	cervical carcinoma	human	DMEM	CCL-2
U2OS ATRIP-*wt*	epithelial	osteosarcoma	human	DMEM	(Mordes et al. 2008)
U2OS ATRIP-*top*	epithelial	osteosarcoma	human	DMEM	(Mordes et al. 2008; Mohni et al. 2010)
U2OS Empty vector	epithelial	osteosarcoma	human	DMEM	(Mordes et al. 2008)

2.1.2. Tissue Culture Media

All tissue culture reagents were pre-sterilized and purchased from Invitrogen unless otherwise stated. Reagents were stored at 4 °C and pre-warmed to 37 °C unless

otherwise stated. Cell lines were maintained in DMEM (Dulbecco's modified Eagles medium) supplemented with 2 mM L-glutamine and 8% (v/v) foetal calf serum (FCS).

2.1.3. Maintenance and passage of cell lines

Cells were grown in humidified incubators set at 37 °C and supplied with 5% (v/v) CO_2. All tissue culture techniques were performed in specialised flow hoods under sterile conditions. Cell lines were grown in monolayers and passaged as follows. Existing medium was removed from cells and then washed twice with phosphate-buffered saline (PBS). Adherent cells were then detached from the plates by adding 1 ml of trypsin followed by a 5 minute incubation at 37 °C. Detachment of cells was confirmed by microscopy, after which cells were washed with media containing FCS to inactivate the trypsin and then pelleted by centrifugation at 1400 rpm for 5 minutes. Pelleted cells were then resuspended in fresh medium and re-plated at the required density before being transferred back to the humidified incubators.

2.1.4. Cryopreservation of cell lines

Cells were trypsinised and pelleted as outlined above before being resuspended in DMEM growth medium containing 10% dimethyl-sulphoxide (DMSO). Typically a confluent plate was divided into 4 aliquots of 1 ml that were cooled to -80 °C at a controlled rate of 1 °C/min in isopropanol. Frozen samples were then transferred to liquid nitrogen tanks for long term storage at -180 °C.

2.1.5. Recovery of cells from liquid nitrogen

Cells taken from liquid nitrogen storage were thawed rapidly in a 37 °C water bath followed by immediate drop wise transfer to a centrifuge tube containing 10 ml of fresh culture medium. Cell were pelleted at 1400 rpm, washed in fresh culture medium,

resuspended in fresh culture medium, and then re-plated and incubated at 37 °C in a humidified incubator.

2.2. CELL BIOLOGY TECHNIQUES

2.2.1 Viruses

Wild-type (*wt*) Ad5 and Ad12 viruses were obtained from the American Type culture Collection (ATCC). Several Ad5 and Ad12 mutants were used in this study. Ad5 *dl*1520 is an E1B-55K null virus generated by deletional mutagenesis, with two stop codons at nucleotides 3 and 3336, as well as an another deletion from nucleotides 2496 to 3323 (Barker and Berk 1987). Ad12 *dl*620 contains an in-frame deletion in the large E1B open reading frame from nucleotides 2129 to 2825 (Byrd et al. 1988). Ad12 *hr*703 contains a point mutation which converts amino acid 134 from a Gln to a stop codon (Byrd et al. 1988). FLAG-Ad12, a mutant Ad12 virus that expresses FLAG-tagged E4orf6, was constructed in collaboration with Professor Thomas Dobner (Blackford et al. 2010). *wt* Ad5 and Ad5 *d1*1520 viruses were propagated on permissive HEK293 cells. *wt* Ad12, *hr703* and *dl620* viruses were propagated on permissive HER3 cells. Stock titres were determined by plaque assay on HER911 and HER3 cells, as appropriate.

2.2.2. Viral infections

Ad infection was carried out using subconfluent monolayers of cultured cells in DMEM without FCS grown on either 6 or 10 cm dishes, or wells of multispot glass microscope slides (Hendley-Essex). Cells were washed twice in PBS, before addition of the virus at 10 plaque forming units per cell (p.f.u) diluted in DMEM without FCS. Cells were kept

at 37 °C, with agitation at 15 minute intervals to ensure even dispersal of the virus. After 2 hours incubation, medium containing virus was removed and replaced with DMEM supplemented with 8% (v/v) FCS.

2.2.3. Transient DNA transfections

Transfections were performed on HeLa cells that were grown to 90% confluence on 6 cm dishes. 4 µg of plasmid DNA was added to 200 µl of Opti-MEM (Invitrogen) and left for 5 minutes at room temperature. In a separate universal, 10 µl of Lipofectamine 2000 was added to 190 µl of Opti-MEM and also left for 5 minutes. The transfection reagent and the DNA mixture were then combined, mixed gently, and left for 30 minutes at room temperature to allow DNA-liposome complexes to form. During this time, cells were washed twice with Opti-MEM. 2.4 ml of Opti-MEM was then added to the DNA-Lipofectamine mix and then subsequently added to the appropriate dish, incubated for 6 hours at 37 °C. After this time fresh DMEM supplemented with 8% (v/v) FCS was then added to the transfected cells and incubated at 37 °C, before being harvested at the appropriate times. Constructs used in this study are listed in Table 2.2.

Table 2.2. Plasmids used in this study

Plasmid	Vector	Tag	From
Ad12 E4orf6	pcDNA3	HA	T. Dobner
Ad12 E4orf6	pGEX 4T-1	GST	PCR from cDNA
Ad5 E4orf6	pcDNA3	HA	T. Dobner
Ad12 E4orf3	pcDNA3	HA	T. Dobner
Ad5 E4orf3	pCMV	HA	T. Dobner

Cul2	pcDNA3	HA	P. Branton
Cul5	pcDNA3	HA	P. Branton
TopBP1	pcDNA3	myc	J. Chen
TopBP1 2-258	pBIND	GAL4	I. Morgan
TopBP1 258-914	pBIND	GAL4	I. Morgan
TopBP1 789-1435	pBIND	GAL4	I. Morgan
TopBP1 1169-1435	pBIND	GAL4	I. Morgan

2.2.4. RNA interference

All small interfering (si)RNAs used in this study are displayed in Table 2.3. siRNA transfections were performed on HeLa cells that were grown to 40% confluency on 6 cm dishes. 20 µl of Oligofectamine (Invitrogen) was mixed with 8 µl of siRNA (40 µM stock) in 972 µl of Opti-MEM medium and left for 30 minutes at room temperature to form oligofecatmine-siRNA complexes. During this time, cells were washed twice with Opti-MEM. 1 ml of Opti-MEM was then added to the Oligofectamine-siRNA mix and then subsequently added to the appropriate dish, incubated for 6 hours at 37 °C, before being replaced with fresh DMEM supplemented with 8% (v/v) FCS. Cells were then incubated in humidified incubators at 37 °C in 5% (v/v) CO_2. Any subsequent infection or transfection was carried out 48 hrs post-RNAi treatment.

Table 2.3. siRNAs used in this study

Target	Sense sequence	Supplier
control/non-silencing	Proprietary	Qiagen

Cul2	5’ GGAAGUGCAUGGUAAAUUU 3’ 5’ CAUCCAAGUUCAUAUACUA 3’ 5’ GCAGAAAGACACACCACAA 3’ 5’ UGGUUUACCUCAUAUGAUU 3’	Dharmacon
Cul5	5’ GACACGACGUCUUAUAUUA 3’ 5’ GCAAAUAGAGUGGCUAAUA 3’ 5’ UAAACAAGCUUGCUAGAAU 3’ 5’ CGUCUAAUCUGUUAAAGAA 3’	Dharmacon
Elongin C	5’ AAACCAAUGAGGUCAAUUU 3’	Dharmacon
RBX1	5’ GAAGCGCUUUGAAGUGAAA 3’	Dharmacon
WDR62	5’ GGAAGUCUCUCAGUGCUCU 3’	Ambion
ATRIP	5’ GGUCCACAGAUUAUUAGAU 3’	Ambion

2.2.5. Ultra Violet irradiation and Ionizing Radiation treatment

Prior to ultra-violet (UV) irradiation, medium was removed from the cells, and cells were washed twice with PBS. The cells were then mock-irradiated, or irradiated at the required dose of UV light from a 254 nm UV light source. The cells were then reincubated with the original medium and incubated at 37 °C. In the case of ionizing radiation (IR) treatment, cells were either mock-irradiated, or irradiated with ionizing γ-rays from a ^{137}Cs source at a dose rate of 2.5 Gy/min.

2.2.6. Drug treatment

Where indicated, cells were treated with caffeine at a final concentration of 5 mM to inhibit caffeine-sensitive kinases, proteasome inhibitor MG132 (N-carbobenzoxy-L-leucinyl-L-leucinyl-L-leucinal) at a final concentration of 10 μM, or the DNA damaging agent, hydroxyurea (HU) at a final concentration of 1 mM.

2.2.7. Flow cytometric analysis.

Flow cytometry was used to assess the cell cycle distribution of UV-irradiated HeLa cells transfected with non-silencing or WDR62 siRNA. Cells were harvested by trypsinisation at appropriate times post UV-irradiation, washed twice with ice-cold PBS, before being resuspended in 600 µl of ice cold PBS. Cells were then fixed by adding ice cold ethanol up to a final total volume of 2 ml whilst placed on slow vortex to avoid cell clumping, and then stored at -20 °C.

On the day of analysis cells were pelleted at 1600 rpm for 5 minutes and then washed twice in 10 ml of ice-cold PBS to dilute the excess ethanol. Cells were then permeabilized in ice-cold PBS with 0.25% (v/v) Triton X-100 for 15 minutes with agitation at 4 °C, then rinsed with 10 ml of PBS with 1% (w/v) Bovine Serum Albumin (BSA), before being incubated with an anti-phosphohistone H3 (Ser10) polyclonal antibody diluted in PBS with 1% (w/v) BSA for 1 hour at room temperature. Samples were then washed twice with PBS containing 1% (w/v) BSA and then incubated with an Alexa-488 anti-Rabbit antibody diluted in PBS containing 1% (w/v) BSA for 30 minutes in the dark. Cells were then washed again once in PBS containing 1% (w/v) BSA and once with PBS alone before being resuspended in 1 ml of PBS containing 25 µg/ml propidium iodide and 0.1 mg/ml RNAse A, and then left in the dark for a further 30 minutes. Cell cycle analysis was carried out using a BD Accuri C6 flow cytometer.

2.2.8. Colony survival assays

Cell sensitivity to DNA damaging agents following the siRNA-mediated knockdown of WDR62 was measured in HeLa cells by the loss of colony forming ability. Cells were plated at a low density on 6 cm dishes in triplicate and then mock treated or treated with appropriate dose of the DNA damaging agent. The cells were then incubated for 14

days in a humidified incubator at 37 °C supplied with 5% (v/v) CO_2 to allow the colonies to develop. Cells were fixed and stained with a 50% ethanol solution containing 2% (v/v) methylene blue. Experiments were repeated three times to ensure reproducibility.

2.3. PROTEIN BIOCHEMISTRY TECHNIQUES

2.3.1. Preparation of total cell lysates

Cell culture medium was removed from cells and then washed twice in ice-cold saline. Cells were then lysed in buffer containing 9 M urea, 50 mM Tris (pH 7.3), and 0.15 M β-mercaptoethanol. Cells were detached from the tissue culture dish using a plastic scraper, and then sonicated for 20 seconds on ice, at setting 4, using a Misonix microsin ultrasonic cell disruptor. Lysates were then spun down at 13000 rpm and then transferred to fresh tubes for protein determination or stored at -80 °C.

2.3.2. Protein concentration quantification

Protein concentrations were determined by Bradford assay (Bio-Rad) using 6 standards using known quantities of BSA diluted in deionised H_20 ranging from 0-30 µg/ml. 10 µl of each standard was added to separate well of a flat bottomed 96 well microplate. 5 µl of protein lysate was added to 45 µl of deionised H_20, of which 10 µl was added to a well. Standards and lysates were measured in quadruplicates. Bradford reagent (Bio-Rad) was diluted 1:4 with deionised H_20, of which 200 µl was added to each well and mixed by gently pipetting. Protein concentrations were calculated by generation of a standard curve using a microplate reader that measures absorbance at a wavelength of 595 nm.

2.3.3. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples or cell lysates were separated according to their size by SDS-PAGE. Polyacrylamide gels were made using a 30% (w/v) Acrylamide/Bis-acrylamide (37.5:1) stock solution (Severn Biotech Ltd). Gels were prepared in the presence of 100 mM Tris, 100 mM Bicine, 0.1% (v/v) SDS and 0.3% (v/v) TEMED. Large proteins were separated on 6-8% (w/v) acrylamide gels, whereas small proteins were separated on 10-12% (w/v) acrylamide gels. Ammonium persulphate was added to a final concentration of 0.06% (w/v) to initiate acrylamide polymerization. Gel apparatus was assembled as per the manufacturer's instructions (Hoeffer Scientific), and run in buffer containing 100 mM Tris, 100 mM Bicine, and 0.1% (v/v) SDS. Protein samples were diluted in an equal volume of Laemmli sample buffer (Bio-Rad), boiled for 5 minutes at 95 °C, and then loaded onto the gel. Gels were generally run over night at a constant current which was dependent on the size of the proteins of interest.

2.3.4. Staining of the polyacrylamide gels.

Following electrophoresis, gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 (Sigma) in 20% (v/v) methanol, 10% (v/v) glacial acetic acid in water, and left on a shaker at room temperature for 20 minutes. Stained gels were then destained in 10% (v/v) glacial acetic acid and 20% (v/v) methanol in water until protein bands were clearly visible.

2.3.5. Detection of radioactive proteins by fluorography

Following sufficient destaining, stained gels were immersed in Amplify™ reagent (Amersham Bioscience) for 2 hours at room temperature with agitation. Gels were then

dried under a vacuum at 80 °C for 2 hrs before being exposed for autoradiography at -20 °C for the appropriate time.

2.3.6. In vitro GST-protein binding assays

L-α-[^{35}S]-methionine-labelled proteins were expressed individually *in vitro* using a TNT T7-Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's guidelines. Typically 10 µl [^{35}S]-labelled proteins were incubated with 10 µg GST-fusion proteins for 1 hr on ice followed by equalisation of the volumes by adding the appropriate amount of GST lysis buffer. Protein complexes were then isolated by incubation with glutathione-agarose beads (Sigma) for 1 hr at 4 °C with rotation followed by the beads being washed three times with GST lysis buffer and twice in GST wash buffer. GST-protein complexes were then isolated by addition of GST elution buffer (25 mM reduced glutathione in 50 mM Tris (pH 8.0)), before addition of SDS-PAGE sample buffer. After separation by SDS-PAGE, [^{35}S]-labelled proteins were visualised by fluorography and autoradiography.

2.3.7. Mass spectrometry

Gel slices were excised under sterile conditions. Slices were washed with 400 µl of 50% (v/v) acetonitrile and 50 mM ammonium bicarbonate by agitation for 1 hour at 37 °C. Proteins were then reduced in 250 µl of a solution containing 50 mM dithiothrietol (DTT), 10% (v/v) acetonitrile and 50 mM ammonium bicarbonate for 1 hour at 56 °C. Proteins were then alkylated using a solution containing 200 mM iodoacetamide, 10% (v/v) acetonitrile, and 50 mM ammonium bicarbonate and incubated for 1 hour in the dark at room temperature.

Proteins were then washed by agitation for 30 minutes in a buffer containing 10% (v/v) acetonitrile and 40 mM ammonium bicarbonate. Gel slices were then dried by rotary evaporation and proteins were then digested by rehydration of the gel slice in modified trypsin overnight at 37 °C (50 µl of 12.5 µg/ml; Sigma-Aldrich). The digested proteins were eluted from the gel slice with a buffer containing 3% (v/v) formic acid, 10% (v/v) acetonitrile and 40 mM ammonium bicarbonate. Peptides were then separated using a Bruker AmaZon ion trap mass spectrometer, and processed and analyzed by the ProteinScape central bioinformatic platform.

2.4. IMMUNOCHEMISTRY TECHNIQUES

2.4.1. Antibodies

All antibodies used in this study are displayed in Table 2.4

Table 2.4. Antibodies used in this study

Antigen	Antibody	Origin	Use	Supplier
Ad12E1A	13	Mouse	WB	In-house
Ad12E1B54K	XPH9	Mouse	WB, IF, IP	In-house
Ad5E1A	M73	Mouse	WB	Ed Harlow
Ad5E1B55K	2A6	Mouse	WB, IF, IP	Arnold Levine
ATM	11G12	mouse	WB, IP	In-house
ATR	N-19	Goat	WB	Santa Cruz
ATRIP	BL733	Rabbit	WB	Gene Tex

Chk1	G-4	Mouse	WB	Santa Cruz
Cul2	Ab1870	Rabbit	WB	Abcam
Cul5	a302-173a	rabbit	WB	Bethyl
Cyclin B1	V152	mouse	WB	CR-UK
DNA ligase IV	LIGIV	Rabbit	WB	Stephen Jackson
Flag	Anti-Flag M2	Mouse	WB, IF	Stratagene
H2AX	07-627	Rabbit	WB	Millipore
HA	Anti-HA 12CA5	Mouse	WB, IF	Sigma-Aldrich
Mre11	12D7	Mouse	WB, IF	GeneTex
NBS1	1C3	Mouse	WB	GeneTex
p53	DO-1	Mouse	WB, IF	David Lane
p-Chk1 S345	133D3	Rabbit	WB	Cell Signalling
PML	PG-M3	Mouse	IF	Santa Cruz
PML	H-238	Rabbit	IF	Santa Cruz
p-ATM	10H11.E12	mouse	WB	Cell Signaling
p-RPA S468	A300-245	Rabbit	WB	Bethyl
p-SMC1 S966	A300-050A	Rabbit	WB	Bethyl
p-TIF1β S824	5824	Rabbit	WB	Bethyl
RAD9	M-389	Rabbit	WB	Santa Cruz
RPA70	Ab-1	Mouse	WB	Santa Cruz
RPA32	Ab-2	Mouse	WB, IF	Calbiochem
RPA32	ab10359	Rabbit	IF	Abcam

SMC1	A300-055A	Rabbit	WB	Bethyl
TIF1β	A300-274A	Rabbit	WB, IF	Bethyl
Timeless	anti-Tim	Rabbit	WB, IF	(Yoshizawa-Sugata and Masai 2007)
Tipin	anti-Tipin	Rabbit	WB, IF	(Yoshizawa-Sugata and Masai 2007)
TopBP1	(5H)52	Rabbit	WB, IF	Iain Morgan
WDR62	A301-559A	Rabbit	WB, IF	Bethyl
β-actin	AC-74	Mouse	WB	Sigma-Aldrich
γ-H2AX	JBW301	Mouse	WB, IF	Millipore
Mouse IgG	Anti-mouse-HRP	Goat	WB	Dako
Rabbit IgG	Anti-rabbit-HRP	Swine	WB	Dako
Rabbit IgG	Alexa Fluor® 488 anti-rabbit	Goat	IF	Invitrogen
Rabbit IgG	Alexa Fluor® 555 anti-rabbit	Goat	IF	Invitrogen
Mouse IgG	Alexa Fluor® 488 anti-mouse	Goat	IF	Invitrogen

2.4.2. Immunoprecipitation

Cells were washed twice in saline before addition of 1 ml ice-cold immunprecipitaion (IP) buffer (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA- pH 8.0), 1% (v/v) Nonidet P-40, 25 mM NaF and 25 mM β-glycerophosphate). Cell lysates were then homogenised or sonicated on ice followed by centrifugation at 40000 rpm for 30 minutes at 2 °C to remove cell debris. Appropriate antibodies were then added to cell lysates and incubated overnight

with rotation at 4 °C to form antigen-antibody complexes. The next day, 30 µl of Protein G-sepharose (Sigma-Aldrich) was added to each sample and incubated for a further 2 hours at 4 °C with rotation. Immunocomplexes bound to the beads were then spun down at 4000 rpm and washed 5 times in ice cold IP buffer followed by addition of 30 µl Laemmli sample buffer to elute the proteins. Samples were then boiled and spun down in preparation for SDS-PAGE and Western blotting.

2.4.3. Western blotting

Following electrophoresis, proteins were transferred onto a nitrocellulose membrane (PALL) using a Hoeffer Scientific transfer system. Nitrocellulose membrane was soaked in blotting buffer (50 mM Tris, 190 mM glycine, 20% (v/v) methanol) before being laid on top of the gel. The gel and nitrocellulose was then sandwiched between two pieces of pre-soaked Whatman 3 MM blotting filter paper and two blotting sponges, before being closed in a plastic cassette and placed into Hoeffer transblot electrophoresis apparatus that was filled with blotting buffer with the nitrocellulose facing the anode. Electro transfer was performed at 280 mA for 6 hours. Upon completion of the transfer, the membranes were stained for 2 minutes with a solution containing 0.1% (w/v) Ponceau S (Sigma-Aldrich) and 3% (w/v) trichloroacetic acid and then rinsed with deionised water several times to detect protein bands. The stain was then removed by washing with 0.1% (v/v) Tween 80 in Tris-buffered saline (TBS) containing 150 mM NaCl and 20 mM Tris-HCl (TBST), pH 7.3.

The membranes were then blocked in 5% (w/v) dried-milk powder in TBST for 40 minutes at room temperature with agitation, and then incubated with the appropriate antibody diluted in TBST containing 5% (w/v) milk overnight at 4 °C with agitation. The membranes were then washed in TBST 3 times for 10 minutes per wash, before

being incubated at room temperature for 1 hour with the appropriate horseradish peroxidase-conjugated secondary antibody (Dako) diluted in TBST containing 5% (w/v) milk. The membranes were then washed again in TBST 3 times for 10 minutes per wash, followed by antigens being visualised by enhanced chemiluminescence (ECL, Amersham Pharmacia) reagents and autoradiography film (Kodak).

2.4.4. Immunofluorescence

Cells were grown on 12-well multispot microscope glass slides (Hendley-Essex) at a density of 2 x 10^4 cells/well. The slides were washed twice in PBS followed by incubation in pre-extraction buffer (10 mM PIPES [piperazine-N,N′-bis(2-ethanesulfonic acid)] pH 6.8, 20 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 0.5% (v/v) Triton X-100) for 5 minutes, and then fixed in 4% (w/v) paraformaldehyde in PBS for 10 minutes. Cells were then washed again in PBS before being blocked in 10% (v/v) FCS in PBS for 45 minutes at room temperature. Cells were then incubated with appropriate primary antibody diluted in 10% (v/v) FCS in PBS for 2 hours at room temperature, after which cells were washed a further three times in PBS before being incubated with appropriate fluorescent secondary antibody diluted in 10% (v/v) FCS in PBS for 1 hour at room temperature in the dark. Cells were then washed again three times in PBS and mounted in Vectashield mounting medium (Vector Laboratories) containing 4',6-diamidino-2-phenylindole (DAPI), and protected with 20-70 mm glass coverslips (Menzel-Gläser). Cells were viewed on an LSM 510 Meta confocal laser sanning microscope (Carl Zeiss), with a UVA 364 nm laser, and visible line 488 and 543 nm lasers.

2.5. MOLECULAR BIOLOGY TECHNIQUES

2.5.1. Preparation of media and plates

Luria broth (LB) was made with 10 g/L bactotryptone (Difco), 5 g/L bacto-yeast extract (Difco) and 10 g/L NaCl (pH 7.2) in water. LB-agar was made by adding 15 g/L to LB. Broth and agar were sterilized by autoclaving at 121 °C and 15 psi for 30 minutes. When needed, the LB-agar was melted down to liquid form and then 100 µg/ml of ampicillin was added to it once it was cooled down to approximately 50 °C. LB-agar was then aliquoted onto plates and left to cool inside a fume hood before being stored at 4 °C until required for bacterial transformations. Prior to use, plates were dried for 30 minutes in a fume hood.

2.5.2. Transformation of bacteria

Bacterial transformations were carried out in DH5α competent cells for plasmid production, or in BL21 competent cells to generate recombinant proteins. Typically, 50 ng of DNA was added to a 25 µl aliquot of the appropriate bacteria. Samples were incubated on ice for 30 minutes followed by heat shock at 42 °C for 1 minute, and then cooled for 5 minutes on ice. 300 µl of super optimal catabolite (SOC) medium (Invitrogen) was then added to the samples before incubation for 1 hour at 37 °C with shaking at 220 rpm. Cells were then pelleted at 13000 rpm, resuspended in 280 µl of LB, and then spread onto LB-agar plates containing ampicillin. Plates were then air-dried and then incubated overnight at 37 °C.

2.5.3. Small scale DNA preparation

Initially, 5 ml of LB, supplemented with 100 µg/ml ampicillin, was inoculated with a single bacterial colony containing the desired plasmid and then incubated overnight at

37 °C with shaking at 200 rpm. The following morning cells were pelleted by centrifugation at 3000 rpm and then resuspended in 250 µl of P1 buffer (Qiagen) and transferred to 1.5 ml microfuge tubes. The cells were then lysed for 5 minutes following addition of 250 µl of P2 buffer, which was then neutralised by adding 350 µl of N3 buffer. The solution was centrifuged at 13000 rpm for 10 minutes, and then the supernatant was transferred to a QIAprep column followed by further 1 minute centrifugation at 13000 rpm to adsorb the DNA. The column was then washed with 750 µl of PE buffer, before addition of 50 µl of nuclease-free water (Ambion) and centrifugation to elute the DNA. DNA concentration was measured using a BioPhotometer (Eppendorf), and then stored at -20 ^{o}C.

2.5.4. Large scale DNA preparation

Initially, 5 ml of LB, supplemented with 100 µg/ml ampicillin, was inoculated with a single bacterial colony containing the desired plasmid and then incubated 6-8 hours at 37 °C with shaking at 200 rpm. The culture was then added to a flask containing 250 ml of LB supplemented with 100 µg/ml ampicillin, and then incubated overnight at 37 °C with shaking at 200 rpm. Cells were then pelleted the next day at 6000 rpm for 10 minute at 4 °C, and then resuspended in 12 ml of resuspension buffer followed by being lysed with 12 ml of lysis buffer (Sigma). The lysis reaction was stopped after 5 minutes by the addition of 5 ml of neutralisation buffer, with the resulting cell lysis suspension then being added to 9 ml of binding solution and poured into a filter syringe and left to stand for 5 minutes. During this time, 12 ml of binding buffer was added to the binding column and spun at 3000 x g for 2 minutes to prepare it for DNA adsorption. The contents of the syringe was then added to binding column and centrifuged at 3000 x g for 2 minutes, and then the column was washed with wash buffer 1 and 2. The DNA

bound to the column was then eluted with 3 ml of elution buffer, before being precipitated with 0.7 volumes of room temperature isopropanol and 0.1 volumes of 3M Sodium Acetate pH 5.5 at 13000 x g for 30 minutes at 4 ^{o}C. The pellet was then washed twice with 70 % ethanol, centrifuged at 13000 x g for 10 minutes at 4 ^{o}C, air dried, and then resuspended carefully in nuclease-free water (Ambion). DNA concentration was measured using a BioPhotometer (Eppendorf), and then stored at -20 ^{o}C.

2.5.5. PCR of gene sequences flanked by restriction sites

25 pmol of the appropriate forward and reverse primers containing the desired restriction sites (Alta Biosciences Ltd- see Table 2.4) was mixed with 10-100ng of template DNA, 5µl of 10X buffer, 1µl of dNTPs (10mM), 1µl of *pfu* DNA polymerase (2.5 U/µl) (Promega), and SDW up to total volume of 50µl. This was then incubated at 95 ^{o}C for 5 minutes in a thermal cycler (GeneAmp PCR Systems 9600), before addition of DNA polymerase (Roche). The reaction solutions were mixed and placed back in the thermal cycler and subjected to the following conditions: 1 cycle of 95 ^{o}C for 5 minutes, 55 ^{o}C for 2 minutes, 72 ^{o}C for 2 minutes; 15-30 cycles of 95 ^{o}C for 1 minute, 55 ^{o}C for 30 seconds, 72 ^{o}C for 1 minute/Kb; and 1 cycle of 72 ^{o}C for 7 minutes. The resulting DNA products were resolved by agarose gel electrophoresis and purified using a Qiagen gel extraction kit as described below.

2.5.6. Ligation

In order to generate GST-fusion proteins, the gene of interest was cloned into pGEX 4T-1. Ligation reactions were made up in a 30 µl volume containing 1 unit of T4 DNA ligase, 3 µl of 10X buffer, 20 ng of vector cut with the appropriate restriction enzymes, 500 ng-2 µg of digested insert, and sterile deionized water, and then incubated overnight at 16 ^{o}C. The reactions were heated at 65 ^{o}C for 15 minutes the following

morning, before being placed on ice for 5 minutes. The ligation mix was then added to 25 µl of DH5α cells, and transformed as previously described. The resulting DNA was purified using a Qiagen miniprep kit and sequenced.

2.5.7. Agarose gel electrophoresis

Agarose gels were prepared by dissolving agarose in 50 ml of TBE (100mM Tris, 100mM Boric Acid, 2mM EDTA, pH 8.3) to a final concentration of 1 % (w/v) . The agarose was dissolved by heating the mixture to boiling. This was then allowed to cool before adding ethidium bromide to a final concentration of 0.5 µg/ml. Samples were then diluted with 6X loading buffer (30 % (v/v) of glycerol, 0.25 % (v/v) of bromophenol blue in a 10 mM Tris-HCl, 1 mM EDTA, pH 8 solution) and then loaded. Gel electrophoresis was carried out in 1X TBE for upto 1 hour at 60 V. DNA was visualized with a UV transilluminator.

2.5.8. Gel extraction of DNA

DNA generated by PCR was resolved by agarose gel electrophoresis prior to purification. The bands were excised and the DNA was purified using QIAquick gel extraction kit (Qiagen) as per the manufacturer's instructions.

2.5.9. Sequencing

Sequencing was performed to validate the DNA sequence of the coding regions generated by PCR. Each reaction was carried out using Big Dye ^{TM} terminator V3.1 cycle sequencing kit (Applied Biosystems) in a volume of 20 µl containing 4µl of 5X buffer, 5pmol of primer (see Table 2.3 for primers used), 1µl of terminator ready reaction mix (Big Dye™ terminator V3.1), and 200ng-500ng of DNA. Reaction solutions were placed in a thermal cycler (GeneAmp PCR Systems 9600) for the

sequencing reaction for a total of 25 cycles using the following conditions: 96 ^{o}C for 10 seconds, 50 ^{o}C for 5 seconds, and 60 ^{o}C for 4 minutes. The PCR products were precipitated by incubating them with 80 µl of 75 % isopropanol at room temperature 30 minutes, before being pelleted at 13000 rpm for 20 minutes. The pellet was then rinsed with 200µl of 75 % isopropanol, centrifuged for a further 5 minutes, and then air dried at 90 ^{o}C to remove the residual isopropanol. 10µl of formamide was then added to the mixture and heated for 5 minutes at 95 ^{o}C, quenched on ice, and briefly centrifuged before loading in a 3130xl Genetic Analyzer (Applied Biosystems) for sequencing. Sequences were analysed using the NCBI nucleotide-nucleotide BLAST analysis obtained online at http://www.ncbi/nlm/nih.org.

Table 2.5. Primers used in this study

Primer	Sequence
pGEX 4-T1 forward	**5' GGGCTGGCAAGCCACGTTTGGTG 3'**
pGEX 4-T1 Reverse	**5' CCGGGAGCTGCATGTGTCAGAGC 3'**
Eco RI Ad12 E4orf6 forward	**5' ATAGCGAATTCATGCAGCGCGACAGACGGTATCGC 3'**
Xho I Ad12 E4orf6 reverse	**5' ATCATCTCGAGTCAGTGTCCATCAGCCGCCCAAGG 3'**

2.5.10. Production of recombinant proteins from bacteria

Plasmids encoding glutathione S-transferase upstream of the region of interest were used to transform BL21 bacteria as previously described. Colonies from successful transformations were picked and used to inoculate 20 ml of LB broth supplemented with 100 µg/ml ampicillin, and incubated overnight at 37 ^{o}C with shaking at 200 rpm.

The next morning, the cultures were added to 500 ml of LB medium supplemented with 100 μg/ml ampicillin and incubated at 37 ^{o}C with shaking at 200 rpm for a further 2.5 hours or until the cultures had reached the optimal density of A600 = ~0.6. At this point, isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma) was added to a final concentration of 1 mM to induce protein production. Cultures were incubated for a further 3 hours at 30 ^{o}C with shaking at 200 rpm, before being pelleted at 6000 rpm for 10 minutes at 4 ^{o}C, and then stored at -80 ^{o}C until required for the next stage of purification.

In order to purify the GST-fusion proteins, bacteria were thawed and resuspended in 20 ml of ice-cold GST lysis buffer (1 mM EDTA (pH 8) and 1 % Triton X-100 (v/v) in PBS), before being sonicated on ice 3 times for 45 seconds each time. The lysates were then centrifuged for 20 minutes at 20000 rpm at 4 ^{o}C, with the resulting supernatant being transferred in to a fresh tube and incubated with glutathione-agarose beads at 4 ^{o}C for 1 hour with rotation. The beads were collected by centrifugation at 3000 rpm, before being washed three times in lysis buffer and twice in wash buffer (1 mM EDTA (pH 8) in PBS). GST-fusion proteins were then released following incubation of the glutathione-agarose beads in GST elution buffer (25 mM glutathione, 50mM Tris-HCl, pH 8.0) for 2 hours with rotation at 4 ^{o}C. Meanwhile, dialysis tubing was hydrated in a warm solution of 3 % (w/v) $NaHCO_3$, 2 mM EDTA pH 8.0 for 20 minutes to chelate metal ions. The beads were then remove by centrifugation at 1500 rpm for 3 minutes at 4 ^{o}C, with the resulting supernatant containing eluted GST-fusion protein being transferred to the dialysis tubing. The supernatant was then subjected to overnight dialysis at 4^{o}C in 5L of 25 mM Tris pH 8.0, 100 mM NaCl, 1 mM DTT (Sigma) and 10 % (v/v) glycerol. The following morning, dialysis tubing that contained the GST proteins was placed in fresh dialysis buffer for an additional 6h. GST-fusion proteins

were then collected, protein concentration was determined, and the purity of the proteins was assessed by SDS-PAGE electrophoresis and Coomassie blue staining. Purified proteins were stored in aliquots at −80 ^{o}C.

2.5.11. In vitro transcription/translation (IVT)

L-α-[^{35}S]-methionine-labelled proteins were expressed using a TNT T7 Coupled Reticulocyte Lysate System (Promega) as per the manufacturer's instructions. Briefly, the reaction was carried out in a volume of 50 µl containing 25 µl rabbit reticulocyte lysate, 0.5 µg plasmid DNA, 2 µl reaction buffer, 1 µl T7 polymerase, 1 µl amino acid mix (minus methionine), 2 µl [^{35}S]-methionine (1000Ci/mmol at 10mCi/ml; Perkin Elmer) and 1 µl RNase inhibitor (Roche Diagnostics Ltd), and Nuclease-free water. The reaction was carried out 30 ^{o}C for 90 minutes, before being centrifuged and stored at –80 ^{o}C until required. The efficiency of translation was assessed by fluorography following SDS-PAGE.

2.6. STATISTICAL ANALYSIS

To determine whether results were statistically significant, a two-tailed T-test was performed, which is typically used to establish whether the difference between two means is significant, or not. Probability (P) values are depicted in the Figure legends where appropriate. The test equation is as shown below, where x-bar = mean; $^{\sigma}_{d}$ = the standard deviation (S.D) of the difference between the means.

$$T = \frac{\text{x-bar}^1 - \text{xbar}^2}{\sigma_d}$$

CHAPTER 3

REGULATION OF THE ATR SIGNALLING PATHWAY BY ADENOVIRUS E4ORF6

3.1. INTRODUCTION

ATM and ATR are kinases that function as key transducers of signals initiated in response to DNA damage. ATM is activated through its recruitment to sites of DNA damage by the MRN complex (Kim et al. 2006). ATM responds primarily to DSB, and controls processes such as apoptosis and activation of cell cycle checkpoints through its ability to phosphorylate downstream effector proteins, such as Chk2, BRCA1, and p53 (Shiloh 2003). In contrast, ATR responds to ssDNA that occurs as intermediates during the processing of damaged DNA, or during DNA replication in S-phase. The ATR cognate binding partner, ATRIP, in conjunction with the 9-1-1, replicative sliding clamp complex, serves to recruit ATR to RPA-coated ssDNA (Petermann and Caldecott 2006). Once recruited to ssDNA, ATR directs the phosphorylation of a number of downstream effector proteins such as Chk1, RPA32, SMC1, and Rad9 in order to regulate pathways that prevent further origin firing, block cell cycle progression, stabilize stalled replication forks and facilitate replication fork restart (Cimprich and Cortez, 2008). There are other proteins in addition to ATRIP that participate in the activation of the ATR pathway, such as TopBP1. TopBP1 is a multifunctional BRCT-containing protein, which is involved in transcriptional regulation, DNA replication, and checkpoint signalling (Garcia et al. 2005). The latter function may primarily be due to the ability of TopBP1 to directly activate ATR by stimulating its kinase activity through interactions with both ATR and ATRIP (Kumagai et al. 2006; Mordes and Cortez 2008). In support of this idea, TopBP1 has been shown to be essential for certain ATR-dependent signalling events, including Chk1 and Nbs1 phosphorylation (Kumagai et al. 2006).

Viruses have evolved a number of mechanisms in order to negate and/or selectively activate genotoxic stress response pathways in order to facilitate the replication of their genomes efficiently in host cells (Weitzman et al. 2004; Lilley et al. 2007). Upon entering the nucleus, the linear dsDNA adenovirus genome has the potential to mimic a DNA DSB, whilst adenoviral DNA replication generates ssDNA intermediates. As such, both of these processes are capable of eliciting cellular DDR pathways which have the potential to limit adenovirus replication. A fully functioning DDR system is, therefore detrimental to adenovirus replication (Weitzman et al. 2004; Lilley et al. 2007).

Adenoviruses have therefore evolved to inactivate proteins involved in DDR signalling pathways, principally by targeting them for proteasome-mediated degradation. The first protein to be identified as a genuine target for Ad-mediated degradation, was p53 in a study which showed that the Ad early region proteins, E1B-55K and E4orf6 function in concert to recruit p53 to a CRL complex containing Cul5, RBX1, and Elongins B and C, whereupon it is ubiquitylated and targeted for proteasomal degradation (Querido et al. 2001; Harada et al. 2002). Other DNA damage proteins targeted by E1B-55K and E4orf6 for degradation include; DNA ligase IV, Mre11, and BLM (Stracker et al. 2002; Liu et al. 2005; Baker et al. 2007; Orazio et al. 2011).

It has been determined that the E1B-55K/E4orf6-dependent degradation of MRN is sufficient to prevent ATM but not ATR activation during infection (Stracker et al. 2002; Blackford et al. 2008; Carson et al. 2009; Karen et al. 2009). Moreover, our laboratory has shown recently that different Ad species differentially regulate ATR activity during infection (Blackford et al. 2008). Specifically, Ad12 infection results in ATR activation and the ATR-dependent hyperphosphorylation of RAD9 and RPA32,

whilst Ad5 infection does not readily activate ATR (Blackford et al. 2008). Interestingly, however, both of these virus serotypes inhibit ATR-dependent Chk1 phosphorylation, suggesting that full ATR activation is prevented by both viruses, but by different strategies. Until recently, it was not clear how Ad5 prevents ATR activation, but now it has been shown that the E4orf3-mediated relocalization and immobilization of MRN subunits to aggresomes is required to inhibit ATR activation during Ad5 infection (Carson et al. 2009). Interestingly, although the E1B-55K/E4orf6-mediated degradation of p53 and the MRN complex appears to be conserved between Ad5 and Ad12 (Stracker et al. 2005; Blackford et al. 2008), the E4orf3-mediated inactivation of ATR is specific to group C adenoviruses, including Ad5 (Carson et al. 2009). Other human adenoviruses such as Ad12 lack a key isoleucine residue in their E4orf3 proteins required for MRN relocalization (Carson et al. 2009); however, as ATR is not fully activated during Ad12 infection, this implies that this serotype must have evolved other means of inactivating ATR. Given all of these findings, the principal aim of the study described in this chapter therefore was to investigate how Ad12 inhibits the ATR-dependent activation of Chk1 during infection.

3.2. RESULTS

3.2.1. TopBP1 is degraded during Ad12 but not Ad5 infection

The MRN complex is targeted for proteasomal degradation by adenoviral proteins E1B-55K and E4orf6 in order to inhibit ATM signalling during adenovirus infection (Stracker et al. 2002). We hypothesised therefore that Ad12 might target components of the ATR signalling pathway for proteasomal degradation as a means of inactivating the ATR pathway during infection. To investigate this, we mock-infected or infected HeLa cells with *wt* Ad5 or *wt* Ad12 at an m.o.i of 10 p.f.u./cell and then harvested cell lysates at intervals over a 48 hour period. The protein lysates were then quantified and subjected to SDS-PAGE. Following transfer to nitrocellulose, levels of proteins involved in ATR signalling pathways were assessed by Western blotting (Fig. 3.1).

In accordance with previous findings we observed that both Mre11 and DNA ligase IV were targeted for degradation during both Ad5 and Ad12 infection. Interestingly however, we observed that the protein levels of TopBP1 were decreased 24 hours post Ad12 infection, yet TopBP1 levels remained constant during Ad5 infection (Fig. 3.1). We noted however, that the protein levels of ATR, ATRIP, and RPA70 remained constant during infection (Fig. 3.1). Consistent with previous findings by our laboratory, ATR targets RPA32 and Rad9 were phosphorylated following Ad12, but not Ad5 infection (Blackford et al. 2008). These data suggest that TopBP1 might be a target for proteasomal degradation during Ad12 infection (Fig. 3.1)

Given that previous research has shown that Ad5 promotes the proteasome-mediated degradation of Mre11 (Stracker et al. 2002), DNA ligase IV (Baker et al. 2007), and p53 (Querido et al. 2001), we decided to investigate if the decrease in levels of TopBP1 observed post Ad12 infection was also proteasome-dependent. HeLa cells were

therefore, mock-infected or infected with *wt* Ad12, whereafter treated cells were incubated in the presence or absence of proteasome inhibitor, MG132. The cells were then harvested at intervals over a 48 hour time course post infection; lysates were then subjected to SDS-PAGE and protein levels of TopBP1 and Mre11 were assessed by Western blotting (Fig. 3.2). The levels of both Mre11 and TopBP1 were substantially greater in the Ad12-infected cells treated with MG132 than in Ad12-infected cells that were not treated with the proteasome inhibitor (Fig. 3.2). Taken together, these results suggest that TopBP1 is specifically targeted for degradation by Ad12 in a proteasome-dependent manner.

3.2.2. TopBP1 is relocalized to viral replication centres in Ad-infected cells

Our laboratory and others have previously shown that components of the ATR signalling pathway ATRIP, RPA32, Rad9, Rad17, and E1B-AP5, are relocalized to VRCs in Ad-infected cells (Stracker et al. 2005; Blackford et al. 2008). Given that TopBP1 appears to be differentially regulated by Ad5 and Ad12, and the fact that these two serotypes also differentially regulate other components of the ATR signalling pathway, we investigated the cellular localization patterns of TopBP1 in Ad5- and Ad12-infected cells using immunofluorescent confocal microscopy. HeLa cells were therefore seeded onto glass slides and grown for 24 hours prior to mock-infection or infection by *wt* Ad5 or *wt* Ad12 at an m.o.i of 10 p.f.u./cell. The cells were then harvested 24 and 48 hours post infection by treatment with a pre-extraction buffer and fixation in 4% (v/v) PFA in PBS. Fixed cells were then stained with an antibody against TopBP1, as well as antibodies against RPA32 and DBP, which served as markers for VRCs. Finally, the

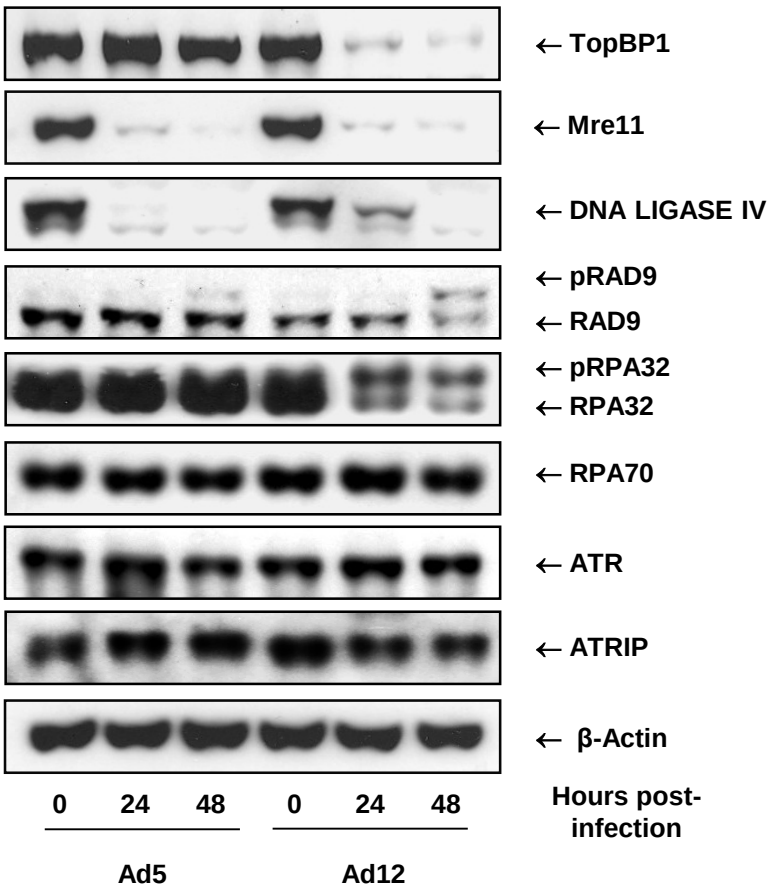

Fig. 3.1. Effect of Ad infection on expression levels of proteins involved in ATR activation. HeLa cells were mock-infected or infected with the indicated viruses at an m.o.i of 10 p.f.u./cell. Cells were harvested and prepared for Western blotting at the indicated time-points using the appropriate antibodies.

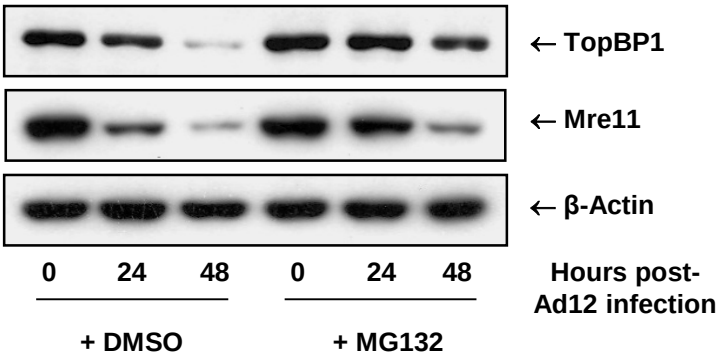

Fig. 3.2. Effects of the proteasome inhibitor MG132 on TopBP1 expression levels in Ad12-infected cells. HeLa cells were mock-infected or infected with Ad12 at an m.o.i of 10 p.f.u./cell, and subsequently incubated in the presence or absence of 10 μM MG132. Cells were harvested and prepared for Western blotting at the indicated time-points, and Mre11 was used as a positive control.

cells were mounted in a DAPI-containing medium to stain the DNA, and visualized using a LSM510 Meta confocal laser scanning microscope.

In the mock-treated cells we found that both TopBP1 and RPA32 were diffusely localised in the nucleoplasm, yet excluded from nucleoli (Fig. 3.3A). In contrast, TopBP1 was found to co-localize with RPA32 at VRCs at 15 and 24 hours post Ad5 infection, as well as with DBP at 24 hours post infection (Fig. 3.3B-C and Fig. 3.4). We also observed TopBP1 co-localization with RPA32 at VRCs at early times post Ad12 infection (Fig. 3.3D), however the staining of TopBP1 foci became distinctively weaker at later times, and TopBP1 did not co-localize with RPA32 at VRCs at this time (Fig. 3.3E). This was consistent with our Western blot analysis in Fig. 3.1. These data indicate that TopBP1 is recruited to VRCs during Ad12 infection prior to degradation.

It has previously been shown that during Ad5 infection, E1B-55K and E4orf3 relocalize MRN and p53 to nuclear track-like structures and cytoplasmic aggresomes prior to degradation by E1B-55K and E4orf6 (Evans and Hearing 2005; Liu et al. 2005). Therefore, to determine if TopBP1 localizes to nuclear track-like structures or cytoplasmic aggresomes during infection, we carried out a similar experiment to that outlined above, where HeLa cells were infected with either Ad5 or Ad12 and then harvested and fixed, 15 hours post infection. Cells were subsequently stained with an antibody against TopBP1, as well as antibodies against PML and γ-Tubulin, which served as markers for nuclear tracks and cytoplasmic aggresomes, respectively. This experiment revealed that TopBP1 is not relocalized to nuclear track like structures in Ad5- or Ad12-infected cells (Fig. 3.5A-B), and neither is it relocalized to cytoplasmic aggresomes in Ad12-infected cells (Fig. 3.5C). These data suggest that TopBP1 degradation by Ad12 is mechanistically different to the degradation of MRN and p53 by Ad5.

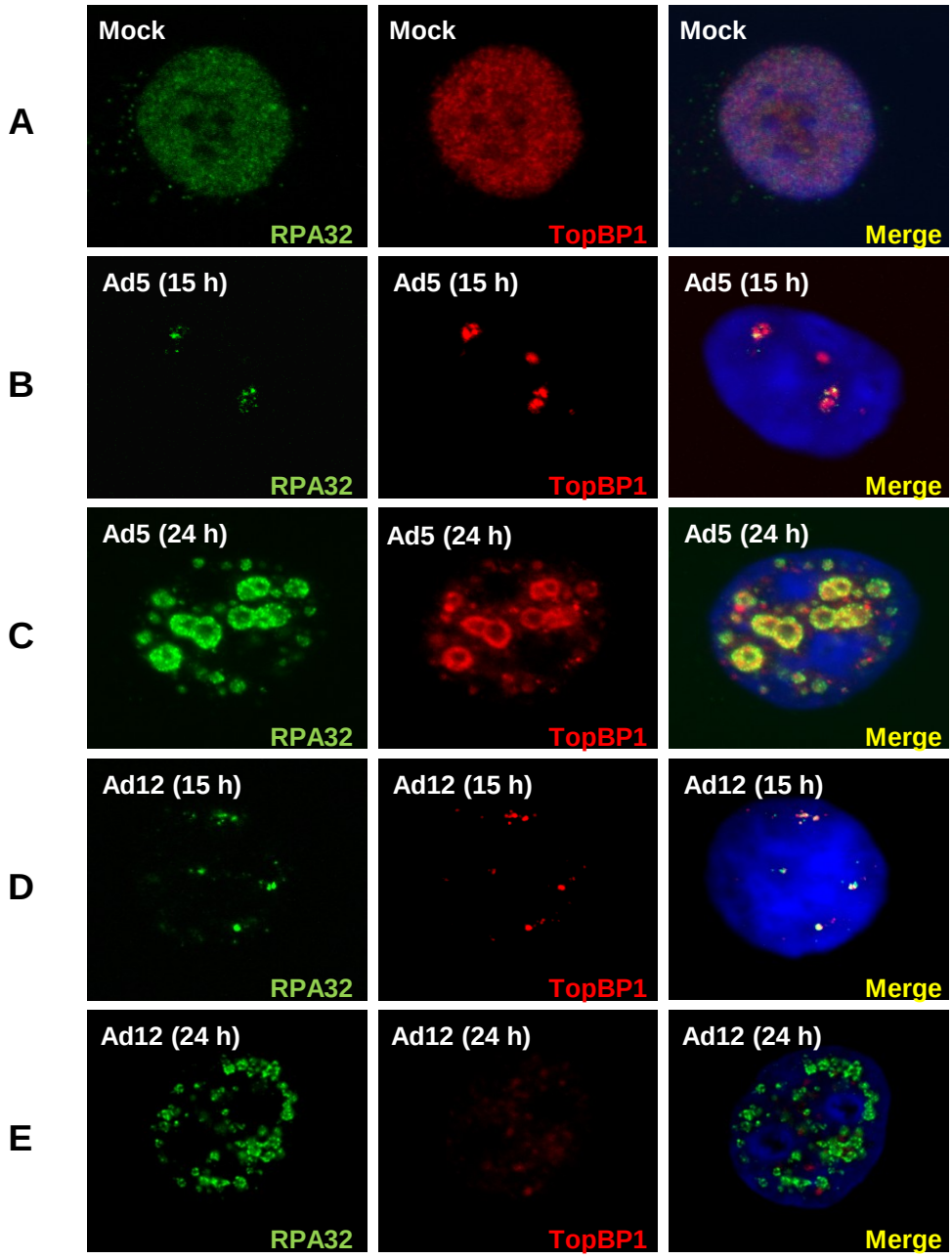

Fig. 3.3 TopBP1 is relocalized to viral replication centres in Ad-infected cells. HeLa cells were grown on glass coverslips and mock-infected (A) or infected with Ad5 (B-C) or Ad12 (D-E) at an m.o.i of 10 p.f.u./cell. At the appropriate time, cells were treated with a pre-extraction buffer and then fixed in 4% (w/v) paraformaldehyde and stained for TOPBP1 (red), RPA32 (green) and DAPI (blue). Images were visualized by confocal microscopy. Colocalization of proteins is shown in yellow.

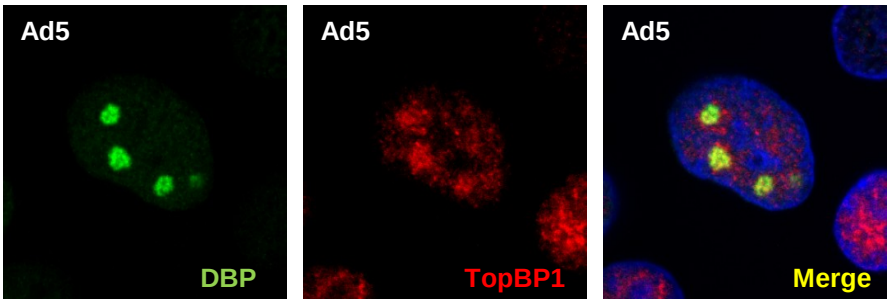

Fig. 3.4 Localization of TopBP1 with DBP in Ad5-infected cells at viral replication centres. HeLa cells were grown on glass coverslips and infected with Ad5 at an m.o.i of 10 p.f.u./cell, treated with a pre-extraction buffer before being fixed in 4% paraformaldehyde and stained for TopBP1 (red), 2A6 E1B-55K, and DBP (both green) and DAPI (blue). Images were visualised by confocal microscopy. Colocalization is shown in yellow.

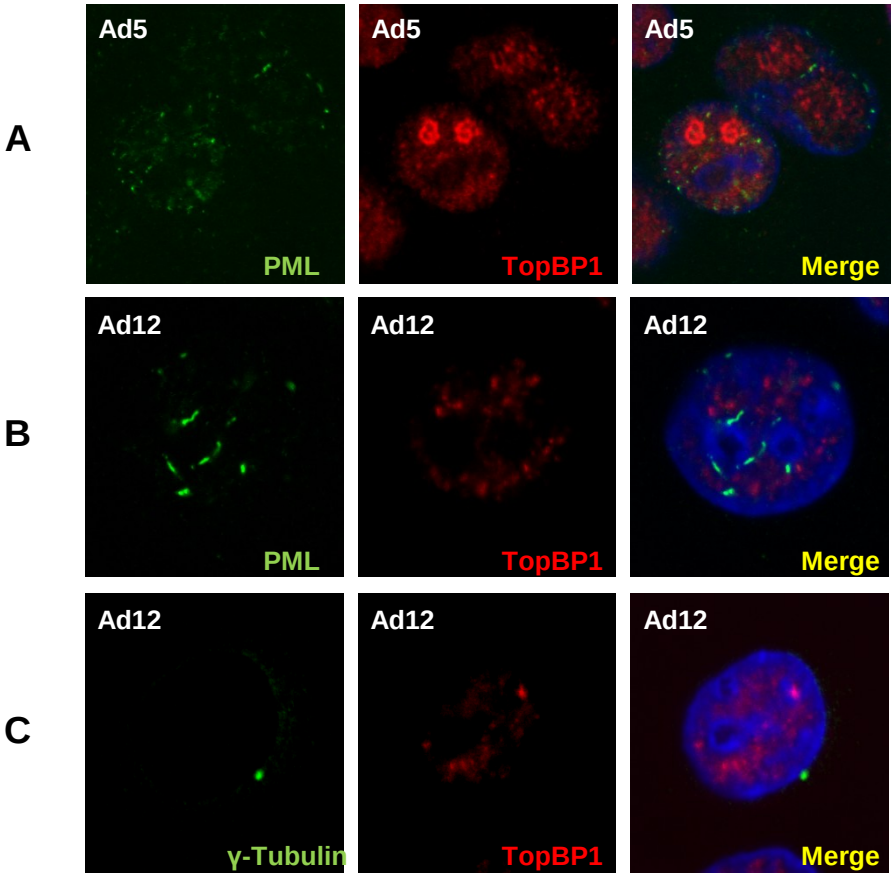

Fig. 3.5. TopBP1 is not relocalized to PML-containing nuclear tracks (A and B) or cytoplasmic aggresomes (C) in Ad5- or Ad12-infected cells. HeLa cells grown on glass coverslips were either mock-infected or infected with Ad5 or Ad12 at an m.o.i of 10 p.f.u./cell. 15 hours post-infection cells were treated with a pre-extraction buffer before being fixed in 4% (w/v) paraformaldehyde and stained for TopBP1 (red), γ-Tubulin (green) and DAPI (blue). Images were visualised by confocal microscopy. Colocalization is shown in yellow.

3.2.3. RBX1 and Elongin C are essential for the Ad12-mediated degradation of p53 and TopBP1.

During Ad5 infection, the viral proteins E1B-55K and E4orf6 function in concert to recruit p53 to an E3 ligase complex containing the cellular proteins Cul5, RBX1, and Elongins B and C, where it is ubiquitylated, and targeted for proteasome-mediated degradation (Querido et al. 2001; Harada et al. 2002). To determine if Ad12 similarly utilizes this complex to promote the degradation of TopBP1, we initially carried out a series of RNA interference experiments targeting Elongin C and RBX1 as these proteins are components of CRL5 complex (Petroski and Deshaies 2005).

To do this, HeLa cells were initially transfected with either non-silencing siRNA oligonucleotides, or specific Elongin C, or RBX1 siRNA oligonucelotides in order to knock-down the expression of Elongin C or RBX1 proteins. Cells were infected 48 hours post knock-down with *wt* Ad12 at an m.o.i of 10 p.f.u./cell, and harvested at the appropriate times post-infection. Lysates were then subjected to SDS-PAGE and protein levels of p53, TopBP1, Elongin C, and RBX1 were assessed by Western blotting. Consistent with previous findings, both p53 and TopBP1 were targeted for proteasomal degradation by Ad12 in cells treated with non-silencing siRNAs (Fig. 3.6A). Consistent with the previously identified role for a CRL in the Ad-mediated degradation of p53, we observed that p53 was not targeted for degradation in Ad12-infected cells where Elongin C had been knocked down by RNAi, demonstrating that the activity of the CRL was compromised (Fig. 3.6A). Moreover, we also determined that TopBP1 was not targeted for proteasomal-mediated degradation by Ad12, in cells where Elongin C had been knocked down by RNAi (Fig. 3.6A). Similarly, TopBP1 was not targeted for degradation by Ad12, in cells that were treated with RBX1 siRNA (Fig.

3.6B). Taken together, these data suggest that like p53, TopBP1 may also be targeted for degradation during Ad12 infection by a cellular CRL complex.

3.2.4. Ad5 and Ad12 utilize different Cullin containing ubiquitin ligase complexes to promote the degradation of cellular proteins during infection

As Elongin C and RBX1 are components of both Cul5 and Cul2 ubiquitin ligases (Petroski and Deshaies 2005), we wanted to determine whether Ad12 utilizes the CRL5 complex, or CRL2 complex to promote the degradation of TopBP1. To do this, we transfected specific Cul2 or Cul5 siRNA oligonucelotides into HeLa cells in order to specifically inhibit the expression of Cul2 or Cul5 proteins. Alternatively, cells were transfected with non-silencing siRNA to serve as a control. Cells were then infected with *wt* Ad5 or *wt* Ad12 at an m.o.i of 10 p.f.u./cell, 48 hours post-transfection and harvested at the appropriate time. Protein lysates were then subjected to SDS-PAGE and protein levels of TopBP1, Cul2, and Cul5 were assessed via Western blotting (Fig. 3.7). We also assessed the level of p53 to gauge adenovirus ubiquitin ligase activity during infection.

Initial Western blot analysis determined that Cul2 and Cul5 expression was reduced significantly following treatment with the appropriate siRNA oligonucleotides (Fig. 3.7). In agreement with previous reports, we found that p53 levels were stabilized in Ad5-infected cells depleted of Cul5. Interestingly however, p53 was still degraded in Ad12 infected cells depleted of Cul5 (Fig. 3.7). As expected, TopBP1 was not degraded in Ad5-infected cells in which Cul5 was knocked down, but it was still degraded in Ad12-infected cells in which Cul5 was knocked down (Fig. 3.7). As anticipated both p53 and TopBP1 were also degraded in Ad12-infected cells treated with control siRNA (Fig. 3.7). Intriguingly, TopBP1 and p53 were not degraded following Ad12 infection

of cells that were depleted of Cul2 (Fig. 3.7) These data suggest that in contrast to Ad5, Ad12 utilizes CRL2 to promote p53 and TopBP1 degradation during infection rather than a CRL5.

CRLs are activated during a process known as Neddylation in which a ubiquitin-like protein NEDD8 becomes conjugated to the Cullin subunit of CRLs (Petroski and Deshaies 2005). Cullin activation can be visualised by SDS-PAGE as an increase in molecular weight. Seeing as Ad5 and Ad12 target different CRLs, we hypothesized that these viruses may differentially neddylate Cul2 and Cul5 during infection. To this end, we mock-infected or infected HeLa cells with *wt* Ad5 or *wt* Ad12 at an m.o.i of 10 p.f.u./cell, and then harvested at intervals over a 48 hour period. The lysates were then subjected to SDS-PAGE and levels of Cul2, Cul5 and TopBP1 were analyzed by Western blotting with Mrell degradation by both virus serotypes being used as a control.

Consistent with earlier findings we observed that 16 hours post-infection, both TopBP1 and Mre11 were degraded in Ad12-infected cells, and Mrell was also degraded in Ad5-infected cells, whereas TopBP1 was not (Fig. 3.8). Interestingly, when we examined the neddylation patterns of Cul2 and Cul5 during Ad12 and Ad5 infection, we observed distinct differences (Fig. 3.8). In Ad5-infected cells we found that Cul5 is converted into its neddylated, high molecular weight form after 16 hours post infection, whereas in contrast to this, Cul2 is increasingly deneddylated 24 hours post Ad5 infection (Fig. 3.8). Consistent with the different requirements for Cul2 and Cul5 during adenovirus infection, the opposite neddylation/deneddylation pattern was observed in Ad12-infected cells (Fig. 3.8). Indeed, Cul2 became increasingly converted to its neddylated state following Ad12 infection (Fig. 3.8). Interestingly, the Cul5 neddylation levels were not altered appreciably following Ad12 infection (Fig. 3.8). Taken together, these

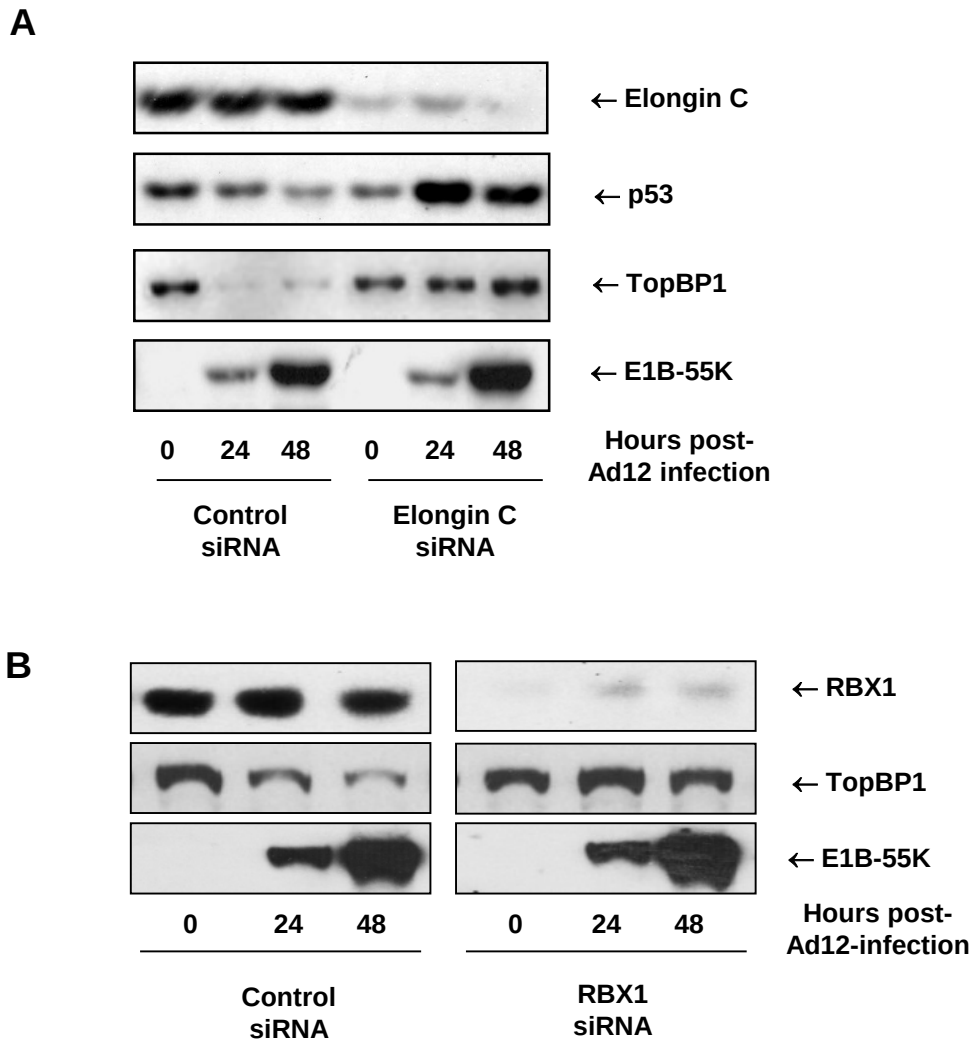

Fig. 3.6. Elongin C (A) and RBX1 (B) are required for p53 and TopBP1 degradation in Ad12-infected cells. HeLa cells were transfected with the indicated siRNAs, before being mock-infected or infected with Ad5 or Ad12 at an m.o.i of 10 p.f.u./cell 48h later. Cells were harvested and prepared for Western blotting at the indicated time points using the appropriate antibodies.

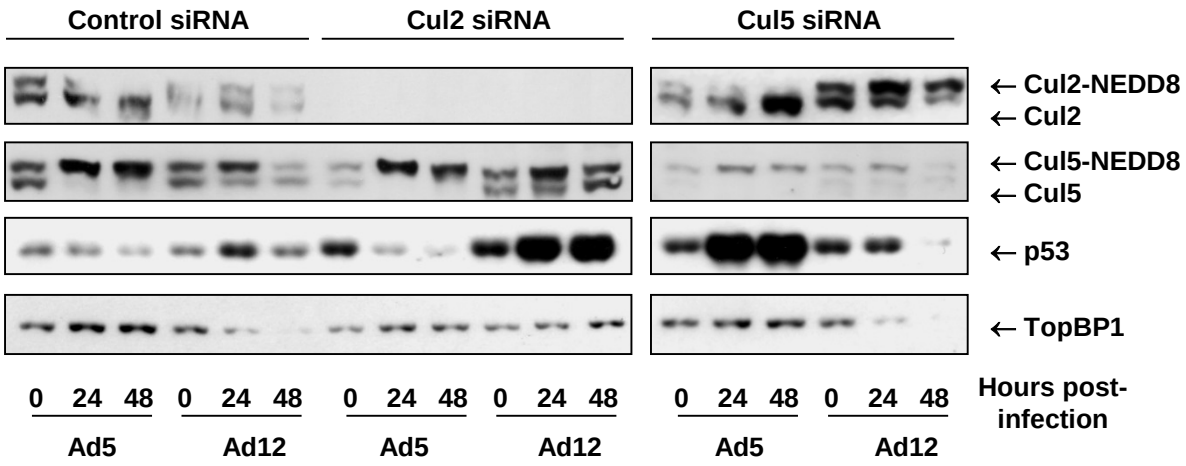

Fig. 3.7. Cul2 is required for the Ad12-mediated degradation p53 and TopBP1. HeLa cells were transfected with the indicated siRNAs, before being mock-infected or infected with Ad5 or Ad12 at an m.o.i of 10 p.f.u./cell 48hrs later. Cells were harvested and prepared for Western blotting at the indicated time points using the appropriate antibodies.

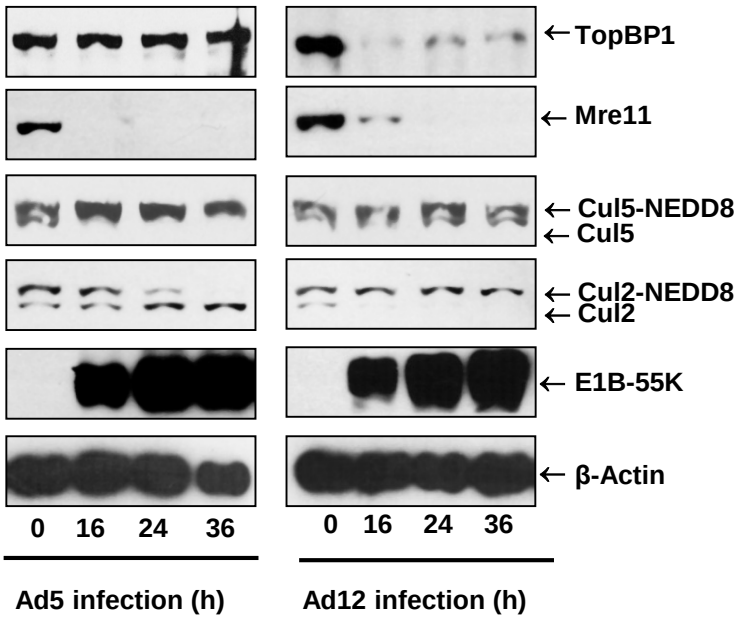

Fig. 3.8 Ad infection modulates Cul2 and Cul5 neddylation status. HeLa cells were mock-infected or infected with the indicated viruses at an m.o.i of 10 p.f.u./cell. Cells were harvested and prepared for Western blotting analysis at the indicated time points using the appropriate antibodies

data suggest that Ad5 promotes the neddylation of the CRL5 complex to facilitate the degradation of p53, whereas Ad12 promotes the neddylation and activation of the CRL2 complex to degrade both p53 and TopBP1.

3.2.5. Ad12 E4orf6 promotes the degradation of TopBP1 independently of E1B-55K

It is known that adenovirus proteins E4orf6 and E1B-55K function in concert to facilitate degradation of target proteins such as p53 and components of the MRN complex (Querido et al. 2001; Stracker et al. 2002). Therefore, we wanted to determine if Ad12 utilizes both of these proteins to promote degradation of TopBP1. To this end, we infected HeLa cells with *wt* Ad12 or an Ad12 *E1B* deletion mutant Ad12 *hr*703 at an m.o.i of 10 p.f.u./cell. We then harvested the cells at appropriate intervals over a 48 hour period. Protein lysates were then quantified and subjected to SDS-PAGE. TopBP1 and Mre11 were then analyzed by Western blotting (Fig. 3.9). Consistent with previous reports, Mre11 was degraded by *wt* Ad12, whilst the E1B-55K deletion mutant virus *hr*703 was unable to promote Mre11 degradation (Fig. 3.9). Interestingly, TopBP1 was degraded following infection with both *wt* Ad12, and the Ad12 E1B-55K mutant, *hr*703 virus (Fig. 3.9). These data suggest that unlike Mre11, the Ad12-mediated degradation of TopBP1 degradation occurs independently of E1B-55K. Thus, TopBP1 degradation by Ad12 is mechanistically different to Ad12-mediated degradation of MRN and p53.

On the basis of these results, we considered the possibility that Ad12 E4orf6 alone may be able to promote degradation of TopBP1. To test this hypothesis we transfected pcDNA3 plasmid alone, or pcDNA3 plasmids expressing Ad12 E4orf6 or Ad5 E4orf6 into HeLa cells. Cells were then harvested 24 hours post-transfection. Protein lysates were then quantified and subjected to SDS-PAGE; the protein levels of TopBP1, p53, and Mre11 were assessed by Western blotting (3.10). As expected the levels of p53 and

Mre11 were not affected by the expression of Ad12 E4orf6 or Ad5E4orf6 (Fig. 3.10). Interestingly, expression of Ad12 E4orf6 promoted a significant decrease in the levels of TopBP1 when compared to the levels of TopBP1 in cells expressing Ad5 E4orf6 or the empty vector (Fig. 3.10). These data are consistent with our hypothesis suggesting that Ad12 E4orf6 alone can promote the degradation of TopBP1.

Given these findings, and those suggesting that the Ad-mediated degradation of TopBP1 was proteasome-dependent, we wanted to investigate whether the Ad12 E4orf6-mediated reduction of TopBP1 levels was also proteasome-mediated. We therefore transfected HeLa cells with pcDNA3 alone or pcDNA3 expressing Ad12 E4orf6. Following transfection cells were treated with 10μM proteasome inhibitor MG132 or DMSO (control), and harvested 24 hours post transfection. Following protein quantification, lysates were subjected to SDS-PAGE and protein levels of TopBP1 were assessed by Western blotting, with Cyclin B1 being used as a positive control for MG132 activity (Fig. 3.11). As expected, MG132 treatment stabilized the levels of Cyclin B in both pcDNA3 and pcDNA3-E4orf6 transfected cells (Fig. 3.11). Consistent with the idea that E4orf6 promotes the proteasome-mediated degradation of TopBP1, treatment with MG132 inhibits the E4orf6-mediated degradation of TopBP1 (Fig. 3.11). These data suggest that the Ad12 E4orf6-mediated reduction in TopBP1 levels is proteasome-dependent. Interestingly, we also observed that the levels of Ad12 E4orf6 were elevated in the presence of the proteasome inhibitor, suggesting Ad12 E4orf6 itself is also a substrate for the proteasome (Fig. 3.11)

Given these findings, and the fact that E4orf6 recruits the CRL as it has been found to contain motifs termed BC boxes, that bind to elongins B and C (Cheng et al. 2007), we considered the possibility that Ad12 E4orf6 alone is able to recruit a CRL2 complex to promote the degradation of TopBP1. To this end we transfected non-silencing siRNA

or specific Cul2 siRNA oligonucelotides into HeLa cells in order to specifically inhibit the expression of Cul2 proteins. 48 hours post-knockdown cells were transfected with pcDNA3 plasmid alone, or pcDNA3 plasmid expressing Ad12 E4orf6. 24 hours post-transfection lysates were harvested, quantified, and subjected to SDS-PAGE; protein levels of TopBP1, and Cul2 were assessed by Western blotting (Fig. 3.12).

As expected, TopBP1 was degraded by Ad12 E4orf6 in cells that were treated with non-silencing siRNA. Interestingly however, Ad12 E4orf6 did not promote degradation of TopBP1 in cells that were depleted of Cul2 by siRNA (Fig. 3.12). Taken together, these results provide evidence that Ad12 E4orf6 alone can promote the proteasome-mediated degradation of TopBP1 in a Cul2-dependent manner.

3.2.6. Ad12 E4orf6 binds to TopBP1 and interacts selectively with Cul2

As Ad12 E4orf6 promotes the proteasome-mediated degradation of TopBP1 independent of E1B-55K, we considered the possibility that Ad12 E4orf6 might also function as a substrate adaptor, and directly bind to TopBP1. To test this hypothesis, we initially performed a GST pulldown assay by incubating 10 µl of in vitro-translated, full length [^{35}S]-TopBP1 with 10 µg of GST-Ad12 E4orf6, or 10 µg GST (Fig. 3.13A) Glutathione-agarose was used to isolate the protein complexes, which were then washed and selectively eluted with glutathione before being separated by SDS-PAGE, and visualised by fluorography and autoradiography. Consistent with our hypothesis that Ad12 E4orf6 also serves as a substrate adaptor, we found that Ad12 E4orf6 binds directly to TopBP1 (Fig. 3.13A). Given this finding, we next attempted to identify the region of the TopBP1 to which Ad12 E4orf6 bound (Fig. 3.13B). GST pulldowns

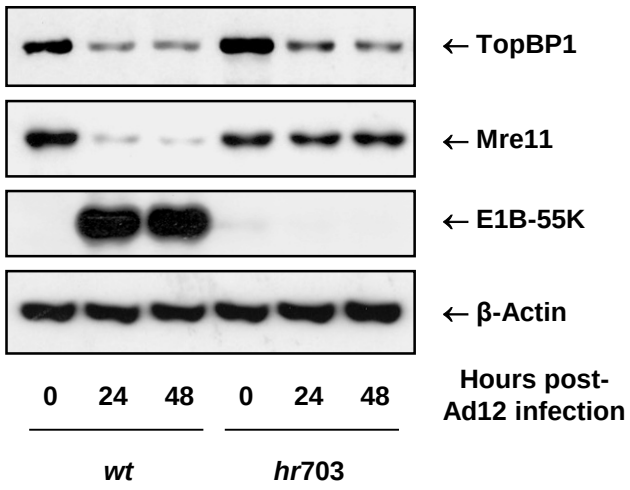

Fig. 3.9 TopBP1 degradation by Ad12 is independent of E1B-55K. HeLa cells were mock-infected or infected with *wt* Ad12 or the E1B-55K mutant virus hr703 at an m.o.i of 10 p.f.u./cell. Cells were harvested and prepared for Western blotting at the indicated times. Mre11 was used as a positive control

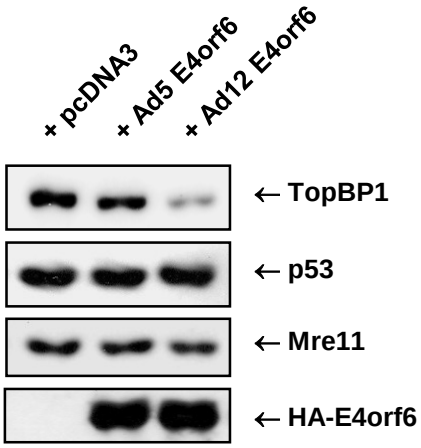

Fig. 3.10. Ad12 E4orf6 is necessary and sufficient for TopBP1 degradation. HeLa cells were transfected with pcDNA3-Ad12-E4orf6, pcDNA3-Ad5-E4orf6, or pcDNA3 vector alone and harvested for Western blotting 24 h later. Mre11 and p53 were used as controls.

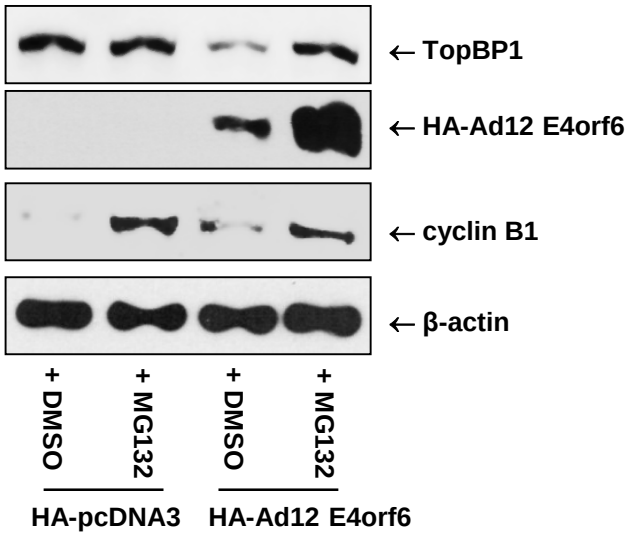

Fig. 3.11. Ad12 E4orf6-mediated degradation of TopBP1 is proteasome-dependent. HeLa cells were transfected with pcDNA3-Ad12-E4orf6 or pcDNA3 vector alone in the presence or absence of 10 μM MG132, added 6h after transfection. Cells were harvested for Western blotting 24h later. Cyclin B1 levels were used as a positive control for proteasome inhibition.

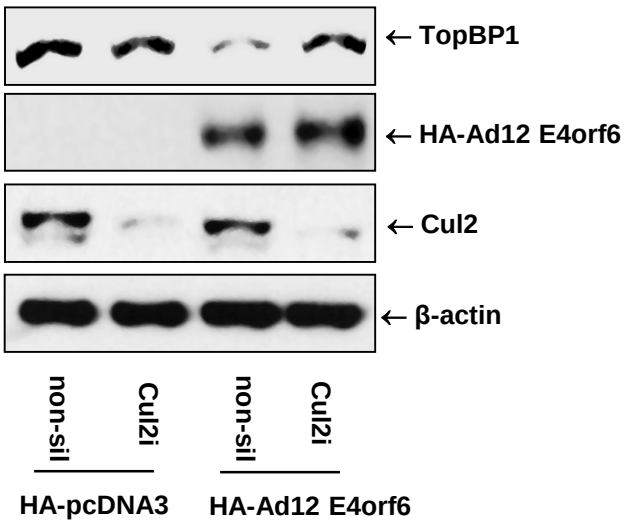

Fig. 3.12 Ad12 E4orf6-mediated degradation of TopBP1 is Cul2-dependent. HeLa cells treated with nonsilencing or Cul2 siRNAs 48h earlier were transfected with pcDNA3-Ad12-E4orf6 or pcDNA3 vector alone, before being harvested for Western blotting 24h later

revealed that the binding site(s) on TopBP1 for E4orf6 were extensive; binding site(s) extended between amino acids 2-1168 (Fig. 3.13B).

As we have provided evidence to show that Ad12 selectively recruits CRL2 complexes to facilitate the degradation of TopBP1, and that the Ad12E4orf6-mediated degradation of TopBP1 requires Cul2, we wanted to establish whether or not Ad12 E4orf6 interacts with Cul2. To this end we performed GST pulldown analyses. We incubated 10 µl of [^{35}S]-Cul2 or 10 µl of [^{35}S]-Cul5 with 10 µg of GST-Ad12 E4orf6 or GST (Fig. 3.14). Glutathione-agarose was used to isolate the protein complexes, which were washed and selectively eluted with glutathione before being separated by SDS-PAGE, and then visualized by fluorography and autoradiography. These analyses revealed that Ad12 E4orf6 had a much greater propensity to bind Cul2 (Fig 3.14A) than Cul5 (Fig 3.14B), consistent with our observations that Ad12 E4orf6-mediated degradation of TopBP1 is Cul2-dependent (Fig. 3.14).

As we determined that Ad12 E4orf6 associates selectively with Cul2 *in vitro* we next decided to establish whether Ad12 E4orf6 has a similar propensity to bind Cul2 *in vivo* (Fig. 3.15). To do this we initially transfected HeLa cells with p-Bind alone, or pcDNA3-HA-Ad12 E4orf6. Twenty four hours post transfection we harvested cellular lysates and performed a co-immunoprecipitation assay. Lysates were incubated with an anti-HA antibody, where after antibody complexes were isolated on protein-sepharose, subjected to SDS-PAGE, and Western blotting for Cul2 (Fig. 3.15A). This experiment revealed that Ad12 E4orf6 bound to Cul2 *in vivo* (Fig. 3.15A).

To address whether Ad12 E4orf6 similarly associates with Cul2 during Ad12 infection, we used an Ad12 mutant virus that expresses FLAG-tagged E4orf6 (Blackford et al. 2010) We infected A549 cells with this mutant virus at an m.o.i of 10 p.f.u./cell, and

then harvested them in NETN lysis buffer 24 hours later. We then performed co-immunoprecipitation assays as described in Fig. 3.15A, before performing SDS-PAGE and Western blot analysis (Fig. 3.15B). Consistent with the transfection studies, we found that Cul2 associated with Ad12 E4orf6 in cells infected with FLAG-Ad12 (Fig. 3.15B). As our data suggests that Ad5 utilizes CRL5 during infection and Ad12 utilizes CRL2, we next performed co-immunoprecipitation assays to gauge the relative binding capacities of Ad5 and Ad12 E4orf6 Cul2 and Cul5 respectively during infection (Fig. 3.15C). To this end we infected A549 cells with *wt* Ad5 or FLAG-Ad12 at an m.o.i of 10 p.f.u./cell. We then harvested the cells in NETN lysis buffer and immunoprecipitated anti-FLAG or anti-Ad5 E4orf6 as described previously. Cells were then subjected to SDS-PAGE and Western blot analysis. Interestingly, these analyses revealed that Ad12 E4orf6 binds exclusively to Cul2 during infection, whereas Ad5 E4orf6 binds exclusively to Cul5 (Fig. 3.15C). Taken together, these data suggest that suggest that Ad12 E4orf6 uses CRL2 to degrade both TopBP1 and p53 during infection, whereas Ad5 E4orf6 utilizes CRL5 to degrade p53 during infection. These data also provides evidence to show that Ad12 E4orf6 can function as substrate adaptor for TopBP1 to recruit it directly to CRL2 for polyubiquitylation and degradation by the proteasome.

3.2.7. Ad12 E4orf6 inhibits ATR-dependent phosphorylation of Chk1 in response to replication stress

It has recently been determined in our laboratory that Ad12 infection results in the partial activation of the ATR-dependent DNA damage response (Blackford et al. 2008). However, despite this Ad12-infected cells fail to properly phosphorylate and activate

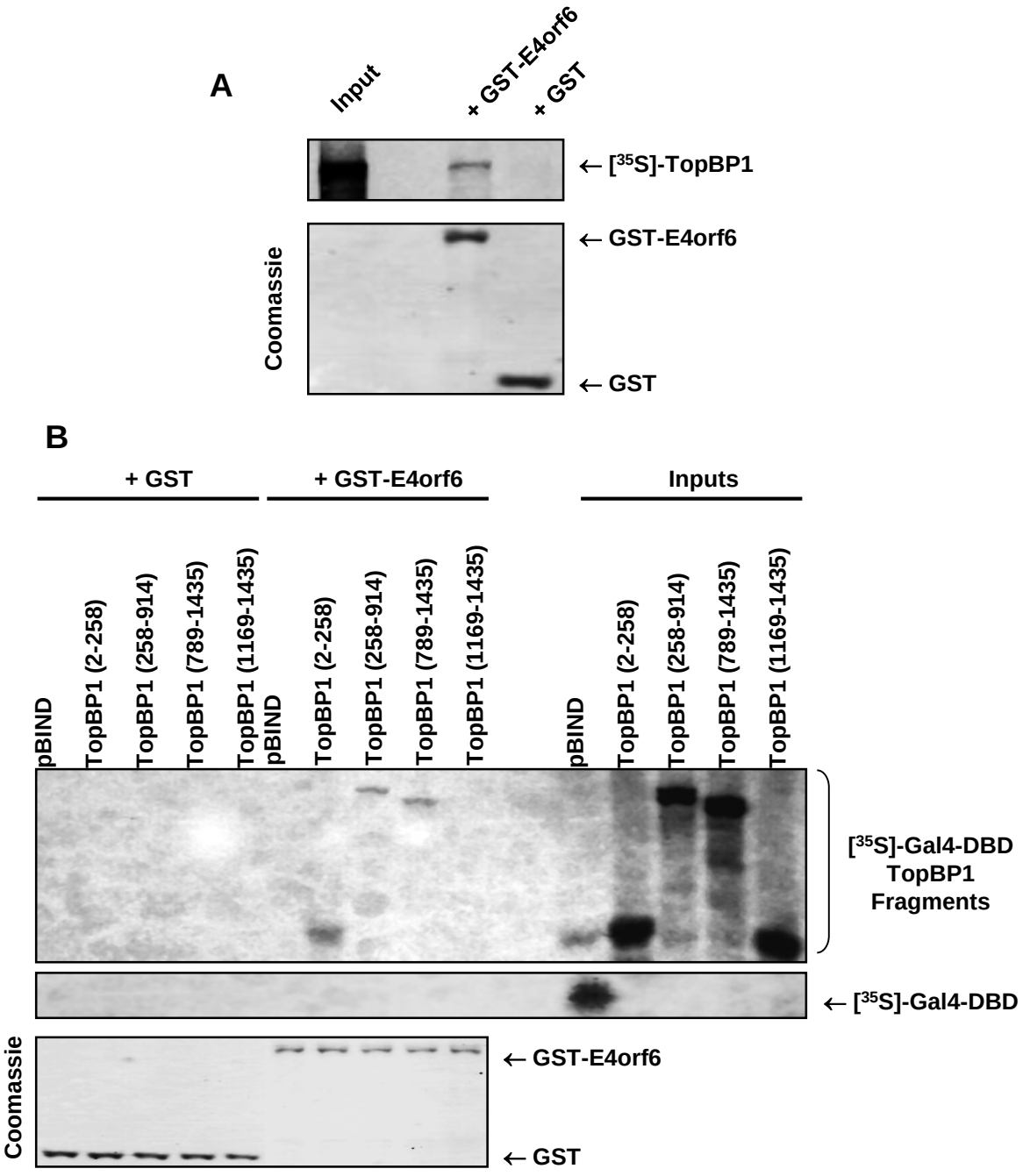

Fig. 3.13 Ad12 E4orf6 associates with TopBP1 *in vitro* through at least two binding sites. GST-Ad12 E4orf6 or GST alone was incubated with [35^S]-methionine-labeled TopBP1 (A) or [35^S]-methionine–labelled TopBP1 fragments fused to Gal4-DBD (B). Following pull-downs and SDS/PAGE, radiolabeled proteins were identified by fluorography and autoradiography

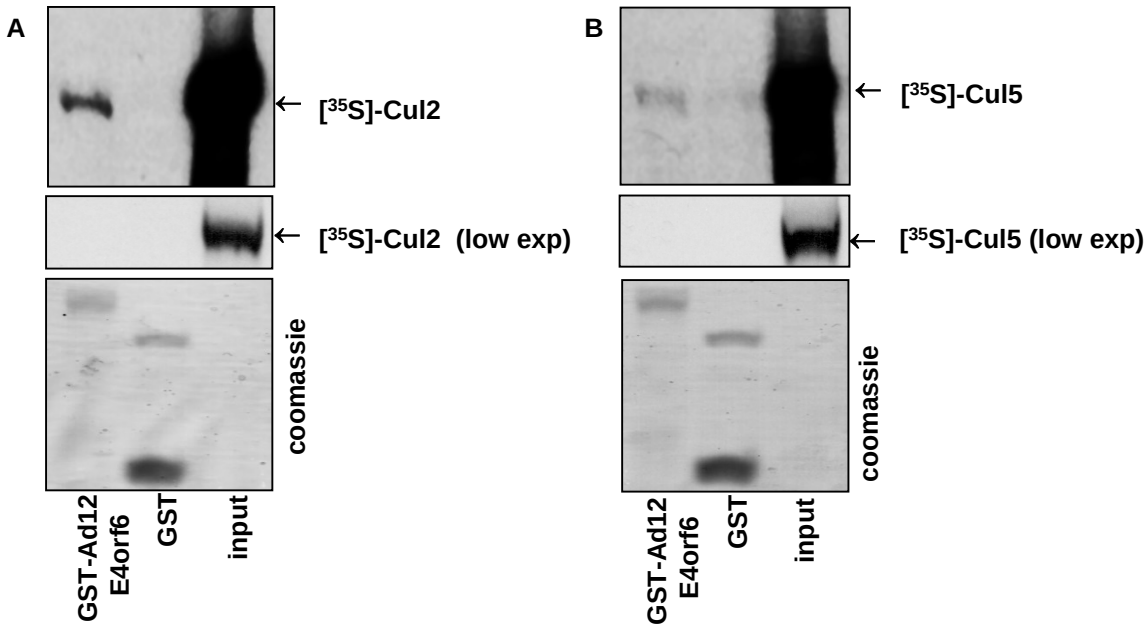

Fig. 3.14 Ad12 E4orf6 associates with Cul2 *in vitro*. GST-Ad12 E4orf6 or GST alone was incubated with [35S]-methionine–labeled Cul2 (A) or Cul5 (B). Protein complexes were isolated using glutathione–Sepharose beads, eluted with glutathione, and separated by SDS/PAGE. Radiolabeled proteins were identified by fluorography and autoradiography.

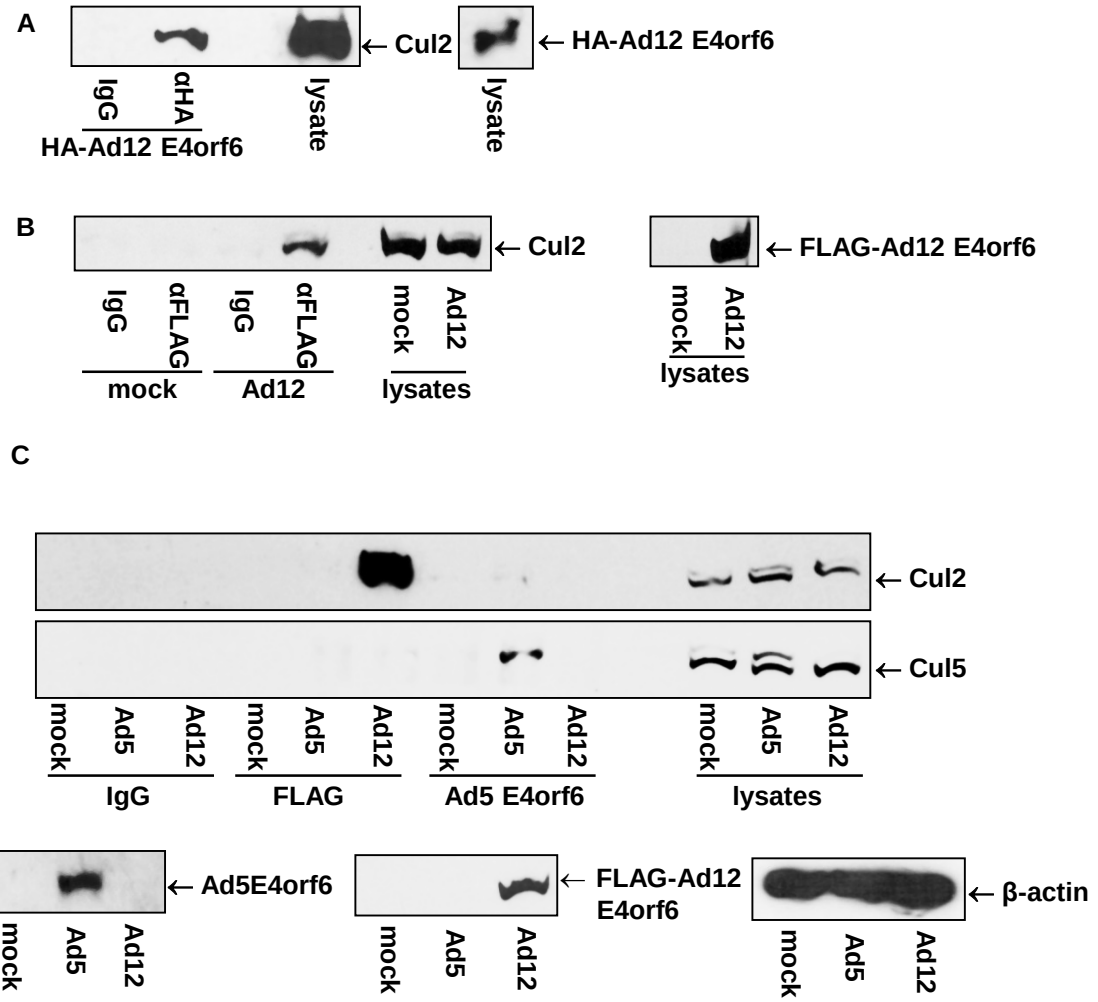

Fig. 3.15 Ad12 E4orf6 associates with Cul2 *in vivo*. HeLa cells were transfected with pcDNA3-Ad12-E4orf6 (A); A549 cells were either mock-infected or infected with FLAG-Ad12 (B). Cells were harvested and subjected to IP–Western blot analyses with the appropriate antibodies 24h later. Ad5 and Ad12 E4orf6 target different Cullins *in vivo* (C). A549 cells were mock-infected or infected with *wt* Ad5 or FLAG-Ad12. Cells were harvested 24h after infection and subjected to IP–Western blot analyses with the appropriate antibodies.

Chk1, a known downstream effector kinase of the ATR pathway (Blackford et al. 2008). As it is known that TopBP1 is specifically required to facilitate the phosphorylation of Chk1 through its ability to regulate the activation of ATR at sites of DNA damage, it might be expected that Ad12 E4orf6 inhibits the ATR-dependent phosphorylation of Chk1 during Ad12 infection by promoting the degradation of TopBP1. We hypothesised therefore that Ad12 E4orf6 could possibly inhibit ATR activation and subsequent downstream Chk1 phosphorylation in the absence of viral infection. To test this hypothesis we transfected HeLa cells with either Ad5 or Ad12 E4orf6, and then subsequently treated them with hydroxyurea (HU; a known activator of ATR) to activate ATR and promote a Chk1 phosphorylation. Cells were harvested 24 hours post-transfection, and then subjected to SDS-PAGE, followed by Western blot analysis to assess the levels of TopBP1 and phosphorylated Chk1. Consistent with our previous data, TopBP1 was degraded in cells expressing Ad12 E4orf6, but not Ad5E4orf6 (Fig. 3.16). Interestingly, we found that levels of phosphorylated Chk1 were reduced significantly in cells expressing Ad12 E4orf6 when compared to cells expressing Ad5 E4orf6 or the empty vector following exposure to HU (Fig. 3.16). These data indicate that, in the absence of other viral proteins, Ad12 E4orf6 is able to inhibit the ATR-dependent phosphorylation and activation of Chk1, most likely by promoting the specific degradation of the ATR activator, TopBP1.

3.2.8. TopBP1 degradation in Ad12-infected cells does not require its binding to ATRIP

Since it is known that TopBP1 recruitment to ATRIP is essential for full activation of ATR (Mordes et al. 2008), we next investigated whether TopBP1 association with ATRIP is essential for the degradation of TopBP1 during *wt* Ad12 infection. In this regard U2OS cells lines stably expressing siRNA-resistant *wt* ATRIP, ATRIP-Δ*top* (an

ATRIP species that does not bind TopBP1), and an empty vector control cell line were obtained from Prof. David Cortez, Vanderbilt University. In order to determine whether Ad-mediated degradation of TopBP1 necessitates TopBP1 association with ATRIP, these cells were transfected with control siRNAs and siRNAs targeting endogenous ATRIP, and then infected with *wt* Ad12 48 hours later. Protein lysates were harvested 24 hours post transfection, quantified, and then subjected to SDS-PAGE, followed by Western blot analysis to assess the levels of TopBP1 and Mrell (positive control). These analyses revealed that Ad12 promoted the degradation of TopBP1 in cells expressing either a *wt* ATRIP species, or an ATRIP species that was no longer able to bind TopBP1 (Fig. 3.17). Mre11 was similarly degraded in cells expressing *wt* ATRIP or ATRIP-Δtop. Taken together these data indicate that the Ad12-mediated degradation of TopBP1 is not dependent upon its recruitment to ATR-ATRIP complexes.

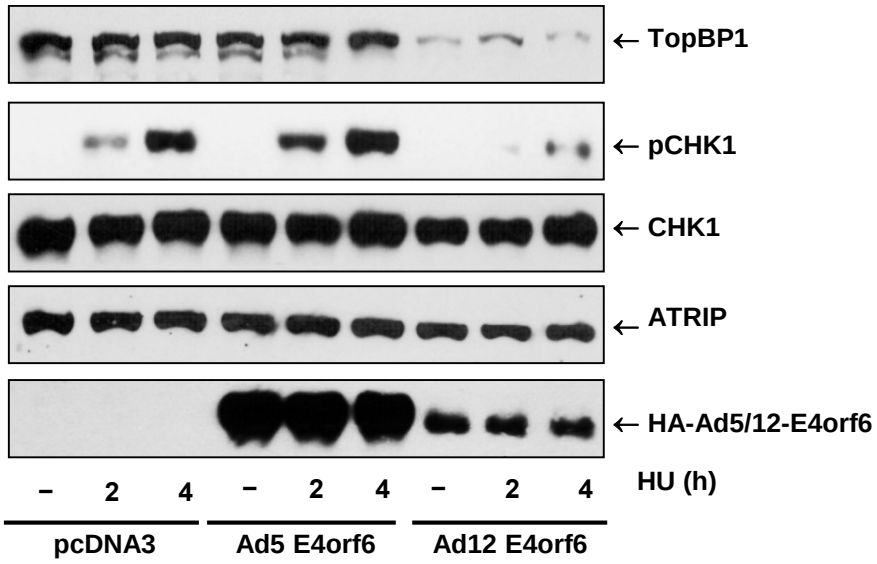

Fig. 3.16 Ad12 E4orf6 inhibits ATR signaling in response to replication stress. HeLa cells were transfected with pcDNA3-Ad12-E4orf6, pcDNA3-Ad5-E4orf6 or pcDNA3 vector alone. After 24 h, cells were mock-treated or treated with HU for the indicated times, before being harvested forWestern blotting analysis.

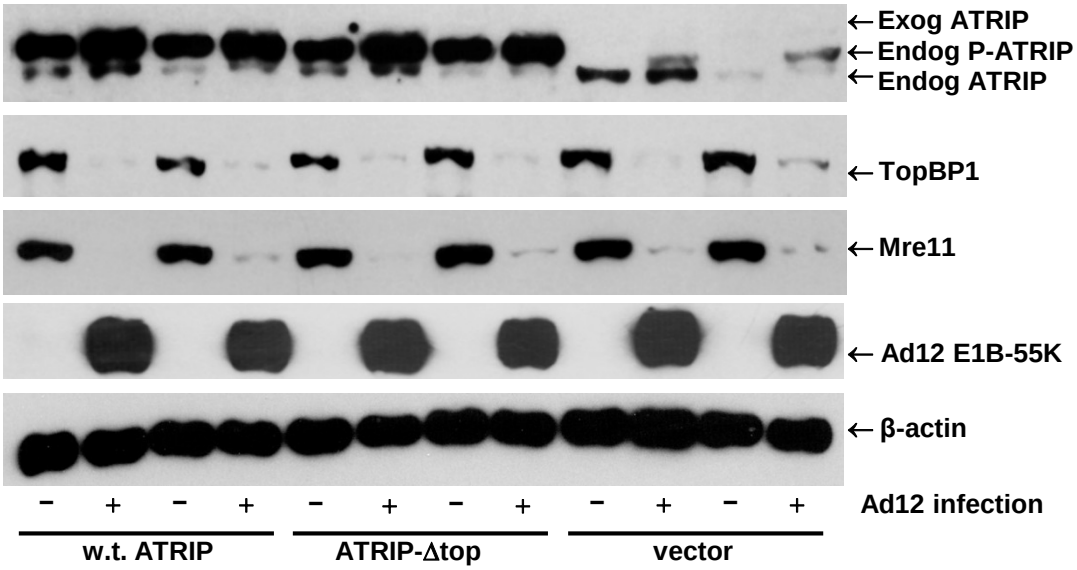

Fig. 3.17 TopBP1 degradation Ad12 infected cells is independent of its binding to ATRIP. U2OS cells stably expressing siRNA-resistant wild-type ATRIP (wt), ATRIP-Δ*top*, or an empty vector (vector) were transfected with either non-silencing (non sil) siRNA control or siRNA targeting ATRIP to deplete endogenous ATRIP. Cells were subsequently infected with Ad12 (at 10 p.f.u./cell). Cells were harvested 24h after infection and subjected to IP–Western blot analyses with the appropriate antibodies.

3.3. DISCUSSION

Both Ad12 and Ad5 are known to inhibit the ATM-dependent DDR by degrading the MRN complex in an E1B-55K/E4orf6 dependent manner (Carson et al. 2003). However, Ad5 and Ad12 only partially inhibit the ATR pathway (Blackford et al. 2008). Both Ad12 and Ad5 inhibit Chk1 phosphorylation although they utilize different strategies. E4orf3-mediated inactivation of ATR is specific to group C adenoviruses, that includes Ad5, whereas other human adenoviruses such as Ad12 lack a key isoleucine residue in their E4orf3 proteins required for MRN relocalization (Carson et al. 2009). The aim of the work presented in this chapter was to investigate the mechanism by which Ad12 inhibits the ATR-dependent activation of Chk1 during infection. In the study described here we have provided evidence to show that Ad12 targets the ATR activator TopBP1 for proteasomal degradation by a novel mechanism in order to negate Chk1 phosphorylation.

We determined Ad12, but not Ad5, promotes the specific proteasome-mediated degradation of TopBP1 during infection (Fig. 3.1-3.2). These findings are interesting because unlike Mrell, p53, and DNA Ligase IV, which are degraded by both Ad5 and Ad12, TopBP1 degradation occurs only in Ad12-infected cells, suggesting that this phenomenon maybe restricted to group A adenoviruses, which incidentally happens to be the most onocogenic. This however awaits clarification.

We strengthened these findings using immunofluorescent confocal microscopy. We showed that TopBP1 localizes at VRCs in both Ad5- and Ad12-infected cells at early times post-infection (Fig. 3.3-3.4). These findings are consistent with previous studies indicating that other components of the ATR signalling pathway: ATRIP, RPA32, Rad9, Rad17, and E1B-AP5, are all relocalized to VRCs in Ad-infected cells (Stracker

et al. 2005; Blackford et al. 2008). Confocal microscopy also revealed that TopBP1 is degraded at later times post infection in Ad12, but not Ad5 infected cells (Fig. 3.3). Furthermore we also determined that TopBP1 is not relocalized to nuclear track like structures in Ad5 or Ad12 infected cells (Fig. 3.5A-B), and neither is it relocalized to cytoplasmic aggresomes in Ad12 infected cells (Fig. 3.5C). This is interesting because in contrast to previous research where it has been reported that p53 and MRN are relocalized to nuclear track like structures and cytoplasmic aggresomes by E1B-55K and E4orf3, before being targeted for degradation by E1B-55K and E4orf6 (Evans and Hearing 2005; Liu et al. 2005), we have shown that TopBP1 is relocalized to viral replication centres in Ad infected cells prior to degradation. These data suggest that TopBP1 degradation by Ad12 is mechanistically different to the degradation of MRN and p53 by Ad5.

It is known that during Ad5 infection, the viral proteins E1B-55K and E4orf6 function in concert to recruit p53 to an E3 ligase complex containing the cellular proteins Cul5, RBX1, and elongins B and C, where it is then ubiquitylated, targeting it for subsequent proteasome mediated degradation (Querido et al. 2001; Harada et al. 2002; Blanchette et al. 2004). RBX1 and elongin C are subunits for both Cul2 and Cul5 containing CRLs. Using RNA interface we have determined that elongin C and RBX1 are both required for the Ad12 mediated degradation of both p53 and TopBP1 (Fig. 3.6A-B). Given these findings we initially assumed that Ad12 most likely utilizes the same CRL complex to degrade TopBP1 and p53 that Ad5 does during infection to facilitate the degradation of p53. However, when used RNA interferace to silence Cul5 expression we found that this had no effect on the Ad12-mediated degradation of both TopBP1 and p53 (Fig. 3.7). Interestingly, we instead determined that Ad12 hijacks CRL2 to promote degradation of both of these proteins (Fig. 3.7), providing the first evidence to show

that adenoviruses can use different CRLs to degrade host proteins. It is becoming increasingly apparent that like adenovirus, many other viruses target CRLs during infection. The HIV-1 protein Vif utilizes a Cul5 CRL to promote ubiquitylation and subsequent proteasomal degradation of APOBEC3G (Yu et al. 2003), whereas HPV16 protein E7 is associated with an enzymatically active Cul2 ubiquitin ligase complex that is required to facilitate degradation of the Retinoblastoma tumour suppressor (Huh et al. 2007). These are just a couple of examples of viruses hijacking CRLs, however it has not been shown before that two viruses from the same family can indeed use different CRLs to degrade host proteins as demonstrated in this study.

We went on to substantiate these findings by studying the neddylation patterns of Cul2 and Cul5 during Ad infection. Neddylation is the process in which a ubiquitin-like protein NEDD8 becomes conjugated to the Cullin subunit of CRLs causing them to become activated (Petroski and Deshaies 2005). Our data indicate that during Ad5 infection, Cul5 becomes increasingly neddylated whereas Cul2 is progressively deneddylated (Fig. 3.8). In contrast, Cul2 becomes increasingly neddylated in Ad12-infected cells whereas Cul5 neddylation is not altered appreciably (Fig. 3.8), further suggesting that adenoviruses also differentially regulate Cullin activity during infection. It is unclear as to how adenovirus regulates Cullin activation and it would therefore be interesting to determine this, especially as it has recently been reported that BPLF1, the Epstein-Barr-virus-encoded member of the large tegument proteins of herpesviruses protease family, is a deneddylase that regulates virus production by modulating the activity of CRLs (Gastaldello et al. 2010). BPLF1 hydrolyses NEDD8 conjugates *in vitro*, acts as a deneddylase in vivo, and binds to Cullins and stabilizes CRL substrates (Gastaldello et al. 2010).

Previous studies have determined that E4orf6 protein is responsible for recruiting the CRL to the substrate that is to be targeted for degradation by adenovirus, and it is understood that this recruitment occurs through its motifs termed BC boxes through which it binds to the scaffold proteins elongins B and C (Blanchette et al. 2004; Cheng et al. 2007). Given that we had already shown that Ad5 and Ad12 target different CRL during infection, we hypothesized that Ad5 and Ad12 E4orf6 proteins might selectively bind to Cul5 and Cul2 respectively. Indeed we confirmed our hypothesis by showing that Ad5 E4orf6 binds Cul5 and Ad12 E4orf6 binds Cul2 *in vitro* (Fig. 3.14A-B). We then went onto show through co-immunoprecipitation studies that Ad12 E4orf6 selectively associates with Cul2 *in vivo*, and that Ad5 E4orf6 selectively associates with Cul5 *in vivo* (Fig. 3.15C). This result was surprising given that Ad5 and Ad12 E4orf6 proteins show 50% identity and 74% similarity, and it would therefore be useful in the future to clarify what determines the Cullin binding selectivity of Ad5 and Ad12 E4orf6 proteins, as this may shed light on how CRLs are regulated in both uninfected and infected cells.

It is known that the early adenoviral proteins E1B-55K and E4orf6 function in concert to promote the proteasomal degradation of a number of proteins involved in the DNA damage response pathways. These include p53, DNA ligase IV, Mre11, and BLM (Querido et al. 2001; Stracker et al. 2002; Liu et al. 2005; Baker et al. 2007; Orazio et al. 2011). Given our findings indicating that Ad12 promotes the degradation of TopBP1, we hypothesized that these two proteins would also play a crucial role in degrading TopBP1. However when we infected cells with a mutant Ad12 E1B deletion virus, that does not express E1B-55K, we found that TopBP1 was still degraded unlike Mre11 which was stabilized. These data suggested that E1B-55K is not required for TopBP1 degradation (Fig. 3.9). Indeed we went onto provide evidence suggesting that

Ad12 E4orf6 alone can promote the degradation of TopBP1 (Fig. 3.10). We also went onto show that the Ad12 E4orf6 mediated degradation of TopBP1 was proteasome dependent and that it also required a CRL2 (Fig. 3.11-3.12). These data suggest provide substantial evidence to indicate that E4orf6 is solely required to promote TopBP1 degradation during Ad12 infection.

To expand upon these findings, we wanted to confirm that Ad12 E4orf6 also serves as a substrate adaptor and binds to TopBP1 directly. We therefore carried out GST pull down assays with Ad12 E4orf6 and radiolabelled full length TopBP1 as well as TopBP1 fragments. This study confirmed that Ad12 E4orf6 binds directly to TopBP1 through extensive binding sites (Fig. 3.13A-B). We additionally went onto confirm by GST pull down and co-immunoprecipitation assays that Ad12 E4orf6 interacts with Cul2 both *in vitro* and *in vivo* (Fig. 3.14-15). These studies clearly indicated that Ad12 E4orf6 is functionally distinct from Ad5 E4orf6 in its abilities to direct protein ubiquitylation, and also that it does not require any other virally encoded proteins to degrade TopBP1. It is also relevant to note that Ad12 E4orf6 is the only viral protein currently known to promote degradation of TopBP1, however it is not the only viral protein to interact with it as it has been shown that HPV16 E2 interacts functionally with TopBP1 (Boner et al. 2002), where TopBP1 has been proposed to act as the chromatin receptor for HPV16 E2 during HPV genome segregation at mitosis (Donaldson et al. 2007). It is yet to be determined if the E2 interaction with TopBP1 affects ATR damage signalling pathways.

In this study we also demonstrated that degradation of TopBP1 in Ad12-infected cells does not require its binding to ATRIP. TopBP1 binds to the ATR binding partner ATRIP, an interaction that is essential for ATR activation. Cortez *et al* constructed a stable cell line that expresses siRNA-resistant *wt* ATRIP and siRNA resistant ATRIP-

top, which is a mutant ATRIP that fails to bind TopBP1, and an empty vector control (Mordes et al. 2008). We depleted these cells of endogenous ATRIP by RNA interference, and infected them with *wt* Ad12 and found that TopBP1 is still degraded after infection, showing that TopBP1 degradation is independent of its binding to ATRIP (Fig. 3.17). These data suggest that Ad effectively negates TopBP1-dependent activation of ATR/ATRIP complexes by promoting TopBP1 degradation and circumventing its recruitment to ATR/ATRIP.

It has been shown that TopBP1 directly activates ATR by stimulating its kinase activity via interactions with both ATR and ATRIP (Kumagai et al. 2006; Mordes and Cortez 2008). Furthermore, TopBP1 has been shown to be essential for certain ATR-dependent signalling events, including Chk1 and NBS1 phosphorylation (Kumagai et al. 2006). Since we have previously observed that cellular infection with both adenovirus serotypes 5 and 12 results in a failure to activate Chk1, it is likely that the functional consequences of Ad12 E4orf6 targeted degradation of TopBP1 is to block the activation of Chk1. Indeed, our data in which expressing E4orf6 in cells in the absence of any other viral proteins resulted in defective HU-induced phosphorylation of Chk1 would support this hypothesis (Fig. 3.16). The underlying reason for viral inactivation of Chk1 remains unclear but it is likely that some aspect of Chk1 function, be it its ability to regulate host cell or viral DNA replication or cell cycle checkpoint activation, is not conducive for completion of the viral life cycle.

Adenovirus is not the only virus in which ATR activation is inhibited during infection. It had initially been suggested that HSV-1 infection disrupts the ATR pathway by a mechanism that prevents the recruitment of repair factors, spatially uncouples ATRIP from ATR and sequesters ATRIP and endogenous hyperphosphorylated RPA within

virus-induced nuclear domains containing molecular chaperones and components of the ubiquitin proteasome (Wilkinson and Weller 2006). However, a more detailed investigation conducted by the same lab has now reported that the ATR-ATRIP interaction remains intact during infection, though it is functionally inactive (Mohni et al. 2010). Furthermore, Immunofluorescent confocal microscope studies have revealed that ATRIP and RPA are recruited, along with ICP8, to prereplicative, stage II microfoci during infection, although, interestingly, RPA that is phosphorylated, and hence activated, by ATR in response to HU-treatment, is excluded from these sites (Mohni et al. 2010). It is interesting to note that a study has shown that ATR signalling has no effect on Ad5 DNA replication, and it therefore remains unclear as to why ATR activation is detrimental to viral replication (Lakdawala et al. 2008). ATM and ATR phosphorylate a number of proteins required mainly for checkpoint activation, apoptosis, and DNA repair, however they also target proteins involved in RNA metabolism which includes splicing (Matsuoka et al. 2007). During infection, adenovirus utilizes the host cell transcription and splicing machinery to produce viral proteins, and interestingly it has been reported that ATM can inhibit protein synthesis (Braunstein et al. 2009). It is however possible that adenovirus has evolved to prevent activation ATM and ATR which could technically inhibit late viral protein expression, and limit the production of viral progeny.

In conclusion, we have described a mechanism by which Ad12 degrades TopBP1 during infection in order to negate Chk1 phosphorylation and activation. We have shown that Ad12 E4orf6 binds directly to TopBP1, and also recruits CRL2, where TopBP1 is then targeted for proteasomal degradation. Thus, we have demonstrated that Ad12 E4orf6 has a novel role as substrate adaptor and can function independent of Ad12 E1B-55K, which may have important ramifications for understanding the role of

E4orf6 during viral infection and the processes of Ad-mediated cellular transformation and oncogenesis. In this study we have also provided evidence to show that Ad5 and Ad12 target different CRLs during infection, further highlighting that closely related human adenovirus species have evolved different strategies to counteract host cell DNA damage signalling pathways activated during infection. It is becoming increasingly clear that viruses have evolved a number of mechanisms in order to negate and/or selectively activate the genotoxic stress response pathways in order to facilitate the replication of their genomes efficiently in host cells. It is important to investigate the mechanisms by which viruses modulate these pathways in order to gain a more complete understanding of how these pathways function at the molecular level.

CHAPTER 4

REGULATION OF THE ATR SIGNALLING PATHWAY BY ADENOVIRUS E4ORF3

4.1. INTRODUCTION

It is known that adenovirus promotes the degradation of a number of proteins involved in DDR pathways during infection. The primary mechanism by which adenovirus accomplishes this involves adenovirus oncoproteins E1B-55K and E4orf6. E1B-55K acts as the substrate adaptor to recruit the target proteins to a Cullin-containing E3 ligase complex that has been hijacked by E4orf6, whereupon the protein is polyubiquitylated and targeted for proteasomal degradation. As described in chapter 3, it is now our understanding that E4orf6 alone can promote the degradation of the ATR activator, TopBP1, by directly interacting with it and recruiting it to a CRL2 complex, independent of E1B-55K, in Ad12-infected cells (Blackford et al. 2010). Interestingly, work from our laboratory has also shown that E4orf3 can degrade TIF1γ in Ad5- and Ad12- infected cells, independently of E1B-55K/E4orf6 and Cullin-containing E3 ligases (Forrester et al. 2012).

Research from our laboratory has shown that Ad5 and Ad12 infection inhibits Chk1 phosphorylation in response to genotoxic stress (Blackford et al. 2008). Ad5 utilizes the Ad oncoprotein E4orf3, which relocalizes and immobilises MRN subunits prior to degradation by E1B-55K/E4orf6 to inhibit Chk1 phosphorylation, whilst Ad12 utilizes E4orf6 to promote the degradation of TopBP1 (Blackford et al. 2010). The mechanism by which ATR contacts and phosphorylates Chk1 is unclear. TopBP1 is known however, to be essential for certain ATR-dependent signalling events, including Chk1 and NBS1 phosphorylation (Kumagai et al. 2006). TopBP1 stimulates ATR kinase activity via interactions with both ATR and ATRIP (Kumagai et al. 2006; Mordes and Cortez 2008). Another protein that has been shown to play a crucial role in the phosphorylation of Chk1 is the mediator protein, Claspin. Claspin was initially

discovered in Xenopus egg extracts as a Chk1-interacting protein; immunodepletion of Claspin from egg extracts inhibits the ATR-mediated phosphorylation of Chk1 (Kumagai and Dunphy 2000). Furthermore, Claspin has been found to contain a 57 amino acid domain that is the Chk1-binding domain, and phosphorylation of Claspin at two sites within this domain is responsible for recruiting Chk1 to Claspin in response to DNA replication stress or DNA damage (Chini and Chen 2003). Claspin has also been shown to interact with Timeless, a protein that has been shown to play a role in Chk1 phosphorylation (Gotter et al. 2007). Timeless has been shown to interact with Chk1, ATR and ATRIP in response to DNA damaging agents HU and UV light (Unsal-Kacmaz et al. 2005). Furthermore, it has also been shown that depletion of Timeless by siRNA reduces phosphorylation of Chk1 in HU-treated cells (Unsal-Kacmaz et al. 2005). Akin to the ATR-ATRIP interaction, Timeless is found associated with its cognate-interacting partner, Timeless- Interacting protein, Tipin (Gotter 2003; Chou and Elledge 2006). RPA2 has been shown to interact with the Timeless-Tipin complex in response to DNA damage, and functions to stabilize Timeless-Tipin complexes and Claspin onto RPA-coated ssDNA, which in turn promotes the Claspin-mediated phosphorylation of Chk1 (Unsal-Kacmaz et al. 2007; Kemp et al. 2010).

Thus, TopBP1, Claspin, Timeless and Tipin play an essential role in the ATR-dependent phosphorylation of Chk1. As adenovirus oncoproteins often target multiple components in the same signalling pathway, other components of the ATR signalling pathway were considered viable targets for adenovirus. Therefore, the aim of the study described in this chapter was to investigate if adenovirus targets other known proteins in the ATR pathway, to specifically prevent Chk1 activation.

4.2. RESULTS

4.2.1. Timeless and Tipin are degraded during Ad12, but not Ad5 infection.

As described in chapter 3, Ad12 targets the ATR activator, TopBP1 for proteasomal degradation in order to inhibit ATR activation during infection (Blackford et al. 2010). We hypothesized therefore that adenovirus might also target other proteins involved in the ATR signalling pathway during infection. To investigate this possibility we mock-infected, or infected, HeLa cells with *wt* Ad5 or *wt* Ad12 at an m.o.i of 10 p.f.u./cell and then harvested cells at intervals over a 48 hour period. The protein lysates were then quantified by Bradford assay and subjected to SDS-PAGE. Following transfer to nitrocellulose, levels of proteins involved in Chk1 phosphorylation pathway were assessed by Western blotting (Fig. 4.1).

In accordance with previous research we observed that both Mre11 and p53 were degraded in adenovirus-infected cells (Fig. 4.1). Furthermore, and in agreement with the data presented in chapter 3, we confirmed that TopBP1 is degraded in Ad12-infected cells (Fig. 4.1). Interestingly, we found that Timeless, and Tipin levels were decreased 48 hours post Ad12-infection, but remained unaffected in Ad5-infected cells (Fig. 4.1). The reduction in Tipin and Timeless levels was observed later during infection, relative to the Ad12-mediated degradation of TopBP1, but paralleled the Ad12-mediated degradation of p53 (Fig. 4.1). However, Claspin levels remained unaffected in both Ad5- and Ad12- infected cells (Fig. 4.1). These data suggest that like TopBP1, the ATR activator proteins Timeless and Tipin might be targeted by Ad12 for degradation during infection.

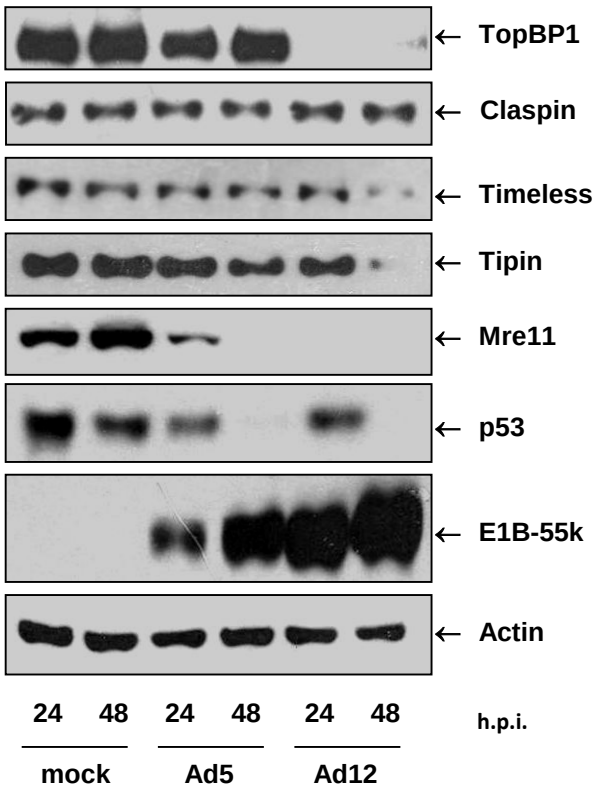

Fig. 4.1. Effect of Ad5 and Ad12 infection on the expression levels of proteins involved in Chk1 phosphorylation. HeLa cells were mock-infected or infected with the indicated viruses at an m.o.i of 10 p.f.u./cell. Cells were harvested and prepared for Western blotting at the indicated time points using the appropriate antibodies (h.p.i., hours post-infection).

4.2.2. Ad12 utilizes a Cul2-containing ubiquitin ligase complex to degrade the Timeless-Tipin complex.

During Ad12 infection, it is postulated that the oncoprotein E4orf6 recruits E1B-55K binding proteins, and TopBP1 to an E3 ligase complex containing the cellular proteins Cul2, RBX1, and elongins B and C, where they are ubiquitylated, and targeted for proteasome-mediated degradation (Blackford et al. 2010). To determine if Ad12 utilizes CRL2 to degrade Timeless and Tipin we transfected specific Cul2 or Cul5 siRNA oligonucelotides into HeLa cells in order to specifically knockdown the expression of Cul2 or Cul5 proteins. Alternatively, cells were transfected with non-silencing siRNA to serve as a control. Cells were then infected with *wt* Ad5 or *wt* Ad12 at an m.o.i of 10 p.f.u./cell 48 hours post-transfection, and harvested at the appropriate times post-infection. Protein lysates were then quantified and subjected to SDS-PAGE; the protein levels of TopBP1, Cul2, and Cul5 were assessed by Western blotting (Fig. 4.2). Initial Western blot analyses indicated that the knockdown was successful, and that Cul2 and Cul5 expression was reduced significantly following treatment with the appropriate siRNA oligonucleotides (Fig. 4.2). In agreement with previous research, as well as data presented in chapter 3, we observed that p53 was not targeted for degradation in Ad5-infected cells depleted of Cul5, or in Ad12-infected cells depleted of Cul2 (Fig. 4.2). Consistent with data presented in Chapter 3, TopBP1 was stabilized in Ad12-infected cells depleted of Cul2 (Fig. 4.2). In accordance with our earlier findings, Timeless and Tipin were not degraded in Ad5-infected cells, but were degraded in Ad12-infected cells depleted of Cul5 or treated with control siRNA (Fig. 4.2). Interestingly however, and akin to p53, Timeless and Tipin were not degraded following Ad12 infection of HeLa cells that were depleted of Cul2 (Fig. 4.2). Taken together these data suggest that Ad12 utilizes a CRL2 complex to facilitate the

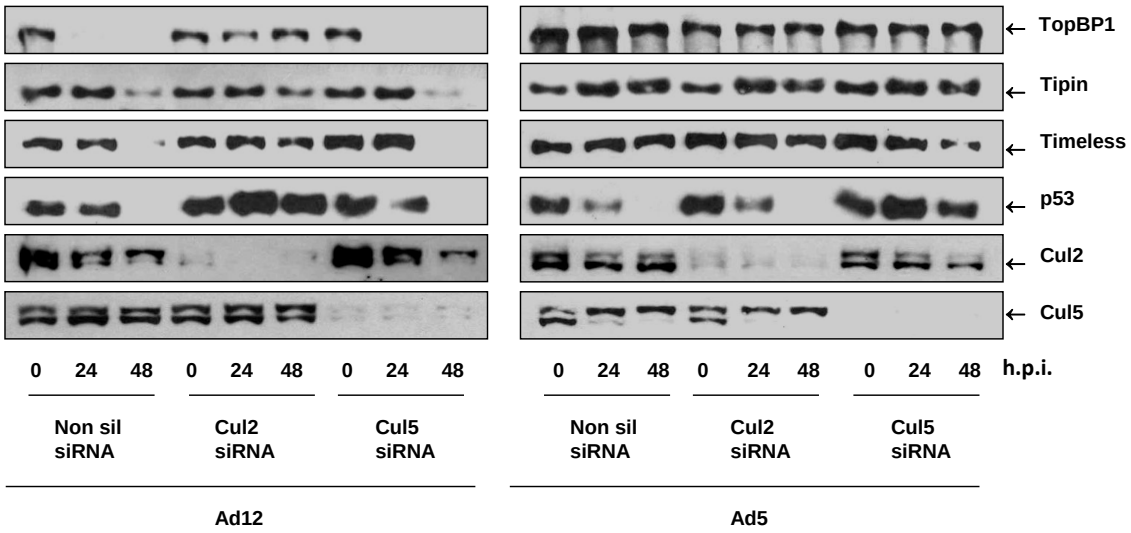

Fig. 4.2. Cul2 is required for the Ad12-mediated degradation of Timeless and Tipin. HeLa cells were transfected with the indicated siRNAs, before being mock-infected or infected with Ad5 or Ad12 at an m.o.i of 10 p.f.u./cell 48hrs later. Cells were harvested and prepared for Western blotting at the indicated time points using the appropriate antibodies (h.p.i. hours post infection).

degradation of Timeless and Tipin during infection.

4.2.3. Timeless and Tipin are degraded independently of E1B-55K.

Given the ever increasing complexity of Ad-mediated degradation of cellular substrates, we wanted to establish the relative contributions of Ad E1B-55K, E1B-55K-E4orf6, E4orf6 and E4orf3 in the degradation of Timeless and Tipin. We initially investigated therefore, whether E1B-55K plays a role in the degradation of Timeless and Tipin. To this end we infected HeLa cells with *wt* Ad5 and an Ad5 E1B-55K deletion mutant, Ad5 *dl*1520, and *wt* Ad12 and the Ad12 E1B-55K deletion mutants, Ad12 *hr*703 and Ad12 *dl*620 at an m.o.i of 10 p.f.u./cell. We then harvested the cells at appropriate intervals over a 72 hour period. Protein lysates were then quantified, subjected to SDS-PAGE and protein levels assessed by Western blot (Fig. 4.3).

Initial Western blot analysis confirmed that cells were infected by either *wt* virus as confirmed by the presence of both E1B-55K and E1A, or mutant virus as confirmed by the absence of E1B-55K, and the presence of E1A (Fig. 4.3). In agreement with previous research, Mre11 was degraded in cells infected with either *wt* Ad5 or *wt* Ad12, but remained stable in cells infected with viruses lacking E1B-55K (Fig. 4.3). In agreement with data presented in chapter 3, TopBP1 was degraded in *wt* Ad12-infected cells and in cells infected with mutant viruses Ad12 *dl*620, and *hr*703, but not in *wt* Ad5- or Ad5 *dl*1520-infected cells (Fig. 4.3).

Consistent with the Ad5 data presented in Fig. 4.1, neither Timeless nor Tipin were degraded in either *wt* Ad5-infected cells or cells infected with Ad5 *dl*1520 (Fig. 4.3). Significantly, Timeless and Tipin were degraded following infection with either *wt*

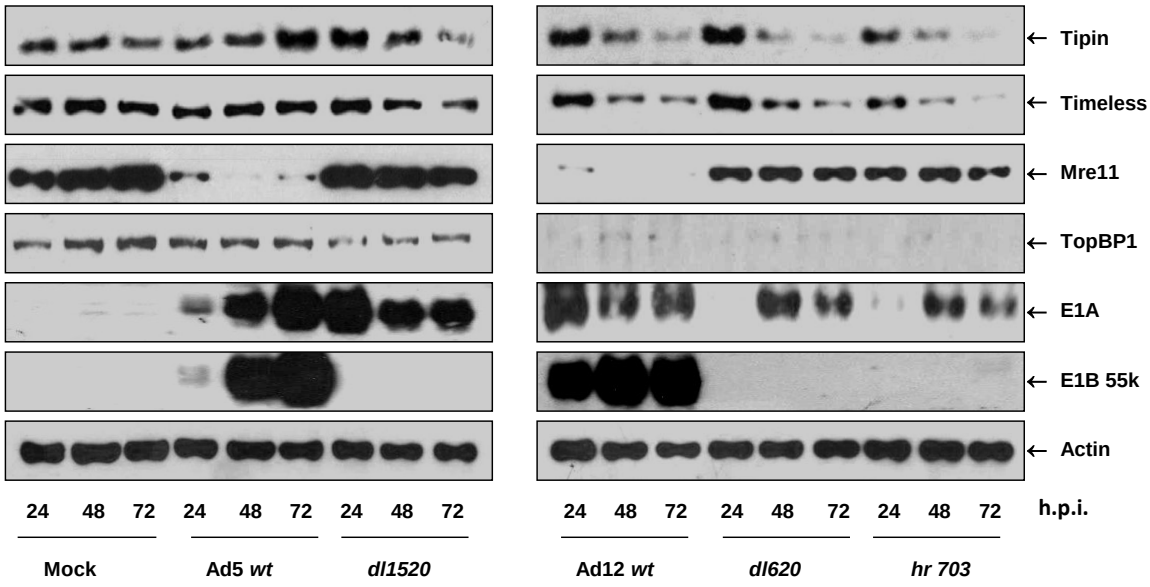

Fig. 4.3. Timeless and Tipin degradation by Ad12 is independent of E1B-55K. HeLa cells were mock-infected or infected with *wt* Ad5 or Ad5 *E1B* deletion mutant, Ad5 *dl*1520, or with *wt* Ad12 or Ad12 deletion mutants Ad12 *hr*703 and Ad12 *dl*620 at an m.o.i of 10 p.f.u./cell. Cells were harvested and prepared for Western blotting at the indicated times. Mre11 was used as a positive control. (h.p.i., hours post infection).

Ad12, or Ad12 E1B-55K deletion mutants (Fig. 4.3). These data suggest that the Ad12-mediated degradation of Timeless and Tipin occurs independently of E1B-55K.

4.2.4. Ad12 E4orf3 alone is sufficient to promote the degradation of Timeless, Tipin and TopBP1.

Given that Ad12 does not utilize E1B-55K to degrade Timeless and Tipin, we next considered the possibility that either Ad12 E4orf3 or Ad12 E4orf6 could promote the degradation of Timeless and Tipin. We therefore transfected pcDNA3 plasmid alone, or pcDNA3 plasmids expressing Ad12 E4orf6, Ad5 E4orf6, Ad12 E4orf3, Ad5 E4orf3 into HeLa cells. Cells were then harvested 24 hours post transfection and protein lysates were then quantified and subjected to SDS-PAGE; the protein levels of TopBP1, p53, Timeless, and Tipin were then assessed by Western blotting (Fig. 4.4).

As expected the levels of p53 were not affected by the expression of any of the Ad oncoproteins expressed in this experiment (Fig. 4.4). Consistent with previous findings, the levels of TopBP1 decreased appreciably in cells expressing Ad12 E4orf6 (Fig. 4.4). Interestingly, TopBP1 levels also decreased appreciably in cells expressing Ad12 E4orf3 (Fig. 4.4). Significantly however, Timeless and Tipin protein levels were only reduced following Ad12 E4orf3 expression; Ad12 E4orf6 expression did not affect Timeless and Tipin protein levels (Fig. 4.4). These data suggest that Ad12 E4orf3 alone, is sufficient to promote the Ad12-mediated degradation of Timeless, Tipin, and TopBP1.

4.2.5. Ad12 E4orf3 mediated degradation of Timeless, Tipin, and TopBP1 is proteasome dependent.

Given that Ad12 E4orf6 promotes the proteasome-dependent degradation of TopBP1

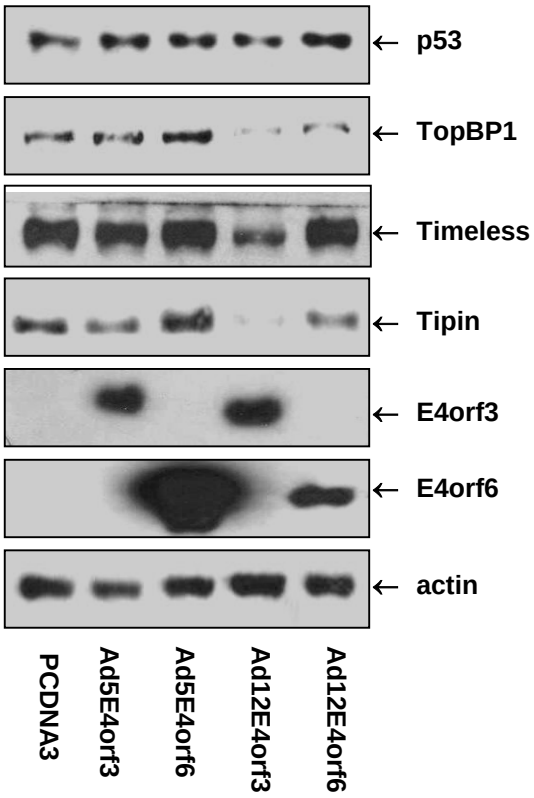

Fig. 4.4. Ad12 E4orf3 is necessary and sufficient for TopBP1, Timeless and Tipin degradation. HeLa cells were transfected with pcDNA3-Ad12-E4orf6, pcDNA3-Ad5-E4orf6, pCMV-Ad5-E4orf3, pCMV-Ad12-E4orf3 or pcDNA3 vector alone and harvested for Western blotting 24 h later.

(Blackford et al. 2010), and E4orf3 promotes the proteasome dependent degradation of TIF1γ (Forrester et al. 2012), we hypothesized that the Ad12 E4orf3-mediated decrease in levels of TopBP1, Timeless, and Tipin was proteasome-dependent. To test this hypothesis we transfected HeLa cells with pcDNA3 alone or pcDNA3 expressing Ad12 E4orf3. Following transfection cells were treated with 10μM proteasome inhibitor, MG132 or DMSO (control), and harvested 24 hours post-transfection. Following protein quantification, lysates were subjected to SDS-PAGE and protein levels of TopBP1 were assessed by Western blotting, with cyclin B1 being used as a positive control for MG132 activity (Fig. 4.5).

Consistent with the idea that E4orf3 promotes the proteasome-mediated degradation of these proteins, treatment with MG132 inhibits the E4orf3-mediated degradation of TopBP1, Timeless, and Tipin (Fig. 4.5). These data suggest that the Ad12 E4orf3-mediated reduction in these protein levels are proteasome-dependent.

4.2.6. E4orf3 does not relocalize Timeless and Tipin to nuclear tracks.

One of the most recognised functions of E4orf3 is that it recruits both viral and cellular proteins to elongated nuclear track-like structures during infection. E1B-55K, PML, TIF1α and TIF1γ all colocalize with E4orf3 in nuclear tracks (Carvalho et al. 1995; Doucas et al. 1996; Leppard and Everett 1999; Yondola and Hearing 2007). We therefore hypothesized that Timeless and Tipin may also be recruited to these nuclear tracks. To this end we transfected HeLa cells with either pcDNA3 expressing Ad5 HA-tagged E4orf3, or pcDNA3 expressing Ad12 HA-tagged E4orf3. Cells were then seeded onto glass slides. The cells were then harvested at the appropriate time by treatment with a pre-extraction buffer and fixation in 4% (w/v) paraformaldehyde. Fixed cells were then co-stained for Tipin and E4orf3, Timeless and E4orf3, and TIF1γ

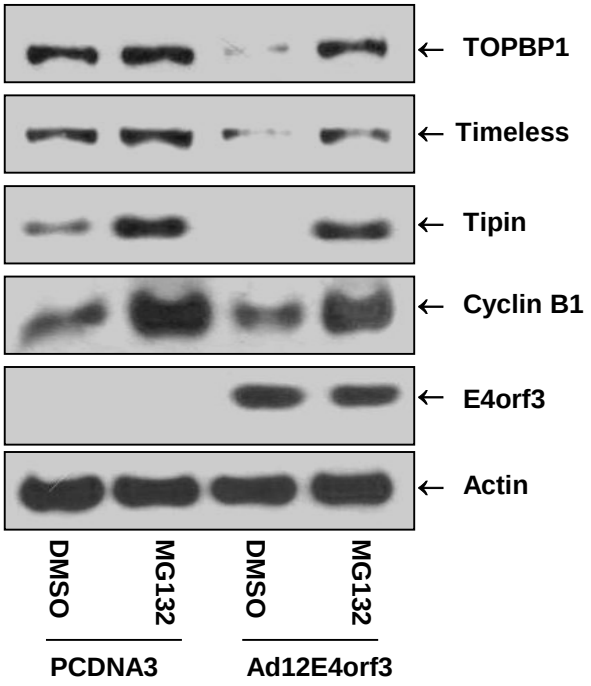

Fig. 4.5. The Ad12 E4orf3-mediated degradation of TopBP1, Timeless and Tipin is proteasome-dependent. HeLa cells were transfected with pCMV-Ad12-E4orf3 or pcDNA3 vector alone in the presence or absence of 10 μM MG132, added 6h after transfection. Cells were harvested for Western blotting 24h later. Cyclin B1 levels were used as a positive control for proteasome inhibition.

and E4orf3, using the appropriate antibodies. Cells were mounted in DAPI to stain DNA and visualised using a confocal microscope.

Consistent with the Western blot data, cells that were transfected with Ad12 HA-E4orf3 showed decreased levels of both Timeless and Tipin when compared to cells that were not transfected (*cf* Fig. 4.6B and D). In contrast, there were no observable differences in the levels of both Timeless and Tipin in cells transfected with Ad5 E4orf3 when compared to cells that were not expressing Ad5 E4orf3 (*cf* Fig. 4.6A and C). These data strengthen our previous findings demonstrating that Ad12 E4orf3, but not Ad5 E4orf3 is able to promote degradation of both Timeless and Tipin. Consistent with previous studies from our laboratory (Forrester et al. 2012) this experiment also revealed that both Ad5 and Ad12 HA-E4orf3 were able to recruit TIF1γ to nuclear tracks (Fig. 4.7A and Fig. 4.8A). Interestingly, some Ad5 E4orf3 expressing cells displayed a cytoplasmic staining pattern reminiscent of aggresomes; TIF1γ colocalized with Ad5 E4orf3 within these sites (Fig. 4.7B). Consistent with the view that Ad5 Eorf3 does not promote the degradation of Timeless or Tipin, neither Timeless nor Tipin were recruited to cytoplasmic aggresomes with E4orf3 (Fig. 4.7, C and D). Further co-staining revealed that Timeless and Tipin do not colocalize with Ad12 E4orf3 at nuclear tracks or cytoplasmic aggresomes (Fig. 4.8).

4.2.7. Ad12 E4orf3 inhibits the ATR-dependent phosphorylation of Chk1 in response to replication stress

Our laboratory has shown previously that Ad12 infection results in the partial activation of the ATR-dependent DDR by promoting the phosphorylation of Rad9 and RPA32, however, despite this, ATR is not able to phosphorylate and activate Chk1 in Ad12-infected cells (Blackford et al. 2008). In chapter 3 we described how E4orf6 inhibits

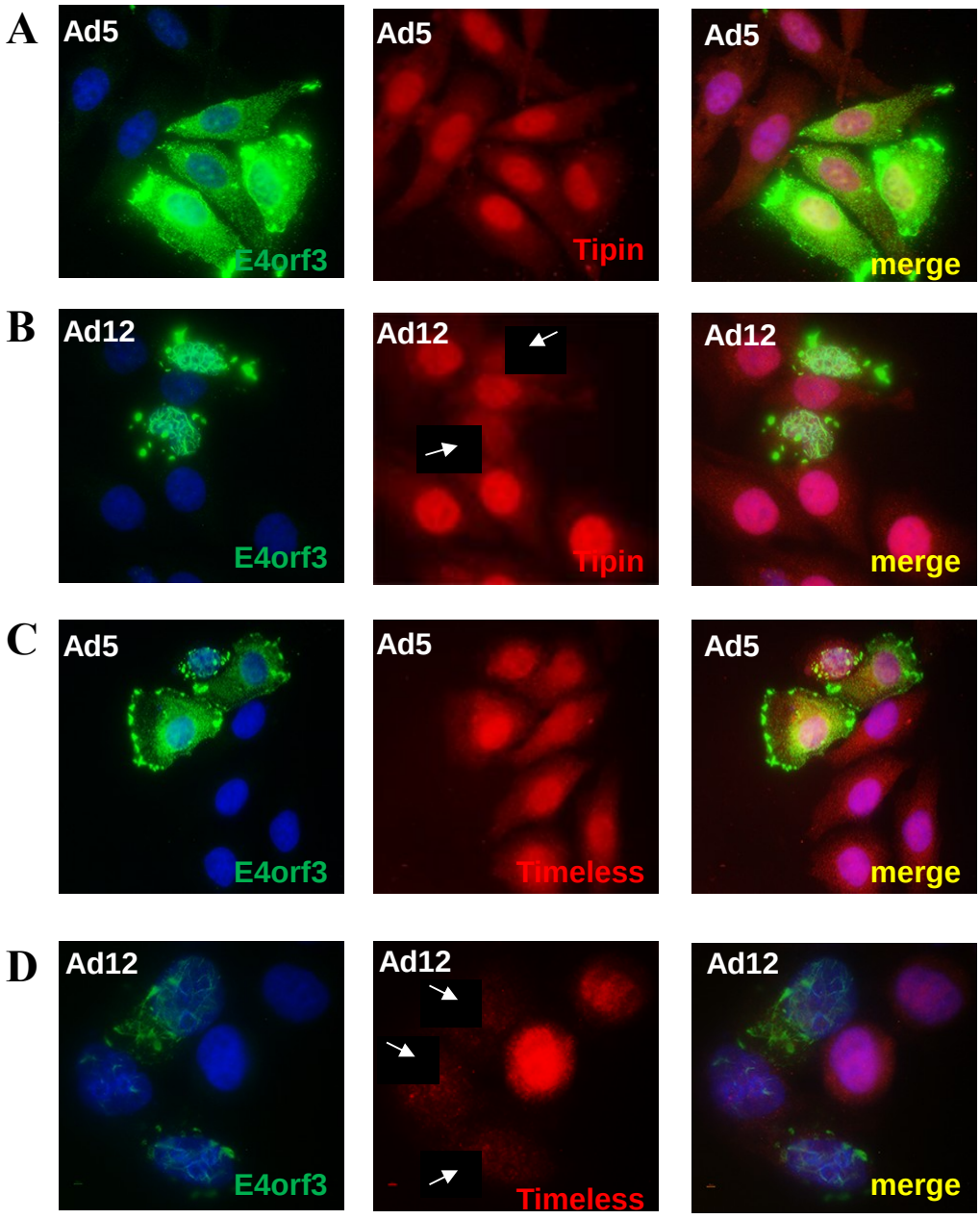

Fig. 4.6. Ad12 E4orf3 is required for Ad12-mediated Timeless and Tipin degradation. HeLa cells were transfected with Ad5 or Ad12 HA-tagged E4orf3 plasmid DNA and harvested after 24 hours. Cells were then treated with a pre-extraction buffer before being fixed in 4% (w/v) paraformaldehyde and stained for E4orf3 (anti HA green), Tipin (A and B, red), Timeless (C and D, red) and DAPI (blue). Images were visualised by confocal microscopy and colocalization of proteins is shown in the right-hand merged column. Arrows indicate transfected cells, showing reduction in Timeless and Tipin staining.

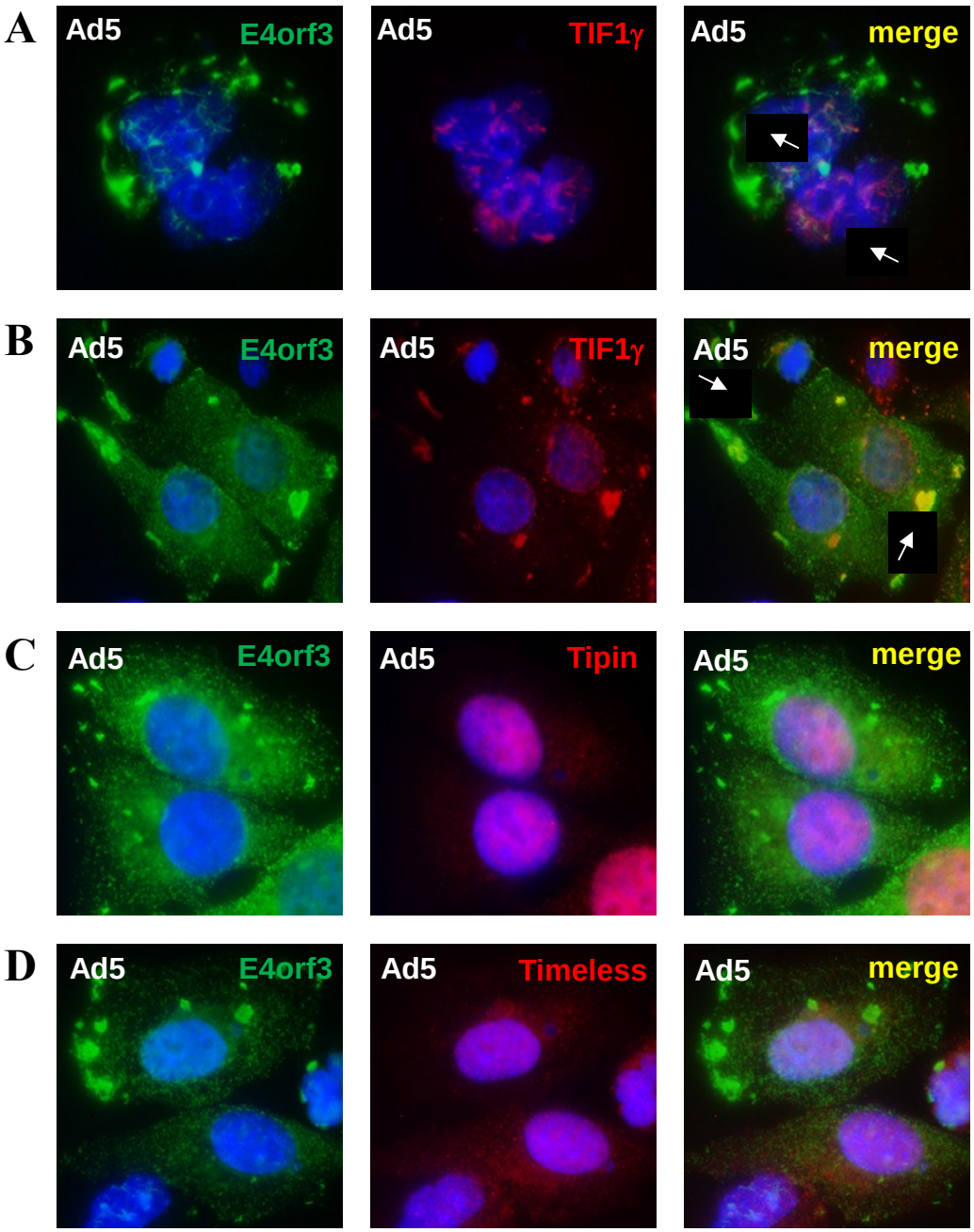

Fig. 4.7. Ad5 E4orf3 does not recruit Timeless and Tipin to nuclear tracks. HeLa cells were transfected with Ad5 HA-tagged E4orf3 plasmid DNA and harvested after 48 hours. Cells were then treated with a pre-extraction buffer before being fixed in 4% (w/v) paraformaldehyde and stained for E4ORF3 (anti HA green), TIF1-γ (A and B, red), Tipin (C, red), Timeless (D, red) and DAPI (blue). Images were visualised by confocal microscopy and colocalization of proteins is shown in the right-hand merged column. Arrows indicate colocalization of E4orf3 and TIF1γ.

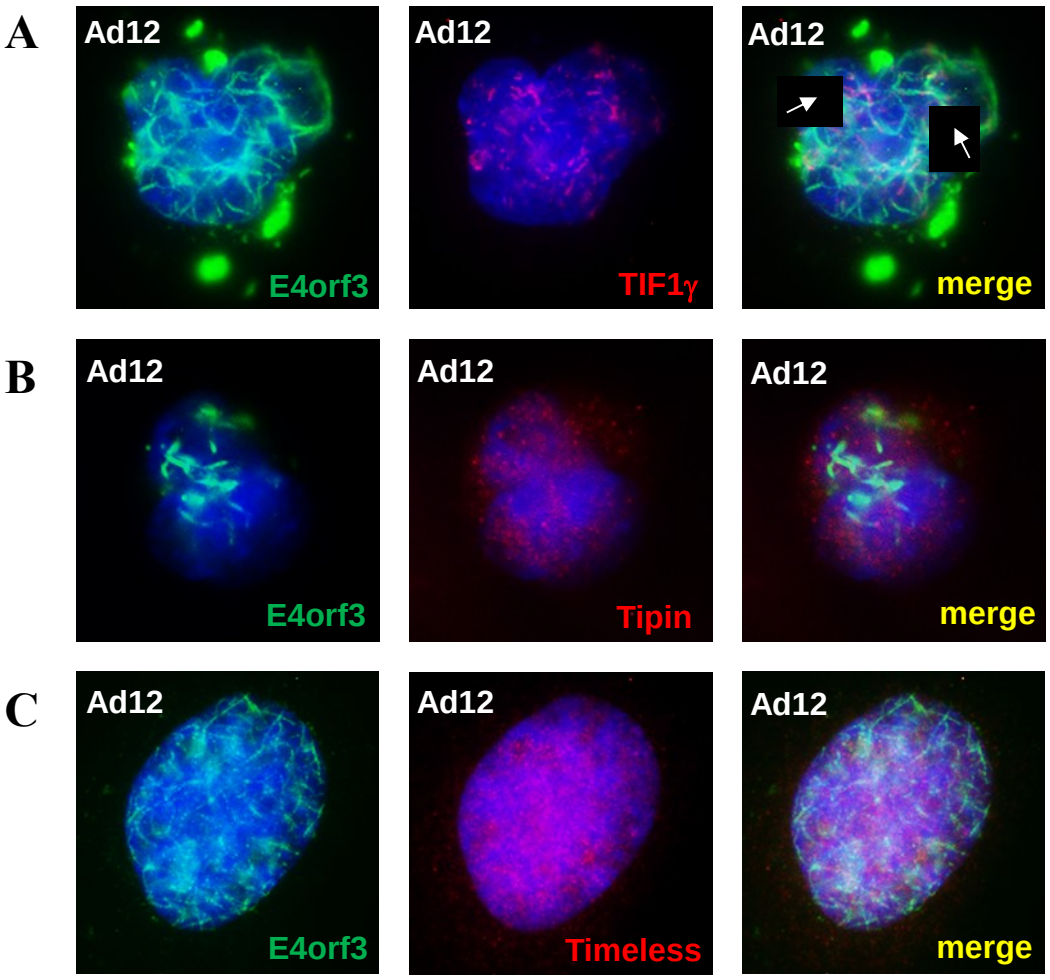

Fig. 4.8. Ad12 E4orf3 does not recruit Timeless and Tipin to nuclear tracks. HeLa cells were transfected with Ad12-HA-tagged E4orf3 plasmid DNA and harvested after 48 hours. Cells were then treated with a pre-extraction buffer before being fixed in 4% (w/v) paraformaldehyde and stained for E4orf3 (A-C; anti HA green) TIF1γ (A; red) Tipin (B; red), Timeless (C; red) and DAPI (A-C; blue). Images were visualised by confocal microscopy and colocalization of proteins is shown in the right-hand merged column. Arrows indicate colocalization of E4orf3 and TIF1γ.

Chk1 phosphorylation by promoting the proteolysis of TopBP1, a protein that is known to be specifically required for the ATR-dependent phosphorylation, and activation of Chk1. Timeless and Tipin are proteins that found in a complex together that play an important role in maintain genome integrity. One mechanism by which these proteins accomplish this is by playing a vital role in the activation of Chk1 (Yoshizawa-Sugata and Masai 2007). Given that we have shown that Ad12 E4orf3 promotes the degradation of Tipin, Timeless and TopBP1, we hypothesized that Ad12 E4orf3 could also potentially inhibit ATR activation and subsequent downstream Chk1 phosphorylation in the absence of viral infection.

To test this hypothesis we transfected HeLa cells with either Ad5 or Ad12 E4orf3, and 24 hours post-transfection treated the cells with the replication inhibitor, HU, to activate ATR and promote Chk1 phosphorylation. Cells were harvested at appropriate times post-treatment, and then subjected to SDS-PAGE, followed by Western blot analysis to assess the levels of TopBP1 and phosphorylated, activated Chk1. Consistent with our previous data, TopBP1 was degraded in Ad12 E4orf3-expressing cells, but was not degraded in cells expressing Ad5 E4orf3 (Fig. 4.9). Interestingly, we found that levels of phosphorylated, activated Chk1 were reduced considerably, following exposure to HU, in cells expressing Ad12 E4orf3 when compared to cells transfected with the empty vector alone (Fig. 4.9). Consistent with previous observations Ad5orf3 also inhibited Chk1 phosphorylation and activation in HU-treated cells (Fig. 4.9; Carson et al. 2009). These data indicate that, in the absence of other viral proteins, Ad12 E4orf3 and Ad5 E4orf3 are able to inhibit the ATR-dependent phosphorylation and activation of Chk1. In consideration of the data presented herein, it is likely that Ad12 E4orf3 inhibits CHK1 activation by promoting the specific degradation of the ATR activators, TopBP1, Timeless and Tipin, whilst Ad5 E4orf3 prevents Chk1

activation by promoting the relocalization of MRN to nuclear tracks (Carson et al. 2009).

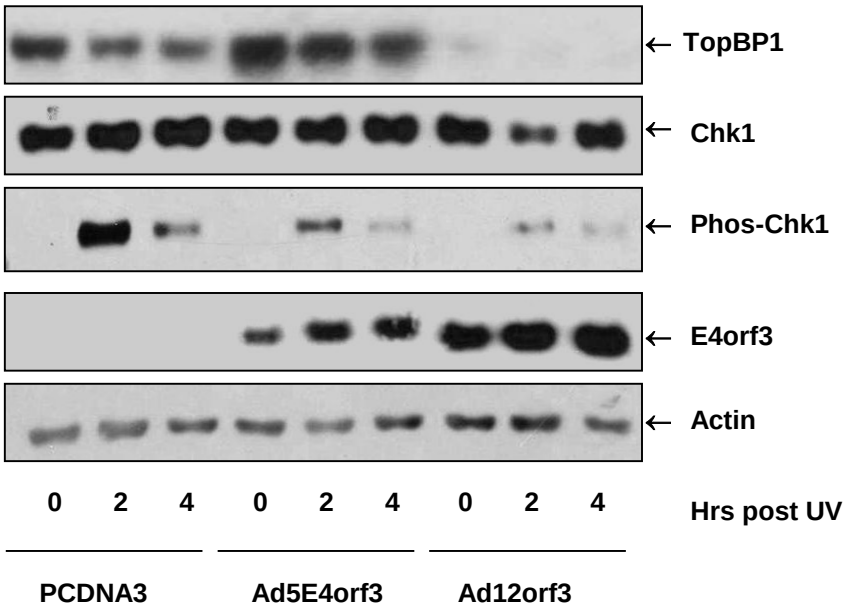

Fig. 4.9. Ad12 E4orf3 inhibits ATR signalling in response to replication stress. HeLa cells were transfected with pCMV-Ad12-E4orf3, pCMV-Ad5-E4orf3 or pcDNA3 vector alone. After 24 h, cells were mock-treated or treated with HU for the indicated times, before being harvested for Western blotting analysis.

4.3. DISCUSSION

Adenoviruses have evolved a number of strategies to inactivate and evade host cell defence mechanisms in order to promote viral replication. One such mechanism is to inactivate the DDR which would normally be activated during infection due to the production of single-stranded viral DNA replication intermediates, and the recognition of the linear double-stranded viral DNA genome as a double-strand break. In order to bypass the host cell response, adenovirus has been shown to promote the degradation of proteins involved in the DDR pathway. Up until recently it was our understanding that the viral oncoproteins E1B-55K and E4orf6 function in concert to promote the degradation of the target proteins, such as p53 and the MRN complex, by recruiting them to a CRL5 complex and stimulating their degradation by the proteasome (Querido et al. 2001; Carson et al. 2003). However, as described in chapter 3, it is now evident that E4orf6 alone is able to promote the degradation of TopBP1, independently of E1B-55K, by recruiting it to a CRL2 complex (Blackford et al. 2010). Furthermore, work presented in this thesis determined that Ad12 inhibited the ATR-dependent activation of Chk1, at least in part, by stimulating TopBP1 proteolysis (Blackford et al. 2010).

The mechanism by which Chk1 phosphorylation occurs is unclear, however research has shown that the Timeless-Tipin complex plays a key role. It has been suggested that Tipin binds to the RPA2 subunit of RPA which then allows for the Timeless-Tipin complex, and then Claspin, to associate with ATR at sites of damage, which in turn allows for Chk1 recruitment, and phosphorylation by ATR (Kemp et al. 2010). The aim of the work presented in this chapter was to further investigate the mechanism by which Ad12 inhibits the ATR-dependent activation of Chk1 during infection. In the study described here we have provided evidence to show that Ad12 targets a number of

proteins for degradation, involved in regulating the ATR-dependent phosphorylation, and activation, of Chk1.

We initially determined that both Timeless and Tipin are degraded in Ad12-infected cells but not in Ad5-infected cells, whereas Claspin levels remained unaffected (Fig. 4.1). These findings are interesting as it appears that Ad5 and Ad12 differentially regulate proteins involved in the phosphorylation of Chk1, further suggesting that this phenomenon maybe restricted to group A adenoviruses as discussed in chapter 3. It is interesting that adenovirus does not target Claspin for degradation as the HPV E7 oncoprotein has previously been shown to bypass the G_2/M checkpoint by accelerating the proteasome-mediated degradation of Claspin (Spardy et al. 2009). These observations highlight once again that although different viruses target the same DDR signalling pathways in order to promote replication, the mechanisms they employ can be very different.

We described in chapter 3 how adenovirus utilizes different Cullin Ring Ligases during infection to promote the degradation of its target proteins. We showed that Ad12 utilizes CRL2, whereas Ad5 utilizes CRL5. Given these findings we reasoned that Ad12 might utilize CRL2 to degrade Timeless and Tipin. We observed that both Timeless and Tipin were degraded in Ad12-infected cells treated with control siRNA or depleted of Cul5 (Fig. 4.2). However in Ad12-infected cells depleted of Cul2, both Timeless and Tipin levels remained unchanged (Fig. 4.2). These data showed that like TopBP1, the Ad12-mediated degradation of Timeless and Tipin requires Ad12 to hijack the CRL2 complex. Work detailed in this chapter is the first report demonstrating that a virus, and more specifically a viral protein, can inactivate ATR signalling pathways by targeting Timeless and Tipin for proteasome-dependent degradation. Interestingly, however, Timeless and Tipin have recently been shown to be required for episomal

maintenance of latent Epstein Barr Virus-EBV (Leman et al., 2010; Dheekollu and Lieberman, 2011). The ability of Timeless and Tipin to stabilize replication forks is utilized specifically by EBV in order to promote viral replication (Leman et al. 2010; Dheekollu and Lieberman 2011). Timeless-Tipin complexes associate with the EBV-encoded nuclear antigen 1 (EBNA1) at viral origins of plasmid replication (OriP) to promote viral replication, such that depletion of Timeless inhibits OriP-dependent viral DNA replication and causes complete loss of the closed-circular form of EBV episomes in latently infected B lymphocytes, as well as DSB accumulation at OriP region (Dheekollu and Lieberman 2011). Given these findings it will be interesting to see if other viruses use, or, inactivate Timeless-Tipin complexes in order to promote viral replication.

E1B-55K has historically been shown to act as the substrate adaptor, whilst E4orf6 recruits the Cullin Ring Ligase to promote degradation of its cellular target. However, it is becoming increasingly evident that adenoviruses can utilize different viral oncoproteins to promote degradation of cellular proteins. Indeed, Ad12 E4orf6 alone is able to promote the proteasomal degradation TopBP1 (chapter 3) and both Ad5 and Ad12 E4orf3 are able to promote the degradation of TIF1γ in an E4orf6/E1B-55K-independent fashion (Forrester et al. 2012). Following infection with E1B-55K deletion mutants, we observed that both Timeless and Tipin levels were still reduced following infection with Ad12 E1B mutants, suggesting that like TopBP1, degradation of both of these proteins occurs in an Ad12 E1B-55K-independent manner (Fig. 4.3). Subsequent studies determined that both Timeless and Tipin were targeted for degradation by Ad12 E4orf3, but not by Ad12 E4orf6, whilst TopBP1 was targeted for degradation by both Ad12 E4orf3 and Ad12 E4orf6 (Fig. 4.4 and Fig. 4.6). The use of the proteasome inhibitor, MG132, established that E4orf3 targeted Timeless and Tipin for proteasomal

degradation (Fig. 4.5). Together, these data show that Ad12 E4orf3 is able to promote the proteasome-mediated degradation of Timeless, Tipin, and TopBP1, independently of E1B-55K and E4orf6 expression. The ability of Ad12 E4orf3 and Ad12 E4orf6 to independently target TopBP1 emphasizes that there is at least some functional redundancy in the ability of Ad oncoproteins to engage the ubiquitin-proteasome pathway and inactivate DDR pathways. In this regard it will be interesting to compare at the whole proteome level, the proteins targeted for degradation specifically by E1B-55K/E4orf6, E1B-55K, E4orf6, and E4orf3. It will also be interesting to see if E4orf3/E4orf6 and E1B-55K/E4orf3 complexes can similarly activate the ubiquitin-proteasome pathway to promote degradation of cellular substrates.

The Ad12 E4orf3-dependent reduction in levels of both Timeless and Tipin was also observed using immunofluorescent confocal microscopy (Fig. 4.6). The most recognised function of E4orf3 is its ability to relocalize proteins to elongated nuclear tracks, examples include PML, p53, TIF1α, TIF1γ, and the MRN complex (Carvalho et al. 1995; Araujo et al. 2005; Stracker et al. 2005; Yondola and Hearing 2007; Forrester et al. 2012). The relocalization of Mre11 into nuclear tracks is an essential step for its Ad5-mediated degradation (Araujo et al. 2005; Liu et al. 2005). Here we were able to replicate previous observations from our laboratory by showing that TIF1γ colocalizes with both Ad12 and Ad5 E4orf3 at nuclear tracks in transfected cells (Fig. 4.7A-B and Fig. 4.8A). Given the known role of E4orf3 in nuclear-track formation we reasoned that Ad12 E4orf3 might also organise Timeless and Tipin in to nuclear tracks prior to degradation. However, we observed that neither Timeless nor Tipin localized at these nuclear tracks, suggesting that the Ad12 E4orf3-mediated degradation of TIF1γ, and Timeless and Tipin occur by different mechanisms (Fig. 4.7 C-D and Fig. 4.8B). Indeed, E4orf3-dependent TIF1γ degradation occurs independently of Cullin Ring

Ligases, whereas CRL2 complexes are required for the Ad12 E4orf3-dependent degradation of Timeless and Tipin (Fig. 4.2; (Forrester et al. 2012). It has been proposed by many laboratories that Cullin Ring ligases are required for the Ad-mediated degradation of Mre11, whilst our laboratory suggest that other E3 ligases might be required for Mre11 degradation (*e.g.* Stracker et al., 2005; Forrester et al. 2011). Irrespective of the E3 ligase(s) required for Mre11 degradation, it is not immediately clear why Timeless and Tipin do not colocalize with E4orf3 within nuclear tracks. It is possible that the pool of Ad12 E4orf3 that associates with Timeless and Tipin does not localize to nuclear tracks, or that Tipin and Timeless associate only transiently with E4orf3 in these structures.

E4orf3 is also able to inactivate the DDR independently of E1B-55K and E4orf6 by relocalizing the MRN complex into nuclear tracks, and this action of E4orf3 appears to be conserved between group C, D, and E serotypes, as Ad4 and Ad12 lack a key isoleucine residue in their E4orf3 proteins that is required to relocalize MRN (Stracker et al. 2005; Forrester et al. 2011) It has been proposed that the Ad5-mediated inactivation of Chk1 during infection is a consequence of the MRN relocalization into nuclear tracks (Carson et al. 2009). Both Ad5 and Ad12 infection results in inactivation of Chk1, but it was unclear how Ad12 achieved this (Blackford et al. 2008). In chapter 3 we described mechanistically how Ad12 E4orf6 inactivates Chk1. Work detailed in this chapter extends these findings to show that Ad12 E4orf3 can also inhibit Chk1 presumably through its ability to target Timeless and Tipin as well as TopBP1.

It has been shown that E4orf3 and E4orf6 can compensate for each other's defects as they have been shown to be functionally redundant, such that both proteins are involved in inhibiting adenovirus DNA concatamer formation, promoting late viral protein synthesis, promoting late viral mRNA export, augmenting viral DNA replication, and

shutting-off host protein synthesis, although they do carry out these functions by different mechanisms (Halbert et al. 1985; Huang and Hearing 1989; Weiden and Ginsberg 1994). Here we have shown that both proteins also function to inhibit Chk1 activation during Ad12 infection by similar mechanisms, as they both recruit CRL2 to target TopBP1 and Timeless and Tipin for degradation. E4orf6 contains 3 functional BC boxes, which are required for recruitment of CRL complexes to facilitate degradation of its cellular substrates that include p53 and BLM (Querido et al. 2001; Cheng et al. 2007; Orazio et al. 2011) Ad12 E4orf3 possesses a putative BC box motif and a Cullin 2 box (amino acids 23-58) but the functional activity of these sites remains to be elucidated. It will be of interest to establish whether these domains are required for engaging with Elongins B and C and Cullin 2, respectively, to promote Tipin and Timeless degradation, or the degradation of other proteins.

In conclusion, we have identified a novel mechanism through which Ad12 inhibits the ATR-dependent phosphorylation and activation of Chk1. Ad12 E4orf3 utilizes CRL2 to promote the degradation of TopBP1, Timeless and Tipin, independently of Ad12 E1B55K and E4orf6. Moreover, Ad12 E4orf3-mediated degradation is mechanistically different to Ad12 E4orf3-mediated degradation of TIFγ as it is not organized into nuclear tracks, and requires a Cullin-containing Ring Ligase.

CHAPTER 5

A ROLE FOR WDR62 IN THE CELLULAR RESPONSE TO DNA DAMAGE

5.1. INTRODUCTION

Viruses are intracellular parasites that hijack host cell functions in-order to facilitate viral genome replication and the production of new virions. They have therefore evolved a number of mechanisms that function to negate the activation of antiviral responses, yet keep the host cell alive long enough to produce viral progeny (Blackford and Grand 2009). DNA tumour viruses express early region genes, the protein products of which serve to disrupt key cellular processes, such as apoptosis and cell cycle checkpoint activation, in the host cell in order to promote viral replication. Given these properties a number of viral early gene products have transforming capabilities, and as such, it has been highly beneficial to study the function of viral oncogenes and the proteins that they encode in order to increase our understanding of the molecular basis of these fundamental cellular pathways.

Ad E1B-55K is a multifunctional protein that uses a number of different mechanisms to facilitate viral replication during infection. A number of the functions carried out by E1B-55K have been shown to require E4orf6. Together they have been shown to assemble a Cullin-containing E3 ubiquitin ligase complex, where E1B-55K acts a substrate adaptor to recruit a number of cellular proteins for proteasomal degradation (Querido et al. 2001; Stracker et al. 2002; Liu et al. 2005; Baker et al. 2007; Dallaire et al. 2009; Orazio et al. 2011). Through their ability to recruit the CRL5 complex, Ad5 E1B-55K and E4orf6 also cooperate to inhibit host cell nuclear mRNA export, and promote viral late nuclear mRNA export to the cytoplasm during the late phase of infection (Woo and Berk 2007).

E1B-55K also has functions independent of E4orf6. The transcription factor, Daxx is degraded in an E1B-55K-dependent manner and, E4orf6-independent manner, during

Ad5 infection (Schreiner et al. 2010). E1B-55K also plays a major role in the transformation of mammalian cells together with E1A and E1B-19K, but independent of E4orf6, though E4orf6 expression does improve transformation frequency (Jochemsen et al. 1982; Gallimore et al. 1985; Moore et al. 1996; Nevels et al. 1999). It has been shown that Ad5 E1B-55K promotes transformation by binding with high affinity to p53, where it blocks its transcriptional activation properties and sequesters it into aggresomes (Yew and Berk 1992). More recently it has been determined that Ad5 E1B-55K promotes the SUMOylation of p53, and sequesters it in PML nuclear bodies before facilitating its nuclear export into cytoplasmic aggresomes (Pennella et al. 2010).

Given the ability of Ad oncoproteins to target cellular tumour suppressor gene products, many researchers have strived to identify novel, cellular Ad oncoprotein-interacting proteins using a wide range of techniques; binding proteins have been identified using conventional immunoprecipitation-Western blotting analyses (Baker et al. 2007; Blackford et al. 2010), utilizing human mutated cell lines (Stracker et al. 2002), and performing immunoprecipitation assays coupled to mass spectrometry screening (Querido et al. 2001; Harada et al. 2002; Forrester et al. 2012). Research from our laboratory has used mass spectrometry to identify novel Ad12 E1B-55K interactors, such as the TIF1 family of proteins (Forrester et al. 2012). This screening method also isolated other potential Ad12 E1B-55K interacting proteins (Forrester, 2011; PhD Thesis, The University of Birmingham); one of these proteins was WDR62.

WDR62 was first described in 2010 when it was found to interact with, and potentiate, JNK kinase activity, and play a possible role in mRNA homeostasis after stress (Wasserman et al. 2010). It was then later discovered that mutation of the WDR62 gene is the second most common cause of MCPH (Nicholas et al. 2010). WDR62 has been identified as a centrosomal protein, whilst mutated WDR62 forms identified in MCPH,

fail to localize at mitotic spindle poles (Nicholas et al. 2010). The functions of WDR62 are relatively unknown as it has only recently been discovered. Given that other centrosomal proteins MCPH1, and Pericentin have been shown to play a role in the ATR signalling pathway (Alderton et al. 2006; Griffith et al. 2008), and that WDR62 was identified as a potential E1B-55K-interacting protein, we hypothesized that WDR62 might also play a role in the DDR pathway. Therefore, the aims of the study described in this chapter were to: confirm the interaction between WDR62 and E1B-55K; investigate the consequence of the interaction during infection; investigate a potential role for WDR62 in the DDR.

5.2. RESULTS

5.2.1. WDR62 interacts with both Ad12 E1B-55K and Ad5 E1B-55K in vivo.

To determine whether Ad12 E1B-55K interacts with WDR62 *in vivo*, we performed immunoprecipitation-Western blot analyses using Ad12 E1-transformed cells. We therefore harvested cellular lysates from Ad12 E1-transformed HER2 and HER10 cells and immunoprecipitated Ad12 E1B-55K and interacting proteins using the anti-Ad12 E1B-55K mAb, XPH9. We collected immunocomplexes on Protein G-sepharose, separated immunoprecipitates by SDS-PAGE, and performed Western blots for WDR62 (Fig. 5.1). Western blotting revealed that WDR62 co-precipitated with Ad12 E1B-55K from both Ad12 E1-transformed HER2 and HER10 cells (Fig. 5.1. A and B). To extend these findings we also investigated whether Ad5 E1B-55K similarly interacted with WDR62. To do this we harvested Ad5 E1-transformed HEK 293 cells, and HER 911 cells and performed immunoprecipitation-Western blot analyses using the anti-Ad5 E1B-55K mAb, 2A6. These analyses revealed that Ad5 E1B-55K also interacts *in vivo*, with WDR62 (Fig. 1 C and D), though Ad12 E1B-55K appeared to bind to a greater proportion of the WDR62 cellular pool, than Ad5 E1B-55K (Fig. 1).

5.2.2. WDR62 protein levels are not affected during adenovirus infection.

The Ad E1B-55K-interacting proteins p53, Mre11, DNA ligase IV and BLM are all degraded during adenovirus infection (Querido et al. 2001; Stracker et al. 2002; Baker et al. 2007; Orazio et al. 2011). Given that we have substantiated earlier findings and established that WDR62 is also an Ad E1B-55K-interacting protein, we wanted to determine whether WDR62 was also targeted for degradation during infection. Hence, we mock-infected, or, infected HeLa cells with *wt* Ad5 or *wt* Ad12 at an m.o.i of 10

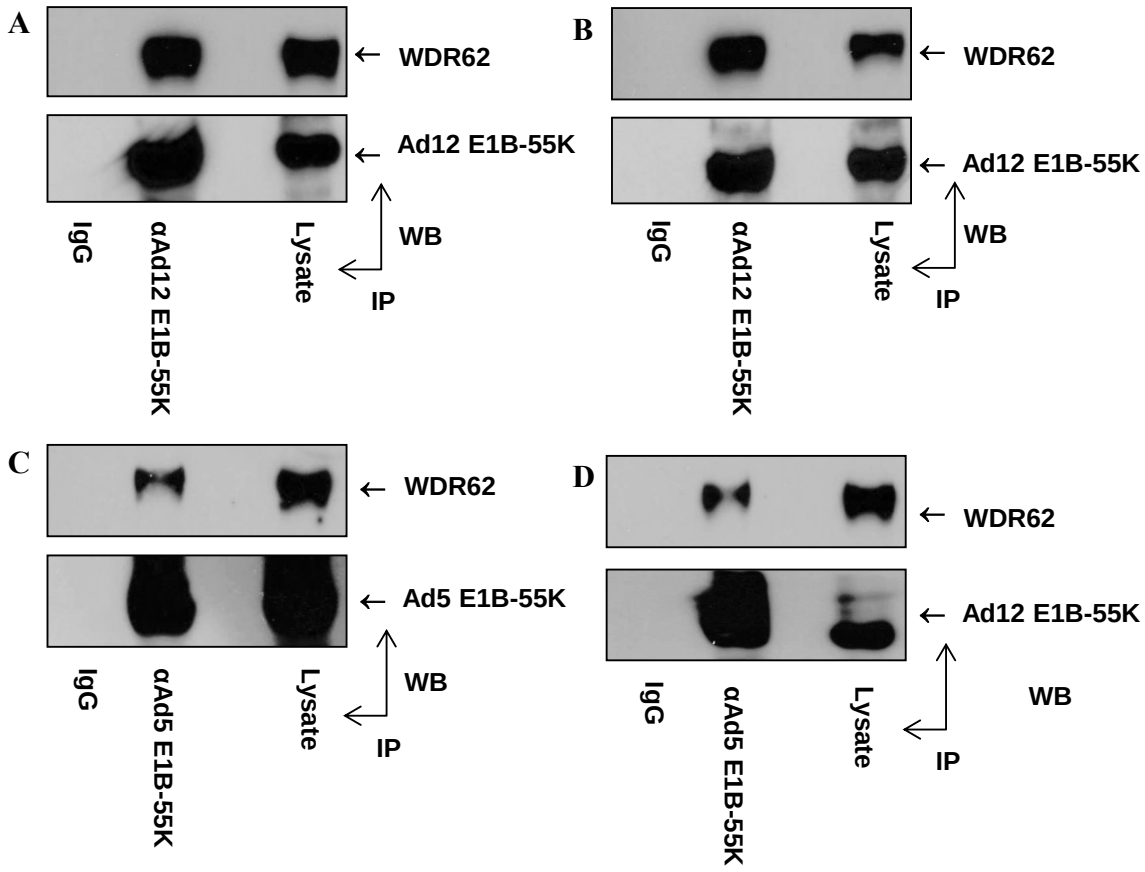

Fig. 5.1. WDR62 and E1B-55K interact in Ad E1-Transformed cell lines. E1B-55K was immunoprecipiated from (A) Ad12 HER2 (B) Ad12 HER10 (C) Ad5 HEK 293 and (D) Ad5 HER 911 cells. Immunocomplexes were isolated on protein-G Sepharose, subjected to SDS-PAGE, and WDR62 and Ad E1B-55K detected by Western blotting.

p.f.u./cell and then harvested cells at intervals over a 48 hour period. The protein lysates were then quantified, subjected to SDS-PAGE, and protein levels were assessed by Western blotting (Fig. 5.2).

In accordance with previous research, we observed that both Mre11 and p53 were degraded in adenovirus-infected cells (Fig. 5.2). Furthermore, we confirmed that TopBP1 is degraded in Ad12-infected cells in agreement with data presented in Chapter 3 (Fig. 5.2). In contrast to Mre11, p53 and TopBP1, the expression levels of WDR62 remained constant in Ad5- and Ad12- infected cells (Fig. 5.2), Interestingly, however, it appeared that WDR62 had reduced mobility on SDS-PAGE at 48 hours post-infection (Fig. 5.2). This apparent increase in molecular weight was more evident in Ad12-infected cells, than Ad5-infected cells (Fig. 5.2). Taken together these data indicate that WDR62 is not targeted for degradation during Ad infection, but might be subject to post-translational modification.

5.2.3. E1B-55K colocalizes with WDR62 during interphase and mitosis in Ad-transformed cells.

WDR62 is a centrosomal protein that localizes at spindle poles during mitosis, and at microtubule-organizing centres in interphase cells (Nicholas et al. 2010). As we had confirmed the interaction between E1B-55K and WDR62 by immunoprecipitation-Western blot analyses, we wanted to determine the localization of E1B-55K-WDR62 complexes within the cell using immunofluorescent confocal microscopy. To this end, Ad5 E1-transformed HEK293 cells were seeded onto glass slides and grown for 24 hours before being fixed with 4% (w/v) PFA. Fixed cells were then co-stained for Ad5 E1B-55K and WDR62, mounted in a DAPI-containing medium to stain the DNA and visualized using a confocal microscope (Fig. 5.3). In interphase cells, WDR62 was

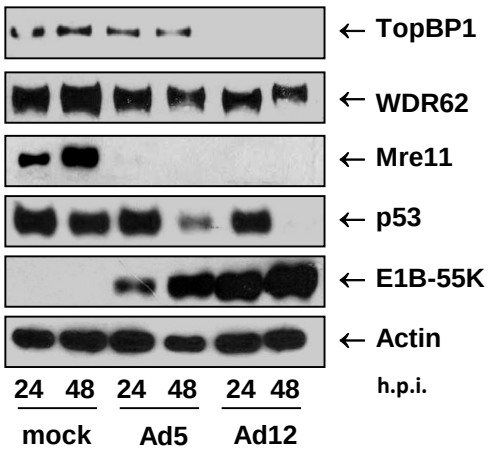

Fig. 5.2. Effect of Ad infection on the protein levels of WDR62. HeLa cells were mock-infected, or, infected with *wt* Ad5 or *wt* Ad12 at an m.o.i of 10 p.f.u./cell. Cells were harvested at the appropriate times post-infection and subject to Western blotting using the appropriate antibodies.

localized predominantly in a single discrete location and appeared as multiple distinct foci within this area (Fig. 5.3). Interestingly, E1B-55K also localized at this site but appeared to have a more homogeneous staining pattern, with the strongest E1B-55K staining appearing to encapsulate WDR62 (Fig. 5.3). As WDR62 localizes specifically at spindle poles during mitosis, we also investigated whether E1B-55K co-localized with WDR62 at these sites. Interestingly, our analyses revealed that a proportion of E1B-55K was always found associated with WDR62 at both spindle poles in mitotic cells, although the majority of E1B-55K was located in cytoplasmic aggresomes; WDR62 was not however found associated with aggresomes (Fig. 5.3).

5.2.4. E1B-55K colocalizes with WDR62 in Ad-infected cells

To extend these observations we next investigated the localization of E1B-55K and WDR62 in Ad-infected cells. To do this we seeded HeLa cells on glass coverslips and then infected them with either *wt* Ad5 or *wt* Ad12 at an m.o.i of 10 p.f.u./cell. Cells were fixed, co-stained with anti-E1B-55K and anti-WDR62 antibodies, mounted in a DAPI-containing solution and visualized by confocal microscopy (Fig. 5.4). In agreement with previous studies, WDR62 localized at the spindle poles in mock-infected mitotic cells (Fig. 5.4A) and as multiple, punctuate foci in discrete locations in interphase cells (Fig. 5.4B). Akin to Ad E1-transformed cells, we observed that E1B-55K formed discrete structures with, and around, WDR62 in both *wt* Ad5- and *wt* Ad12- infected cells (Fig. 5.4 C and D). Consistent with the ability of Ad to promote S-phase arrest in infected cells we did not observe any E1B-55K mitotic staining in either *wt* Ad5-, or *wt* Ad12- infected cells (data not shown). Taken together, these data suggest that Ad E1B-55K colocalizes with WDR62 in both Ad-transformed and Ad-

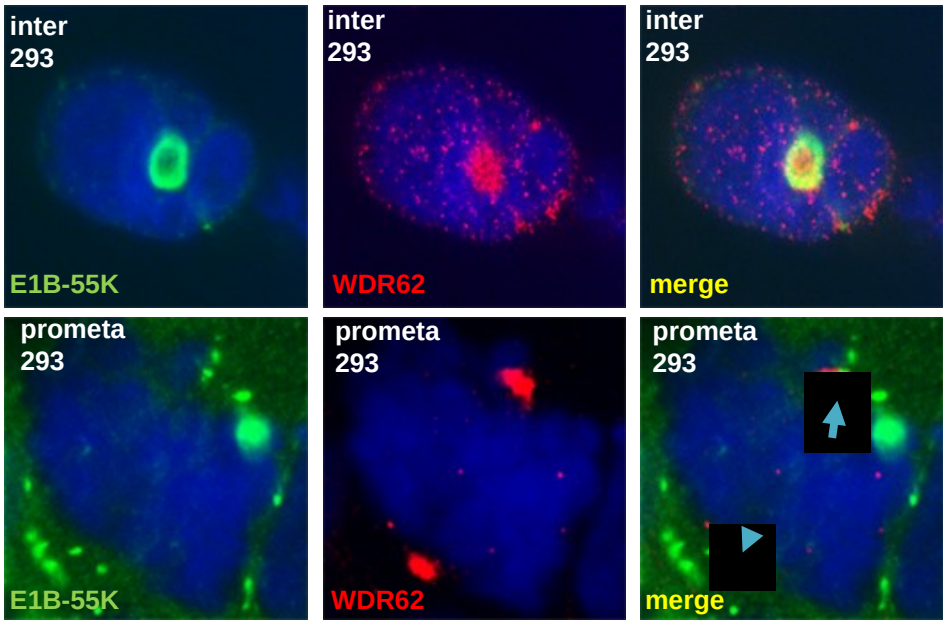

Fig. 5.3. E1B-55K co-localizes with WDR62 at centrosomes in Ad5 E1-transformed cells. Ad5 E1-transformed cells were grown on glass coverslips, treated with a pre-extraction buffer before being fixed in 4% (w/v) paraformaldehyde and stained for WDR62 (red), E1B-55K (green) and DAPI (blue). Images were visualized by confocal microscopy and colocalization of proteins is depicted by the arrowheads in the right-hand merged column (yellow). Inter, interphase; prometa, prometaphase.

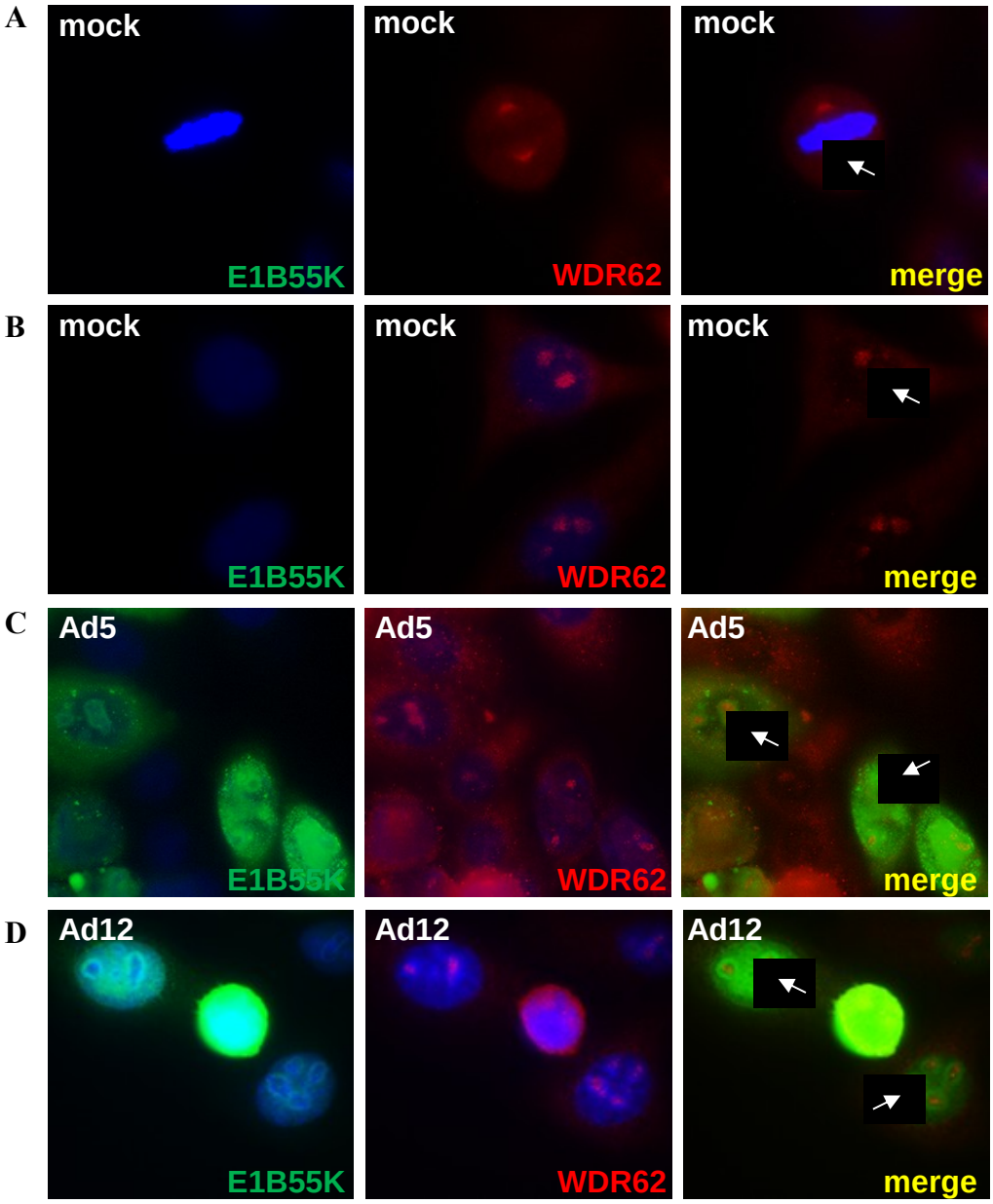

Fig. 5.4. E1B-55K co-localizes with WDR62 in Ad-infected cells. HeLa cells were grown on glass coverslips and (A-B) mock-infected or infected (C) Ad5 (D) or Ad12 at an m.o.i of 10 p.f.u./cell, treated with a pre-extraction buffer before being fixed in 4% (w/v) paraformaldehyde and stained for WDR62 (red), E1B-55K (green) and DAPI (blue). Images were visualized by confocal microscopy and colocalization of proteins is evident in the right-hand merged column (yellow).

infected cells and supports earlier observations indicating that these proteins associate specifically in Ad-transformed cells.

5.2.5. WDR62 regulates RPA32 phosphorylation during adenovirus infection.

Our laboratory has previously reported that E1B-AP5 has a role in ATR signalling pathways during Ad infection, and that it is required for the ATR-dependent phosphorylation of RPA32 in response to adenovirus infection (Blackford et al. 2008). Given the role of E1B-55K-binding proteins in the DDR we wanted to examine if WDR62 might also play a role in RPA32 phosphorylation during adenovirus infection. Given the role of E1B-55K in promoting degradation we also wished to establish whether WDR62 participated in the E1B-55K-dependent degradation of known adenovirus targets. We therefore, transfected specific WDR62 siRNA oligonucelotides into HeLa cells in order to specifically ablate the expression of WDR62. Alternatively, cells were transfected with non-silencing siRNA for comparison. Cells were infected with *wt* Ad5 or *wt* Ad12 at an m.o.i of 10 p.f.u./cell at 48 hours post-transfection and harvested at the appropriate time post-infection. Protein lysates were then subjected to SDS-PAGE and protein levels, and phospho-protein levels, of the relevant proteins were assessed by Western blotting (Fig. 5.5). Initial Western blot analysis indicated that WDR62 knockdown was successful, and WDR62 expression was reduced significantly following treatment with the appropriate siRNA oligonucleotides (Fig. 5.5). WDR62 knockdown did not however affect Ad infection, as E1A and E1B-55K proteins were expressed to similar levels in the absence, or presence, of WDR62 (Fig. 5.5). In agreement with our earlier results, we observed that the WDR62 protein levels remained constant in Ad-infected cells treated with non-silencing siRNA, as well as observing the same apparent increase in molecular weight, 48 hours post-infection (Fig. 5.5). Mre11, p53 and TopBP1 were degraded in Ad5- and/or Ad12- infected cells

treated with non-silencing siRNA, or depleted of WDR62, suggesting that WDR62 does not participate in the Ad-mediated degradation of these proteins (Fig. 5.5). In agreement with earlier observations, RPA32 is phosphorylated in both Ad5- and Ad12-infected cells that were treated with non-silencing siRNA (Fig. 5.5). Interestingly, however, the Ad-induced phosphorylation of RPA32 was reduced appreciably in cells depleted of WDR62 (Fig. 5.5). These data suggest that like E1B-AP5, WDR62 might function in ATR signalling pathways during Ad infection, to promote the phosphorylation of RPA32.

5.2.6. WDR62 is required for efficient ATR signalling in response to DNA damage

ATR responds to single-stranded DNA that occurs as intermediates during the processing of UV-damaged DNA or, during DNA replication during S-phase. Ss-DNA becomes coated with RPA which is required to recruit ATR-ATRIP to sites of DNA damage and promote subsequent activation of the cell cycle checkpoint signalling pathways which lead to cell cycle arrest, DNA repair or apoptosis (Bartek et al. 2004). Because we have provided evidence of a possible role for WDR62 in the phosphorylation of RPA32, we next investigated a more general role for WDR62 in mediating ATR-dependent phosphorylation events initiated in response to DNA damage. To do this HeLa cells were transfected with specific WDR62 siRNA oligonucelotides in order to ablate the expression of WDR62 proteins. Alternatively, cells were transfected with non-silencing siRNA to serve as a control. 48 hours post-transfecton, these cells were exposed to UV irradiation in order to activate ATR, and then subjected to SDS-PAGE, followed by Western blotting (Fig. 5.6).

Initial observations revealed that the siRNA-mediated knockdown of WDR62 was successful (Fig. 5.6). Interestingly, we found that the UV-induced, and ATR-dependent

phosphorylation of Chk1 was reduced dramatically in WDR62-knockdown cells relative to non-silencing controls (Fig. 5.6). In agreement with these findings we also found that the UV-induced phosphorylation of RPA32 was also reduced in WDR62-knockdown cells relative to non-silencing controls (Fig. 5.6). Interestingly, however, WDR62 knockdown did not significantly affect H2AX phosphorylation in response to UV irradiation (Fig. 5.6). In order to expand on these findings we next investigated whether WDR62 was required for ATR-dependent signalling events in response to replication stress. We therefore treated WDR62-knockdown cells, and non-silencing control cells with HU, in order to promote replication stress, and then examined, at appropriate times post-treatment, ATR-dependent phosphorylation events. Consistent with the UV irradiation studies, WDR62 knockdown, reduced considerably the ability of ATR to phosphorylate both Chk1 and RPA32 (Fig. 5.7). These results, suggest, that like E1B-AP5, WDR62 is integral to ATR signalling activated in response to UV irradiation, or replication stress.

5.2.7. WDR62 is not required for ATM signalling in response to DNA damage

As we had demonstrated previously that E1B-AP5 was required for ATR signalling but not ATM signalling, we next investigated whether WDR62 was required for ATM activation and ATM-dependent phosphorylation events in response to IR. We therefore treated WDR62-knockdown cells and non-silencing controls with IR, and at the appropriate times post-treatment harvested cells and subjected protein lysates to SDS-PAGE and Western blotting (Fig. 5.8). Interestingly, although WDR62 expression was efficiently reduced following siRNA treatment, WDR62 knockdown, relative to controls, did not affect the IR-induced activation and autophosphorylation of ATM on S1981 (Fig. 5.8). Consistent with this observation, WDR62 knockdown did not affect the ATM-dependent phosphorylation of KAP1 (TIF1β), MRN component, NBS1, or

cohesin component, SMC1 (Fig. 5.8). Taken together these data indicate that WDR62 is not required for ATM signalling.

5.2.8. WDR62 is required for cell survival following exposure to UV irradiation and treatment with HU, but not in response to IR

Mutations in DNA damage proteins like ATR often cause cells to display increased sensitivity to genotoxic agents which include UV or IR, although this may not always be detectable (Cliby et al. 1998; O'Driscoll et al. 2003). Given that we have shown that WDR62 is required for efficient activation of the ATR signalling pathway, we wanted to examine the ability of WDR62-depleted cells to recover from treatment with DNA damage agents. To do this, we performed colony survival assays using HeLa cells that were treated with either control siRNA, or WDR62 siRNA, and then exposed to varying doses of UV, HU, or IR. Briefly, HeLa cells treated with either control siRNA or WDR62 siRNA were plated at a low density, exposed to the indicated doses of the appropriate genotoxic agent, and then left to grow in a humidified incubator set at 37 °C and supplied with 5% (v/v) CO_2 for 14 days. Cells were then stained with a 50% (v/v) ethanol solution containing 2% (w/v) methylene blue. Cell sensitivity to DNA damaging agents following silencing of WDR62 was measured by determining the colony forming ability (*i.e.* cell survival) of control and knockdown cells treated with the various genotoxic agents (Fig.s. 5.9-5.11).

Initial Western blot analysis revealed that the knockdown was successful, and that WDR62 expression was reduced significantly following treatment with the appropriate

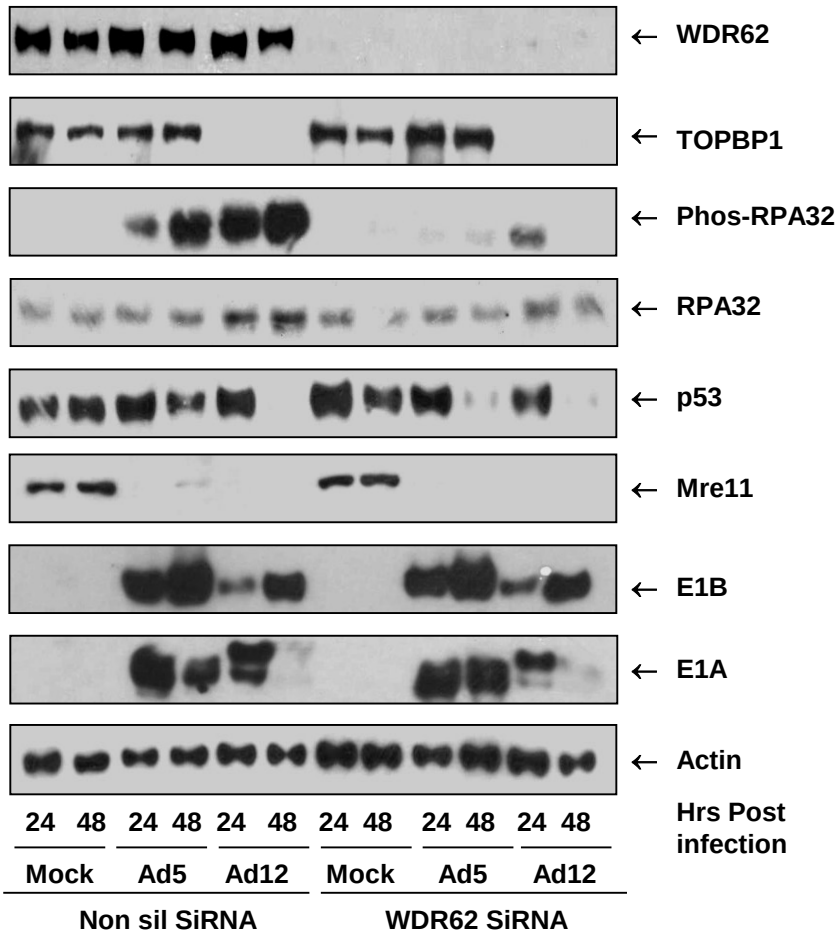

Fig. 5.5. WDR62 is required for RPA32 phosphorylation during adenovirus infection. HeLa cells were transfected with the indicated siRNAs, and subsequently mock-infected or infected with *wt* Ad5 or *wt* Ad12 at an m.o.i of 10 p.f.u./cell, 48 hours post-transfection. Cells were harvested at the specific times post-infection and then subjected to SDS-PAGE and Western blotting using the appropriate antibodies.

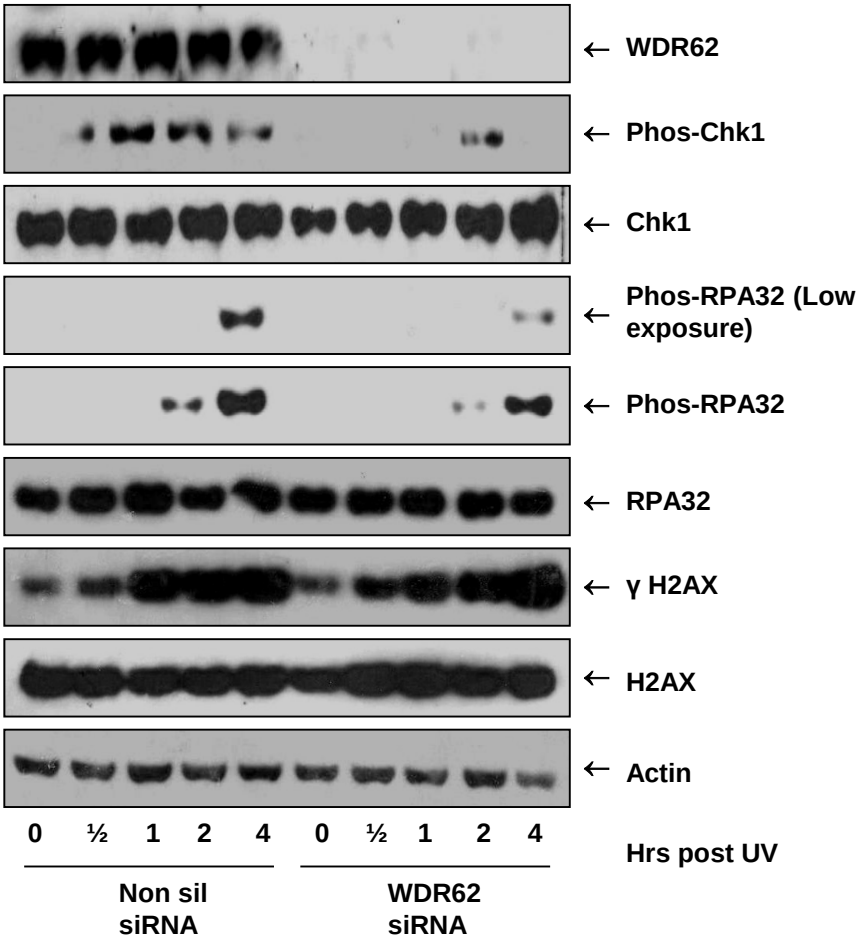

Fig. 5.6. WDR62 is required for ATR-dependent phosphorylation events in response to UV irradiation. HeLa cells were transfected with the indicated siRNAs, before being UV-irradiated at 20 J/m^2, 48 hours later. Cells were then harvested at the indicated time-points, and then subjected to SDS-PAGE and Western blotting using the appropriate antibodies.

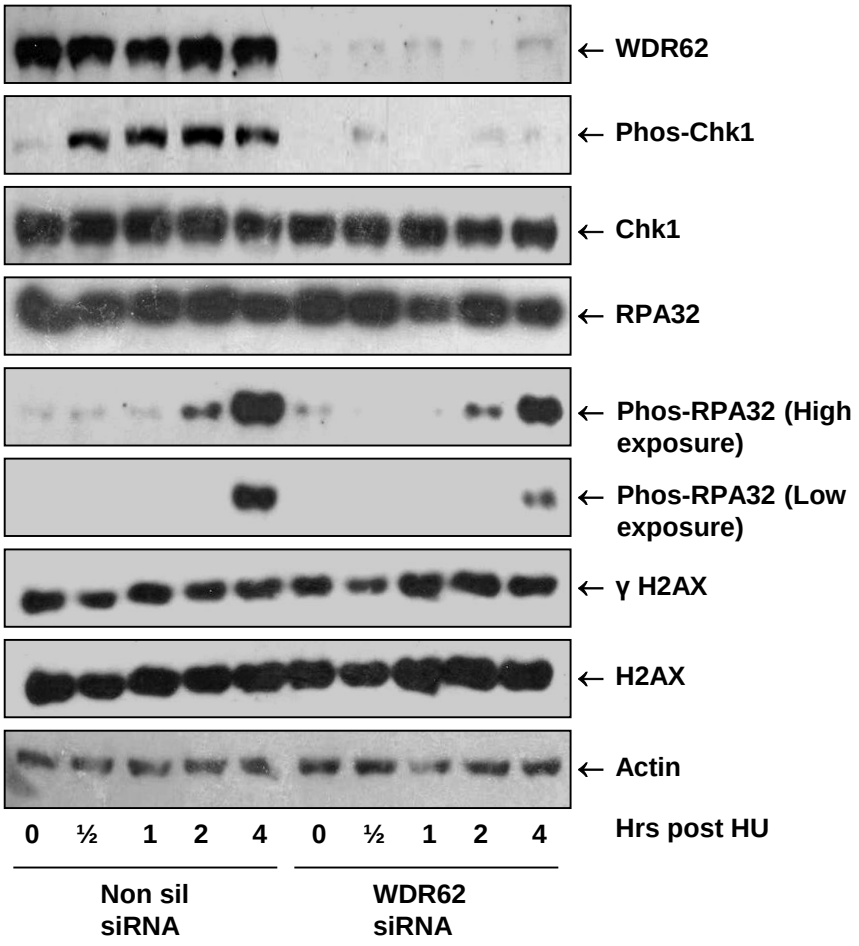

Fig. 5.7. WDR62 is required for ATR-dependent phosphorylation events in response to replication stress. HeLa cells were transfected with the indicated siRNAs, before being treated with HU, 48 hours later. Cells were then harvested at the indicated time-points, and then subjected to SDS-PAGE and Western blotting using the appropriate antibodies.

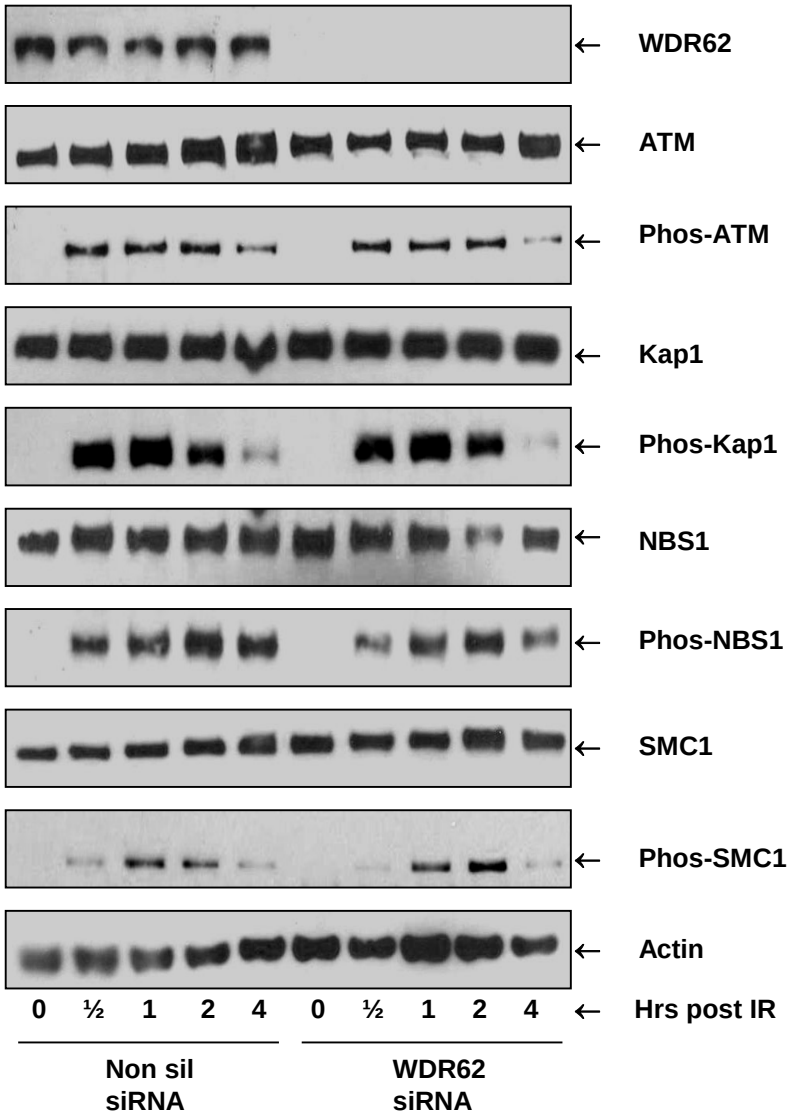

Fig. 5.8. WDR62 is not required for ATM-dependent phosphorylation events in response to IR. HeLa cells were transfected with the indicated siRNAs, before being treated with 10 Gy of IR 48hrs later. Cells were then harvested at the indicated time-points, and then subjected to SDS-PAGE and Western blotting using the appropriate antibodies.

siRNA oligonucleotides (Fig.s. 5.9B, 5.10B, 5.11B). As anticipated, cells depleted of WDR62 showed significant increased sensitivity to the proapoptotic effects of UV and HU when compared to cells that expressed endogenous, WDR62 (Fig.s. 5.9 and 5.10). This was evident even at low doses of UV and HU (Fig.s. 5.9 and 5.10). Consistent with the Western blot analyses, cells exposed to IR showed no increased sensitivity, relative to control cells, when WDR62 was depleted by RNAi (Fig. 5.11). These data demonstrate that loss of WDR62, sensitizes cells to DNA damage-induced killing in response to UV and HU, and suggest that ATR-mediated repair functions are abrogated in WDR62-knockdown cells. In agreement with previous findings these data also indicate that ATM repair functions remain intact in cells depleted of WDR62. Together, these findings substantiate our previous data and indicate that WDR62 might have an important role in the ATR signalling pathway in response to DNA damage.

5.2.9. WDR62 is required for efficient activation of the G2-M DNA damage checkpoint.

Chk1-dependent phosphorylation of cdc25 in response to DNA damage is a vital process required for G2-M checkpoint arrest (Sanchez et al. 1997). As we had shown earlier that Chk1 phosphorylation, and hence activation, was abrogated in WDR62-depleted cells challenged with UV or HU (Fig.s. 5.6 and 5.7), we wanted to establish whether the G2-M checkpoint was also affected by WDR62 knockdown. To investigate this possibility we quantified the number of cells in mitosis in control cells, and WDR62-knockdown cells, prior to, and following exposure to UV irradiation. Briefly, HeLa cells were transfected with non-silencing, or WDR62 siRNA 72 hours prior to UV irradiation. Cells were then harvested by trypsinization at appropriate times post-

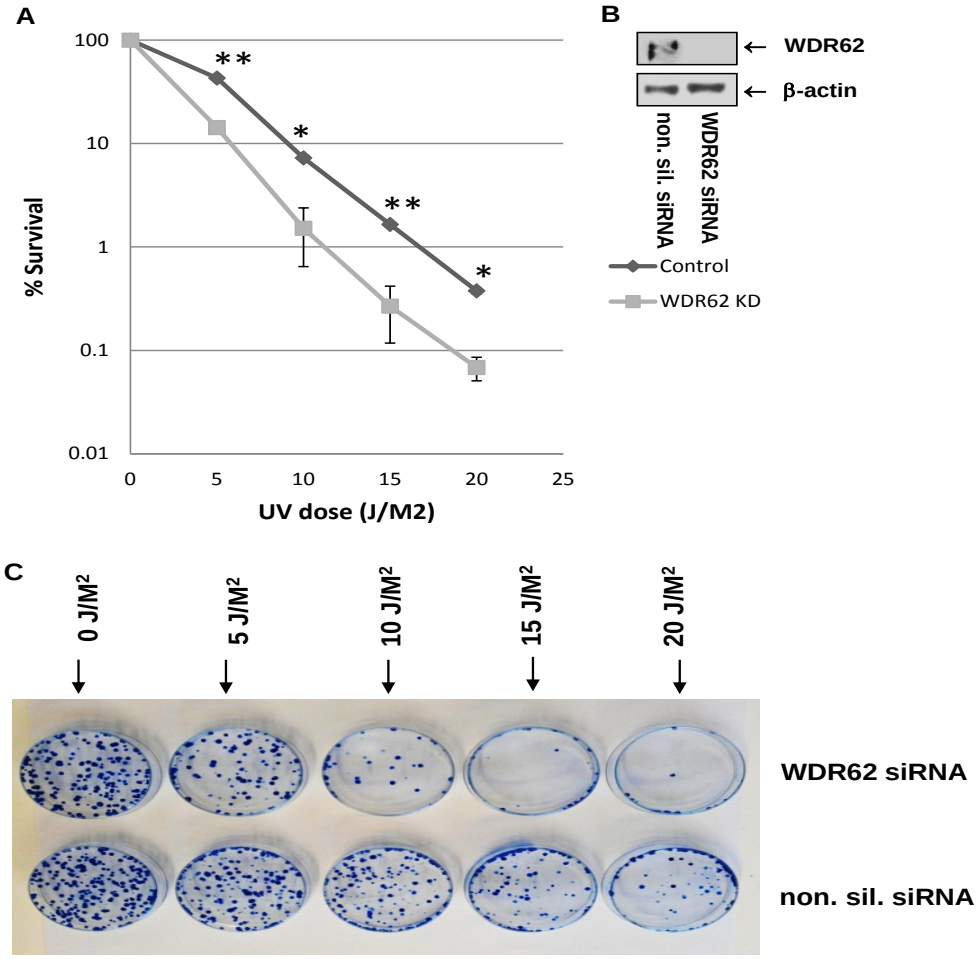

Fig. 5.9. WDR62 depletion sensitizes cells to UV irradiation. HeLa cells were transfected with the indicated siRNAs, before being plated at low density 48 hours later. Cells were then irradiated with the indicated doses of UV irradiation and left to grow. Colonies were then counted 14 days later. (A) Graph indicating % cell survival of control, and WDR62-knockdown cells following exposure to specific doses of UV. (B) Western blot analysis showing efficiency of WDR62 knockdown. (C) Representative Figure showing methylene-blue stained cells following 14 days growth post UV-irradiation. non. sil., non-silencing. This assay was performed three times in triplicate. Error bars are mean +/- S.D. *P<0.05, ** P<0.01.

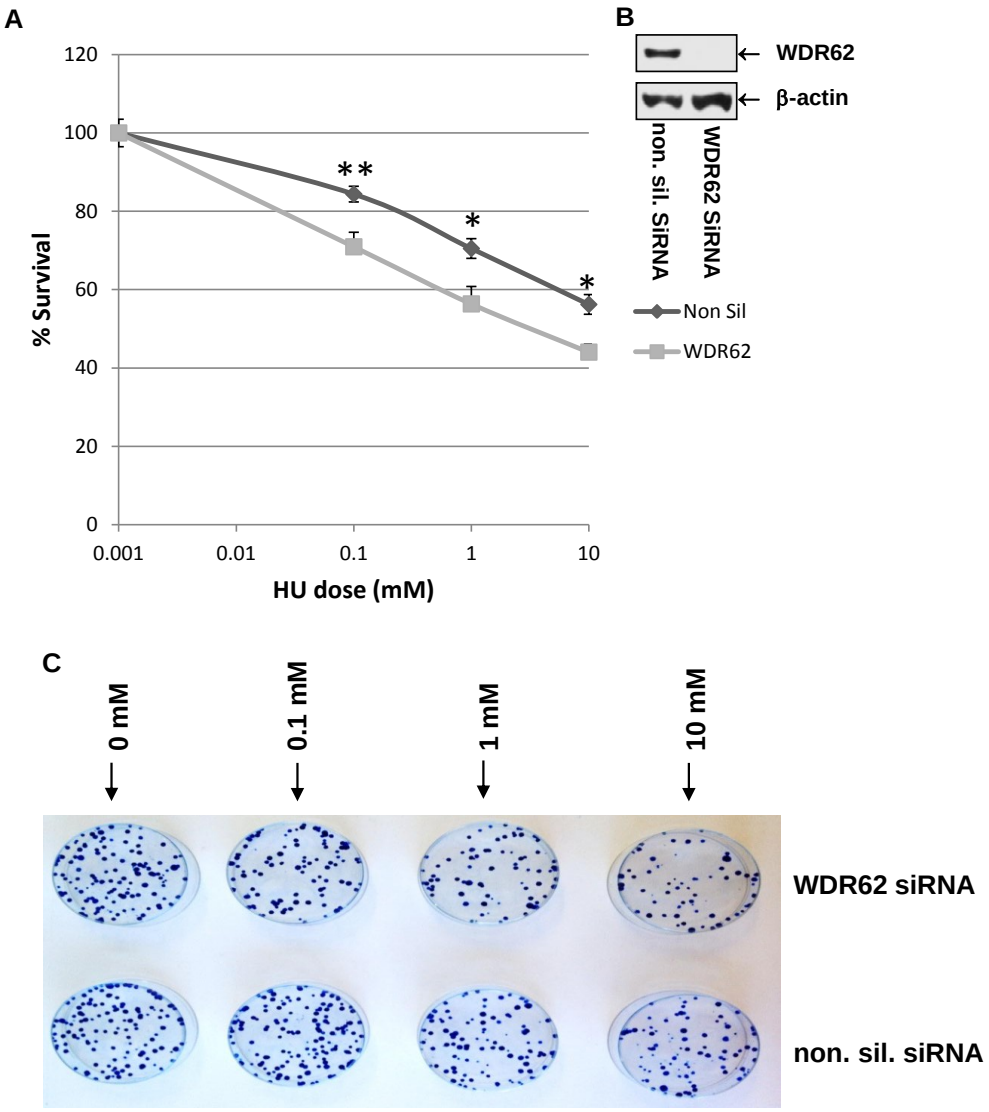

Fig. 5.10. WDR62 depletion sensitizes cells to HU treatment. HeLa cells were transfected with the indicated siRNAs, before being plated at low density 48 hours later. Cells were then treated with the indicated doses of HU and left to grow. Colonies were then counted 14 days later. (A) Graph indicating % cell survival of control, and WDR62-knockdown cells following exposure to specific doses of HU. (B) Western blot analysis showing efficiency of WDR62 knockdown. (C) Representative Figure showing methylene-blue stained cells following 14 days growth post HU-treatment. non. sil., non-silencing. This assay was performed three times in triplicate. Error bars are mean +/- S.D. *P<0.05, ** P<0.01.

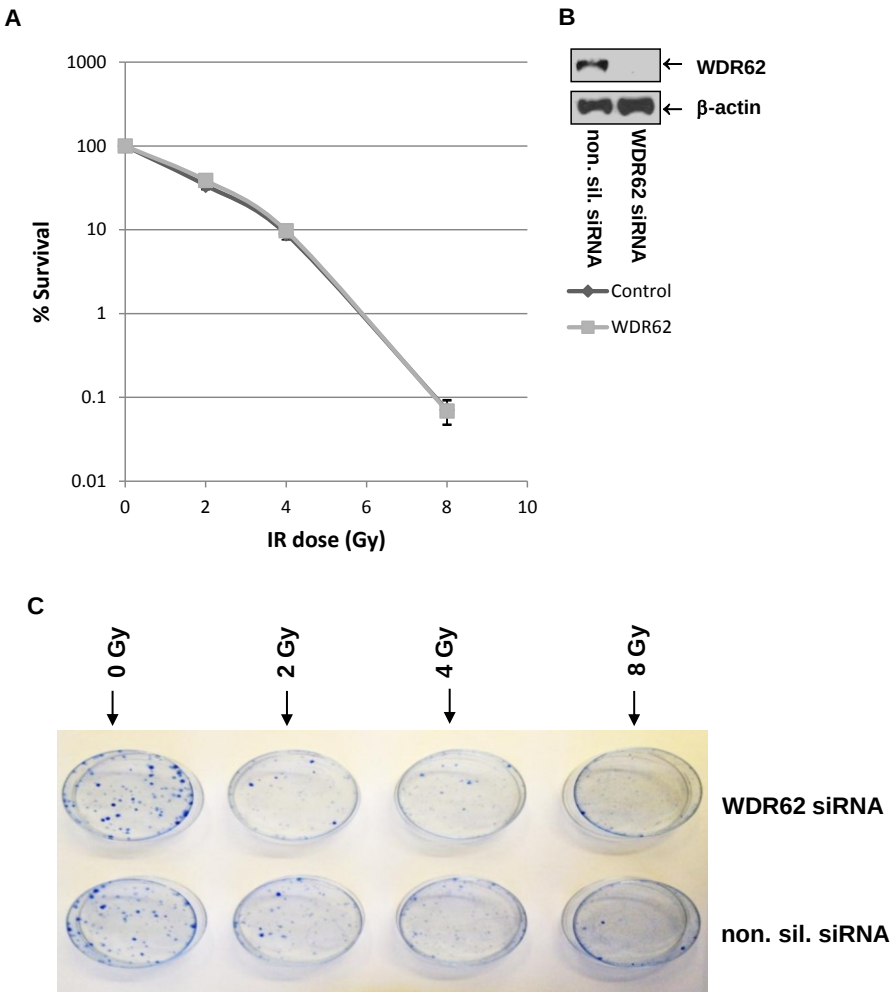

Fig. 5.11. WDR62 depletion does not sensitize cells to IR irradiation. HeLa cells were transfected with the indicated siRNAs, before being plated at low density 48 hours later. Cells were then irradiated with the indicated doses of IR and left to grow. Colonies were then counted 14 days later. (A) Graph indicating % cell survival of control, and WDR62-knockdown cells following exposure to specific doses of IR. (B) Western blot analysis showing efficiency of WDR62 knockdown. (C) Representative Figure showing methylene-blue stained cells following 14 days growth post IR-treatment. non. sil., non-silencing. This assay was performed twice, in triplicate.

irradiation, and fixed by the addition of 70% (v/v) ice-cold ethanol. To determine the number of cells in mitosis we quantified the number of phosphohistone H3 Ser10 (pH3) positive cells by FACS analysis. Cells were therefore incubated with an anti-pH3 polyclonal antibody, followed by incubation with an anti-Rabbit, Alexa-488 secondary antibody, in the dark. Cells were then resuspended in 1 ml of PBS containing 25 μg/ml propidium iodide and 0.1 mg/ml RNAse A, and incubated in the dark again. Cell cycle analysis was then performed using a BD Accuri C6 flow cytometer (Fig. 5.12-5.13).

These analyses revealed that UV irradiation activated a more robust G2-M checkpoint response in control cells, relative to WDR62-knockdown cells, 2 hours post-irradiation; there were more mitotic cells in WDR62-knockdown cells than control cells (Fig. 5.12 B). Indeed, analysis of the numbers revealed that there were 1.6-fold more WDR62-depleted cells, than control cells, in mitosis 2 hours post-irradiation (Fig. 5.13). Further analysis revealed that 4 hours post-irradiation the G2-M checkpoint was still enforced in control cells (Fig. 5.12C). Interestingly, however the maintenance of the G2-M checkpoint in WDR62-knockdown cells was severely compromised (Fig. 5.12C); there were 7.6-fold more mitotic cells in WDR62-knockdown cells, relative to control cells, 4 hours post-irradiation (Fig. 5.13). The differences between control cells and WDR62 cells were statistically significant (Fig. 5.13). Western blot analysis revealed WDR62-knockdown cells were successfully depleted of WDR62 following treatment with appropriate siRNAs (Fig. 5.13B). These data suggest that WDR62 is required for both induction, and maintenance of the G2-M checkpoint, and provides further evidence to indicate that WDR62 participates in ATR signalling pathways in response to DNA damage.

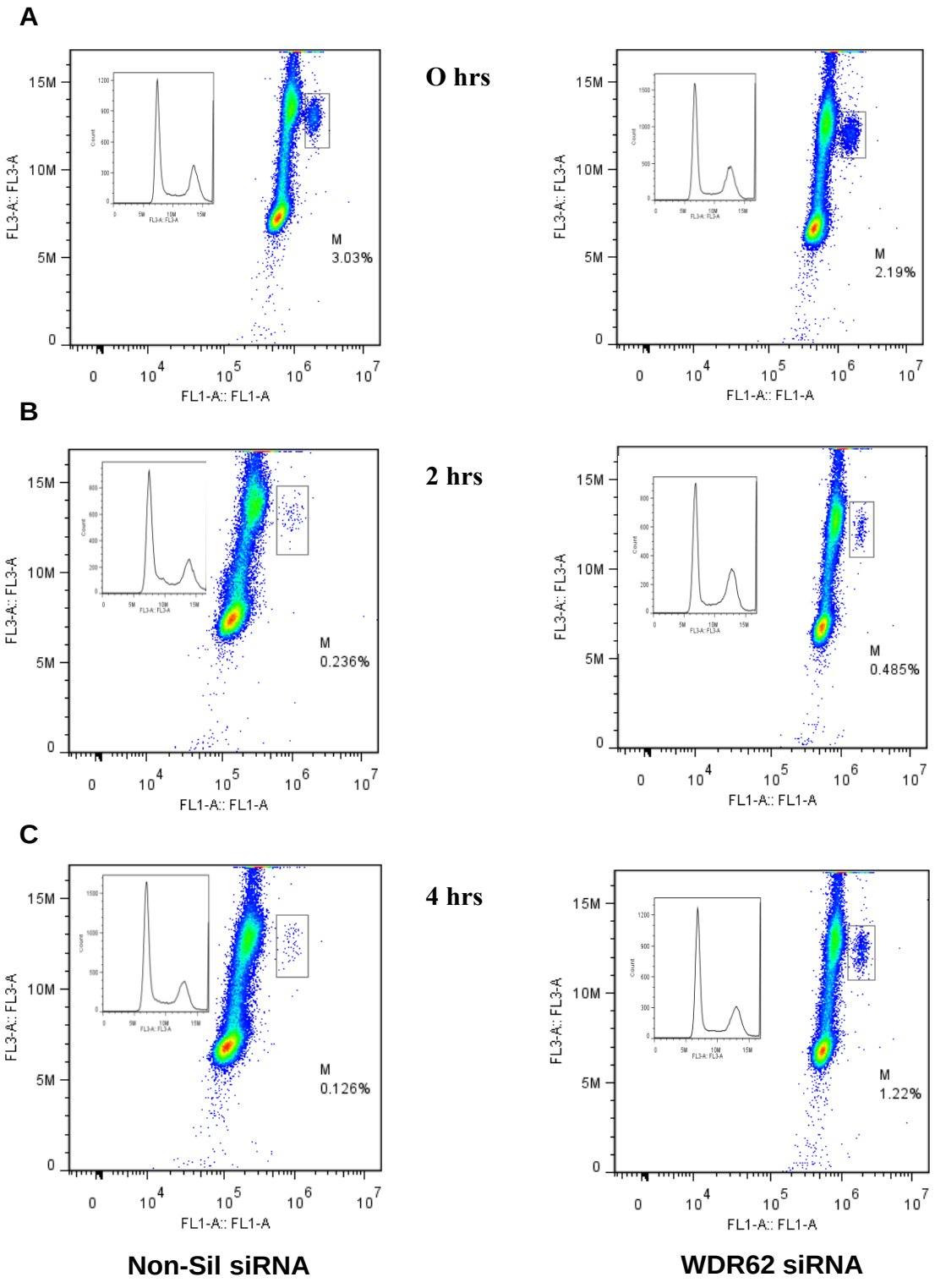

Fig. 5.12. WDR62 is required for efficient activation of the G2-M DNA damage checkpoint. HeLa cells were transfected with the indicated siRNAs, before being irradiated with 20 J/m^2 of UV. Cells were stained with 25 μg/ml propidium iodide (inset profile) and an anti-pH3 antibody, and examined by flow cytometry. The cell cycle distribution is shown by histogram and dot plot. The % of pH3 positive cells (boxed cells in dot plot) is indicated in each panel. (A) cell cycle profile of control and WDR62 knockdown cells, prior to UV irradiation. (B) cell cycle profile, 2 hours post-treatment. (C) cell cycle profile, 4 hours post-treatment.

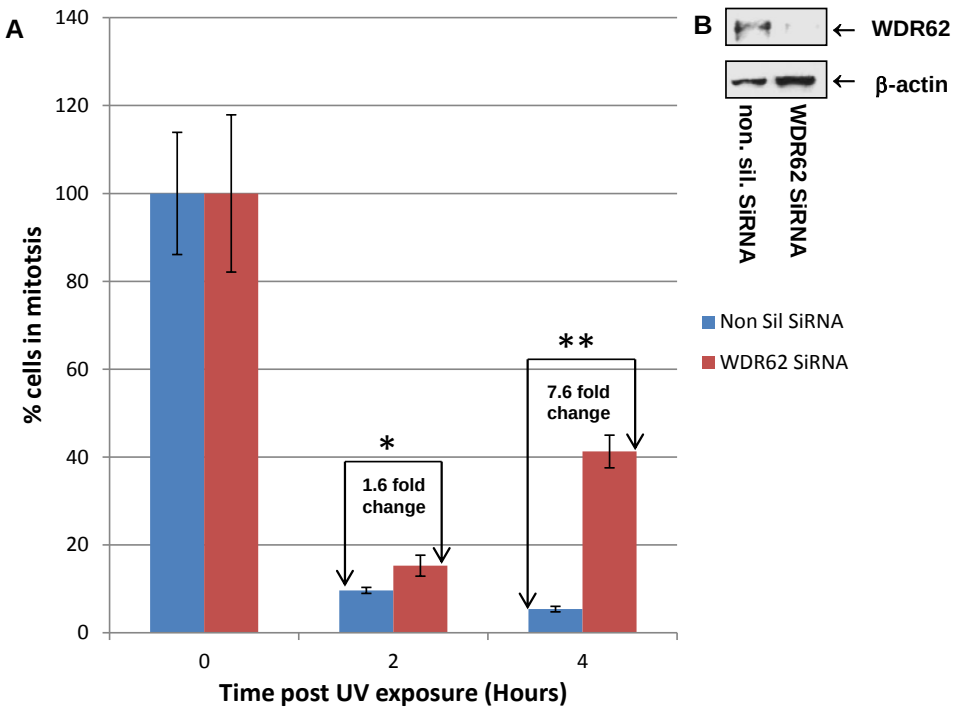

Fig. 5.13. WDR62 is required for efficient activation of the G2-M DNA damage checkpoint. HeLa cells were transfected with the indicated siRNAs, before being irradiated with 20 J/m^2 of UV. Cells were stained with 25µg/ml propidium iodide and pH3 antibody, and examined by flow cytometry. Quantification of the cells in mitosis are presented. The number of cells in mitosis for each condition at time zero, was given an arbitrary value of 100%. (A) Graph indicating % of control, and WDR62-knockdown cells in mitosis following exposure to specific doses of UV. (B) Western blot analysis showing efficiency of WDR62 knockdown This assay was performed three times, in triplicate. *P<0.05, ** P<0.01.

5.2.10. WDR62 is required for the centrosome cycle

Cell lines expressing mutant ATR have been shown to display an abnormal mitotic phenotype, such that cells progressing into mitosis have supernumerary centrosomes that leads to abnormal mitoses and aneuploidy (i.e. >2 centrosomes;(Alderton et al. 2004). Furthermore, cells expressing mutant centrosomal proteins MCPH1 and Pericentrin, which have been shown to play a role in ATR signalling, also display supernumerary centrosomes (Alderton et al. 2006; Griffith et al. 2008). We therefore hypothesized that mitotic cells depleted of WDR62 might also contain supernumerary centrosomes, and hence we performed immunofluorescent confocal microscopy to investigate this possibility. HeLa cells were therefore treated with non-silencing siRNA or WDR62 siRNA for 48 hours, and then seeded onto glass slides and grown for an additional 24 hours. The cells were then harvested by treatment with a pre-extraction buffer and fixation in 4% (w/v) PFA, chapter 2. Fixed cells were then stained with antibodies against WDR62 and γ-tubulin, in order to visualise centrosomes. Finally, the cells were mounted in a DAPI-containing medium to stain the DNA and visualized using a confocal microscope. Cells with supernumerary centrosomes were quantified by counting mitotic cells (*i.e.* prophase to metaphase) with greater than 2 γ-tubulin foci (Fig. 5.14 A-E). Interestingly, we found that there was a statistically significant 2.7-fold increase in the number of WDR62-depleted mitotic cells containing supernumerary centrosomes, when compared to control cells that still express endogenous WDR62 (Fig. 5.14). These analyses suggest that akin to ATR, and other proteins in the ATR signalling pathway that function to control centrosome duplication, WDR62 also participates in this biological process.

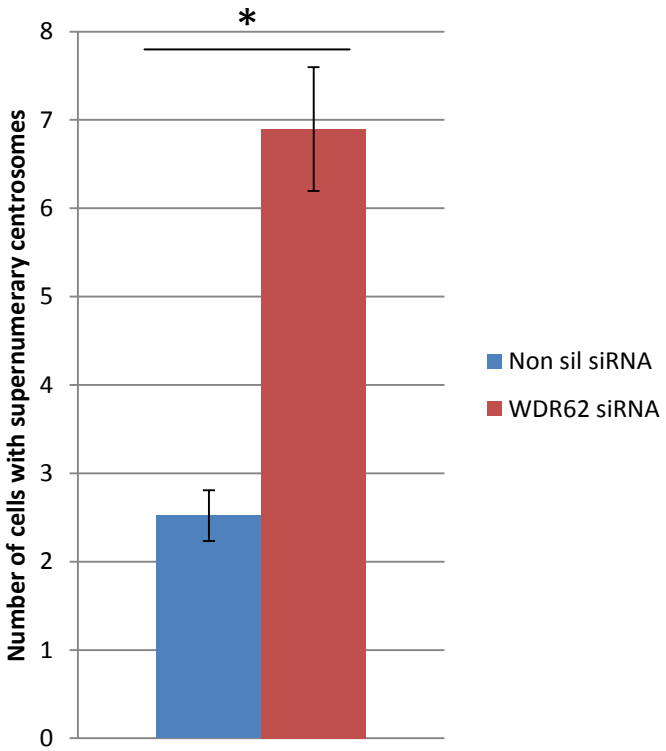

Fig. 5.14. WDR62 is required for centrosome duplication. HeLa cells were transfected with the indicated siRNAs, and then grown on glass coverslips, treated with a pre-extraction buffer before being fixed in 4% (w/v) paraformaldehyde and stained for WDR62, γ-tubulin, and DAPI. Cells with >2 centrosomes were counted. This assay was performed three times counting at least 250 cells for each experiment. *P<0.05.

5.2.11. Mass spectrometric analysis of WDR62-binding proteins

In an attempt to identify potential novel WDR62-interacting proteins, UV-irradiated and non-irradiated HeLa cells were harvested in NETN lysis buffer, and incubated with anti-IgG control, or anti-WDR62 antibody to immunoprecipitate WDR62 and associated proteins from the cell lysate. These immunoprecipitates were then incubated with Protein G-sepharose to isolate immunocomplexes, which were then separated by SDS-PAGE. The gels were then stained with Coomassie Brilliant Blue G-250 solution to visualise protein bands. Given that there were no clear differences in the protein bands between the control and positive lanes, we excised multiple sections of the gel, and then treated them with modified trypsin that cleaves C-terminal arginine or lysine residues. The Bruker AmaZon ion trap ETD-enabled mass spectrometer was used to analyze peptides, which were then identified by the ProteinScape bioinformatic platform.

This screen identified WDR62 and a number of potential interacting proteins for WDR62 both in the absence and presence of UV (Table 5.1). As only one or two peptides were identified for each potential interacting protein, these interactions need to be validated by other methods. However, given that proteins like RPA70, ATR, DNA-PK and BRCA1 were pulled down in this screen, it gives some credence to our data which suggests that WDR62 plays a role in DNA damage signalling.

Protein	MW (kDa)	Number of peptides	Mascot score	Sequence coverage
- UV				
WDR62	165.9	74	4312.3	36.4%
APC2	243.8	1	58.7	1%
KU70	69.8	2	56.3	4.9%
PCNT	377.8	2	52.7	0.5%
BRCA2	384	2	51.9	0.6%
SMC1	143.1	1	32.6	1.3%
PIAS1	71.8	1	28.6	1.4%
XRCC1	69.5	1	28.6	1.4%
BLM	158.9	1	28.4	1.2%
CYCLIN D1	33.7	1	25.4	4.7%
MCM6	92.8	1	25	2.7%
+UV				
WDR62	165.9	39	2160	20.4%
DNA-PKcs	468.8	1	67.2	0.7%
HUWE1	481.6	2	51.3	0.6%
CYCLIN D1	33.7	2	50.8	4.7%
RPA70	68	1	28.9	2.6%
FANCA	163	1	28.4	0.6%
ATR	301	1	28.4	0.4%
MCM4	96.5	1	26.1	1.7%
PML	97	1	26	2.9%
PER2	136.5	1	25.8	1.0%
AP-1	35.7	1	25.2	6.3%
BRCA1	207	1	25.2	0.6%

Table 5.1. Mass spectrometric identification of WDR62 and WDR62-interacting proteins from anti-WDR62 immunoprecipitates. HeLa cells were either mock treated (-UV) or treated with UV irradiation (10 J/m^2) and allowed to recover for 1 hour. Cell lysates were prepared and WDR62 and interacting proteins were identified by mass spectrometry following their immunoprecipitation with an anti-WDR62 antibody and isolation upon SDS-PAGE.

5.3. DISCUSSION

As viruses have evolved a number of strategies to negate the host cell activation of antiviral responses, they have served as useful models to enhance our understanding of fundamental cellular functions (Blackford and Grand 2009). Adenovirus E1B-55K is a multifunctional oncoprotein which has been shown to bind to a number of cellular proteins involved in the DDR, such as p53, Mrell, DNA ligase IV, and BLM (Querido et al. 2001; Stracker et al. 2002; Baker et al. 2007; Orazio et al. 2011). Mass spectrometry data from our laboratory identified a number of novel interacting proteins for Ad12 E1B-55K, one of which was the centrosomal protein, WDR62. Given that other centrosomal proteins, MCPH1, and Pericentrin, have been shown to play a role in ATR signalling after DNA damage, we hypothesised that WDR62 might play a role in DNA damage signalling. The aim of the work presented in this chapter was to confirm the interaction between E1B-55K, and investigate its potential role in viral infection, and its role in the DDR pathway. In the study described here we provide evidence to show that WDR62 binds to E1B-55K *in vivo*, and is required for efficient ATR signalling in response to genotoxic stress.

In addition to demonstrating that E1B-55K does indeed bind to WDR62 *in vivo* in Ad E1-transformed cells (Fig. 5.1), we went on to show that E1B-55K co-localizes with WDR62 at centrosomes in Ad5 E1-transformed cells, which is interesting given that E1B-55K has not previously been described to localize at centrosomes (Fig. 5.3-5.4). This is also the first time that E1B-55K has been shown to interact with a centrosomal protein. Unfortunately, the commercial antibody available for WDR62 is very weak for immunoprecipitation assays, hence we were unable to perform the reciprocal WDR62 immunoprecipitation and E1B-55K Western blot, or identify strong interactors using

mass spectrometry (Table 5.1). One of the future directives would be to produce both mouse monoclonal and rabbit polyclonal antibodies against WDR62 to analyze its biological functions in greater detail; this is considered in chapter 6.

A large number of proteins that interact with E1B-55K have been shown to be targeted for proteasomal degradation during Ad infection (Querido et al. 2001; Stracker et al. 2002; Liu et al. 2005; Baker et al. 2007; Dallaire et al. 2009; Schreiner et al. 2010; Orazio et al. 2011; Forrester et al. 2012). The E1B-55K-binding protein, WDR62 was not however targeted for degradation during infection (Fig. 5.2). Instead, levels were more comparable to those seen for other E1B-55K interacting proteins such as E1B-AP5 and TIF1β (Blackford et al. 2008; Forrester et al. 2012). Interestingly, there appeared to be an increase in the molecular weight of WDR62 48 hours post-infection, suggesting that it had undergone a post translational modification (Fig. 5.2). E1B-55K has been shown to promote the SUMOylation of p53 (Muller and Dobner 2008), though whether WDR62 is SUMOylated, or subject to another form of post-translational modification, during infection requires further investigation. Identification of this modification might give greater insight into WDR62 function.

Our laboratory has previously reported that E1B-AP5 has role in the DDR and that it is also required for RPA32 phosphorylation in response to adenovirus infection (Blackford et al. 2008). Using RNA interference we have determined that like E1B-AP5, WDR62 is also required for efficient RPA32 phosphorylation during adenovirus infection (Fig. 5.5). We then went onto investigate a possible role for WDR62 in the DNA damage response pathway by exposing cells treated with control siRNA, or WDR62 siRNA to a range of genotoxic agents, and then observing DDR pathway activation and phosphorylation of DDR proteins. Interestingly, we found that WDR62 was essential for efficient phosphorylation and activation of Chk1 and RPA32 in

response to UV- or HU- induced DNA damage (Figs. 5.6-5.7). However, in contrast, WDR62 depletion did not appear to have a significant impact on the DNA damage response to IR (Figs. 5.8).

Cells expressing mutant ATR have been shown to display increased sensitivity to exposure with UV and IR irradiation (Wright et al. 1998). Furthermore cells are also hypersensitive to exposure to genotoxic agents when they harbour mutations in other DDR genes, for example *ATM* or *XPA* (Sancar et al. 2004). Given that we had provided evidence for a potential role of WDR62 in the DNA damage response, the possibility that WDR62 depletion may sensitize cells to UV, HU, or IR exposure was examined. Interestingly, we found that cells depleted of WDR62 were indeed hypersensitive to UV irradiation, as well as HU treatment, further substantiating our idea that WDR62 plays a role in the ATR signalling pathway (Fig. 5.9-5.10). In contrast, however we found that depletion of WDR62 did not increase the sensitivity of cells to IR exposure (Fig. 5.11), which was interesting given that molecular cross talk between ATM and ATR has been shown to occur (Cuadrado et al. 2006).

The Chk1 kinase is required for G2-M checkpoint arrest in response to DNA damage (Sanchez et al. 1997). Given that we had shown that cells depleted of WDR62 were not able to phosphorylate Chk1 in response to DNA damage, we also examined the G2-M checkpoint in WDR62-depleted cells in response UV irradiation. As expected, we found that the G2-M checkpoint control was significantly abrogated in cells depleted of WDR62 (Fig. 5.12-5.13). A similar phenotype is displayed when another protein involved in the ATR signalling pathway, TopPB1, is depleted from cells, that are then exposed to genotoxic agents (Yamane et al. 2003). TopBP1 has been shown to be essential for certain ATR-dependent signalling events, including Chk1 and NBS1 phosphorylation (Kumagai et al. 2006). Given that like TopBP1, WDR62 depletion

inhibits Chk1 phosphrylation and subsequent G2-M checkpoint control, our data indicates that WDR62 functions in the ATR signalling pathway. It is also interesting to note that Chk1 has been observed to localize to the centrosome, and this localization is increased after DNA damage due to phosphorylation at Ser317 and Ser345 (Niida et al. 2007). Given these observations, it would be interesting to establish whether WDR62 interacts with TopBP1 and/or Chk1 *in vivo*, or localizes with these proteins at centrosomes.

Cell lines expressing mutant ATR have been shown to display an abnormal mitotic phenotype; cells have supernumerary centrosomes in mitosis (Alderton et al. 2004). Given that we have provided evidence to show that WDR62 may play a role ATR signalling pathways in response to DNA damage, and also the fact that WDR62 is a centrosomal protein, we examined the centrosome cycle in cells depleted of WDR62. Here we observed that WDR62-depleted mitotic cells did indeed display an increase in cells that had supernumerary centrosomes (Fig. 5.14). MCPH1, like WDR62 is a centrosomal protein, where mutations in the genes encoding these proteins lead to primary microcephaly. Interestingly MCPH1 has been found to play a role in the ATR signalling pathway, whereby *MCPH1*-mutant cell lines also have defective UV-induced G2-M checkpoint arrest, as well as containing supernumerary centrosomes in mitosis (Alderton et al. 2006). Also, another centrosomal protein Pericentrin has also been found to play a role in ATR signalling (Griffith et al. 2008). Mutations in this protein give rise to Seckel syndrome which is an autosomal recessive disorder characterized by growth retardation, severe proportionate short stature and marked microcephaly (Griffith et al. 2008). Furthermore, cells that express mutated Pericentrin also have defective UV-induced G2-M checkpoint arrest, as well as containing mitotic supernumerary centrosomes (Griffith et al. 2008). Thus it appears that like other

centrosomal proteins, WDR62 also functions in ATR signalling pathways. Given these similarities with Pericentrin and MCPH1, it would also be interesting to establish the relationship between WDR62 and these proteins.

In this regard we attempted to identify novel WDR62-interacting proteins in both non-stressed cells, and in response to genotoxic stresses that activate the ATR pathway. To do this we performed mass spectrometric analyses following the immunoprecipitation of WDR62 from non-stressed and UV-irradiated cells. In addition to identifying WDR62, we identified a limited number of potential interacting proteins, which include RPA70, ATR, DNA-PK and BRCA1 (Table 5.1). However, given that the WDR62 antibody was not particularly good for immunoprecipitation, this mass spectrometric data is not definitive, and needs to be repeated with other anti-WDR62 antibodies to substantiate these findings.

In conclusion, the studies detailed in this Chapter, have identified novel functions of a cellular E1B-55K interacting protein, and give further credence to the importance of studying the biological functions of adenovirus oncoproteins. Specifically, we identified WDR62 as a new E1B-55K interacting protein, and provided considerable evidence to suggest that it functions in ATR signalling pathways activated in response to DNA damage.

CHAPTER 6

FINAL DISCUSSION AND FUTURE PERSPECTIVES

6.1. ROLE FOR AD12 E4ORF6 AS A NEGATIVE REGULATOR OF ATR

The adenovirus oncoprotein, E4orf6 functions to regulate viral DNA replication, late mRNA export, and host-cell shutoff (Halbert et al. 1985). In addition to these roles, E4orf6 is also essential for negating the host cell DDR, which is required to prevent concatenation of the Ad genome (Weiden and Ginsberg 1994; Stracker et al. 2002). The majority of the functions carried out by E4orf6 require the protein to function in concert with E1B-55K, the most well-known example of which is to promote the degradation of cellular proteins involved in DDR pathways. In chapter 3 we described a novel mechanism where Ad12 E4orf6 was able to promote the proteasome-mediated degradation of TopBP1 in an Ad12 E1B-55K-independent manner. TopBP1 is essential for the ATR-dependent phosphorylation and activation of Chk1 in response to DNA damage (Kumagai et al. 2006). Our laboratory has previously shown that ATR is differentially activated by Ad5 and Ad12 during infection (Blackford et al. 2008). The mechanism by which Ad5 inhibits Chk1 phosphorylation has been shown to be as a result of the relocalization of MRN into nuclear tracks by Ad5 E4orf3, but Ad12 E4orf3 proteins are unable to carry out this function (Stracker et al. 2005; Carson et al. 2009). In chapter 3 we determined that Ad12 inhibits Chk1 activation by promoting the degradation of the ATR activator, TopBP1.

We have shown that the Ad12 E4orf6-mediated degradation of TopBP1 inhibits Chk1 phosphorylation in response to genotoxic stresses that normally activate the ATR signalling pathway. It is unclear however, as to how Chk1 inactivation is beneficial for Ad12 infection, and it would therefore be of great interest to identify a separation-of-function TopBP1 mutant that is resistant to Ad12 E4orf6-mediated degradation, but still

retains its ability to activate ATR. We could then examine how ATR and Chk1 activation is detrimental to Ad12-infected cells, by determining its effects on Ad12 viral DNA replication, viral mRNA synthesis, viral early and late protein synthesis, viral genome packaging and the production of new productive virions. Furthermore, TopBP1 functions in other cellular processes such as DNA replication where it functions as a modulator G_1/S transition (Kim et al. 2005). It would therefore be interesting to see if other functions of TopBP1 are detrimental for Ad12 infection, by overexpressing a non-degradable TopBP1 mutant and inhibiting Chk1 activation with a Chk1 inhibitor such as 7-hydroxystaurosporine (UCN-01), and then observing any affects upon Ad12 infection (Busby et al. 2000).

Adenovirus, HPV, HSV, EBV, and SV40 are just a few examples of DNA viruses that affect ATM/and or ATR signalling pathways during infection, as a result of the host cell recognising viral DNA as damaged cellular DNA, or potentially, due to deliberate activation by the virus or viral proteins (Turnell and Grand 2012). For instance, adenovirus partially activates the ATR signalling pathway, whilst SV40 activates the ATR signalling pathway, and SV40 LTag binds directly to RPA70 which is required for initiation of SV40 viral genome replication (Dornreiter et al. 1992; Melendy and Stillman 1993). Moreover, HSV-1 was originally shown to attenuate ATR activation by disrupting the association between ATR-ATRIP, although a more recent study has now shown that this complex does, in actual fact, remain intact, and instead ATR-ATRIP are sequestered to replication compartments where they are functionally inactivated (Wilkinson and Weller 2006; Mohni et al. 2010). Given that adenovirus has been shown to activate some components of the ATR signalling pathway, as well as recruiting ATR, ATRIP, Rad9, RPA, Rad17, TopBP1, and E1B-AP5 to VRCs, it would be interesting to investigate if, and how this is beneficial for the virus (Carson et al.

2003; Blackford et al. 2008; Carson et al. 2009). One approach would be to deplete cells of these proteins and investigate the consequences of their inactivation upon viral infection as outlined above.

In chapter 3 we also described for the first time that adenovirus differentially regulates Cullin ring ligases during infection. We showed that Ad5 activates and utilizes CRL5 to promote the degradation of p53, and inactivates CRL2, as is evident by its neddylation patterns. In contrast Ad12 increasingly activates a CRL2 and utilizes it to degrade both p53 and TopBP1. Furthermore, other viruses have been shown to target Cullin ring ligases during infection to facilitate the degradation of cellular proteins, however, it has not been shown before that two viruses from the same family can use different Cullin ring ligases to degrade host proteins as demonstrated in this study. As different Ads promote the selective activation of Cullin ring ligases through their ability to interact with these complexes and promote Cullin neddylation it would be interesting to establish whether E4orf6 also interacts with, and activates the NEDD8 -activating enzymes, and -conjugating enzymes responsible for this modification.

In the study described in chapter 3 we have also described a novel role for Ad12 E4orf6 as both a Cullin ring ligase recruiter and, a substrate adapter, by showing that this oncoprotein can bind directly to TopBP1 and recruit it to a CRL2, which then ubiquitylates TopBP1 targeting it for proteasomal degradation. E4orf6 has not been shown to interact directly with many cellular proteins, and instead interaction occurs mainly via E1B-55K, although E4orf6 is able to bind directly to p53 to repress its transcriptional activity (Dobner et al. 1996). Most of the studies carried out on the function of E4orf6 are from the Ad5 serotype, for which there is a panel of monoclonal antibodies. The novel function of Ad12 E4orf6 that we have described appears to be restricted within the group A viruses, as Ad5 E4orf6 is unable to degrade TopBP1. It

would therefore be interesting to confirm which Ad types are able to degrade TopBP1. It would also be interesting to make and characterize monoclonal and polyclonal antibodies raised against Ad12 E4orf6 protein, in order to identify novel Ad12 E4orf6-interacting proteins by Mass spectrometry. As E4orf6 can promote the degradation of cellular proteins, it would be prudent to do these analyses in the absence and presence of the proteasome inhibitor, MG132. Once identified, the role of novel Ad12 E4orf6 proteins in ATM and ATR pathways, and other DDR pathways should be examined, as well as determining whether Ad12 E4orf6 also promotes their degradation.

We have shown that Ad12 E4orf6 alone is able to inhibit Chk1 phosphorylation and activation, which might have some benefit for anti-cancer therapies. In this regard it has been proposed that in normal cells harbouring *wt* p53, Chk1 inactivation does not hinder chemotherapeutic p53-dependent G1 arrest, whilst Chk1 inactivation in tumour cells that lack a functional p53, and hence a G_1/S checkpoint, will undergo mitotic catastrophe and cell death (Chen et al. 2006; Xiao et al. 2006). Given these observations it would be extremely interesting to explore whether we could engineer an Ad12 E4orf6 mutant that retained its ability to inhibit Chk1, yet was unable to repress p53 function (assuming that like Ad5 E4orf6, Ad12 E4orf6 has this property). If this mutant could be made it might promote the selective killing of cancer cells in combination with other therapeutics.

6.2. ROLE FOR AD12 E4ORF3 AS A NEGATIVE REGULATOR OF ATR

The adenovirus E4orf3 oncoprotein shares functionally redundancy with E4orf6, such that these proteins perform overlapping functions (Halbert et al. 1985). In chapter 4 we

presented evidence to indicate that Ad12 E4orf3, like Ad12 E4orf6, can independent of Ad12 E4orf6 and E1B-55K, promote the proteasome-mediated degradation of TopBP1; Ad12 E4orf3 and Ad12 E4orf6 differ in some regards, however as Ad12 E4orf3 can promote the degradation of Timeless and Tipin, whereas Ad12 E4orf6 cannot. The resultant effect of Ad12 E4orf3 and E4orf6 expression is to inhibit the ATR-dependent activation of Chk1, as TopBP1, Timeless and Tipin are all essential for Chk1 phosphorylation and activation in response to DNA damage (Unsal-Kacmaz et al. 2005; Unsal-Kacmaz et al. 2007; Kemp et al. 2010).

Ad12 E4orf3 utilizes CRL2 to degrade to TopBP1, Timeless and Tipin. It would be interesting to confirm if, like Ad12 E4orf6, Ad12 Eorf3 does indeed interact directly with CRL2 using co-immunoprecipitation assays. Interestingly, Ad12 E4orf3, like Ad12 E4orf6 contains a BC-box motif, as well as a Cul2-box motif, though whether these are functional remains to be determined. It would be interesting to mutate these motifs and establish if they are required to degrade TopBP1, Timeless and Tipin.

In chapter 4 we showed that the Ad12 E4orf3-mediated degradation of these three proteins occurs independently of Ad12 E1B-55K and Ad12 E4orf6. Our laboratory has shown that both Ad12 and Ad5 E4orf3 are able to degrade TIF1γ, which also occurs independently of other viral proteins, however there are a few differences between this study and the study presented in chapter 4 (Forrester et al. 2012). Firstly, the ubiquitin ligase required for the degradation of TIF1γ is unknown, whereas we have shown here that CRL2 is required for TopBP1, Timeless and Tipin degradation. Secondly, TIF1γ is reorganized into nuclear tracks by E4orf3 prior to degradation, but this is not the case for TopPB1, Timeless or Tipin. Finally, degradation of TIF1γ occurs in both Ad5- and Ad12- infected cells, whereas degradation of the three proteins described in this study appears to be specific to Ad12. It would be interesting to determine if Ad12 E4orf3 is

able to bind directly to TopBP1, Timeless and Tipin, as well as confirming if the degradation of these proteins is conserved between group A adenoviruses.

Most of the work to date that has contributed to our understanding of the functions of the E4orf3 protein has come from studying the Ad5 serotype. The data presented in chapter 4 further highlights the differences between functions of the E4orf3 protein from the two different serotypes Ad5 and Ad12. Ad5 and Ad12 E4orf3 proteins are only 45% identical and share 69% similarity at the amino acid level, therefore it would be beneficial to study the Ad12 E4orf3 protein in more depth as it may reveal novel functions of this protein, and give further insight into Ad E4orf3 function *per se*. Firstly, we would need to produce and characterize monoclonal and polyclonal antibodies raised against the Ad12 E4orf3 protein. Using these antibodies we could then identify possible novel Ad12 E4orf3-interacting proteins by Mass spectrometry and investigate their roles in Ad infection, and in the DDR pathway.

It would also be of interest to determine, more generally, the role of Ad12 E4orf3 and Ad12 E4orf6 in promoting proteasome-mediated degradation, to see if there are other substrates targeted by these proteins. To do this we could generate adenovirus vectors expressing either Ad12 E4orf3 or Ad12 E4orf6, and assess, by Stable Isotope Labelling by Amino acids in Cell Culture (SILAC) the proteome levels, of cellular proteins using mass spectrometry, before and after expression of these proteins. This would help identify novel proteins that are targeted for degradation by these proteins, and which might function in DDR pathways.

6.3. A ROLE FOR WDR62 IN THE DNA DAMAGE RESPONSE

Viral oncoproteins have been shown to interact with a number of cellular proteins, most commonly tumour suppressors and crucial cell cycle regulators. Indeed, the p53 tumour suppressor gene product, p53 was originally identified as a viral oncoprotein-interacting protein (Lane and Crawford 1979; Linzer and Levine 1979; Whyte et al. 1988) . E1B-55K itself interacts with a wide range of proteins which include p53, the MRN complex, DNA ligase IV, TIF1γ, BLM and Daxx (Sarnow et al. 1982; Liu et al. 2005; Baker et al. 2007; Schreiner et al. 2010; Orazio et al. 2011; Forrester et al. 2012). It is therefore of great value to study the biological functions of proteins that have been identified as novel cellular interactors of E1B-55K. Our laboratory identified WDR62 as a potential E1B-55K-interacting protein using an immunoprecipitation/mass spectrometry approach. In chapter 5 we confirmed the E1B-55K/WDR62 interaction *in vivo* using Ad5- and Ad12- E1 transformed cells.

The fate of many E1B-55K interactors, is proteasome-mediated degradation, however we observed that WDR62 in not a target for Ad-mediated degradation using Western blotting and immunofluorescence. Instead, E1B-55K forms structures that encapsulate WDR62 foci in interphase cells. Furthermore, at late times after infection there is an increase in the molecular weight of the WDR62 protein which is indicative of a post-translational modification. Recent evidence has shown for the first time that WDR62 does indeed undergo a post-translational modification by JNK, such that phosphorylated WDR62 is required to maintain metaphase spindle organization during mitosis (Bogoyevitch et al. 2012). There is also evidence to suggest that E1B-55K can engage with cellular E3 SUMO ligase activity (Muller and Dobner 2008), and it would therefore be interesting to investigate if WDR62 is subject to post-translational

modification following Ad infection, either by SUMOylation or phosphorylation, and determine the consequences of WDR62 modification upon its role in DDR pathways.

E1B-55K interacts with a number of proteins involved in many cellular processes, including the DDR, and it is therefore easy to postulate that novel interactors may function in one of these pathways. There are also numerous examples of centrosomal proteins that have been shown to function in the DDR pathways. One such example is MCPH1, which is mutated in primary autosomal microcephaly, and has been implicated as a possible tumour suppressor, where it functions in both ATM and ATR signalling pathways in response to DNA damage (Rai et al. 2006). Indeed, in chapter 5 we presented evidence to indicate that WDR62 is required for phosphorylation of ATR targets in response to DNA damage, and is also required for efficient G_2/M damage checkpoint activation. Further work revealed that cells depleted of WDR62 also displayed increased sensitivity to genotoxic stress. Using Mass spectrometry, we attempted to identify novel WDR62-interacting proteins; using this approach we putatively identified amongst others, ATR, RPA70, BRCA1, DNAPK and cyclin D1 as WDR62-interacting proteins. Other studies have shown that MCPH1 co-localizes with ATR and RPA in response to UV-induced DDR, and is essential for the ATR-mediated phosphorylation of RPA (Rai et al. 2006). Furthermore, MCPH1 has been shown to act as a transcriptional regulator of BRCA1 and Chk1, and is thus required for the regulation of the intra-S and G_2/M DNA damage checkpoints (Xu et al. 2004; Alderton et al. 2006). It would therefore be interesting to investigate further whether WDR62 interacts with MCPH1 and also functions in these processes. For this it would be extremely beneficial to create a new panel of anti-WDR62 antibodies as the current, commercially available antibody, although it appears to immunoprecipitate WDR62, does not co-precipitate many other proteins. By creating a GST-WDR62 construct we

could generate anti-WDR62 mouse monoclonal and rabbit polyclonal antibodies. We could then use these antibodies for immunoprecipitation and binding assays to re-evaluate the WDR62 interactome and define the role of these proteins in the DDR and cell cycle checkpoints. It would also be intriguing to see if we could reverse the phenotype that is observed when cells are depleted of WDR62 and subjected to genotoxic stress agents by making a *wt* WDR62-siRNA resistant construct, and then transfecting it into cells depleted of WDR62. Furthermore, it would also be interesting to examine whether WDR62 functions to promote Ad replication by determining the effects of WDR62 knockdown on all aspects of Ad replication, as described above. In conclusion, the studies presented in this thesis further highlight the importance of studying adenoviruses in the 21st century, and that investigation into adenovirus oncoprotein function still has much to offer in terms of understanding the molecular basis of fundamental cellular processes.

CHAPTER 7

REFERENCES

Abraham, R. T. (2001). "Cell cycle checkpoint signaling through the ATM and ATR kinases." Genes Dev **15**(17): 2177-2196.

Abuelo, D. (2007). "Microcephaly syndromes." Seminars in Pediatric Neurology **14**(3): 118-127.

Adams, K. E., A. L. Medhurst, et al. (2006). "Recruitment of ATR to sites of ionising radiation-induced DNA damage requires ATM and components of the MRN protein complex." Oncogene **25**(28): 3894-3904.

Ahmed, A., J. Yang, et al. (2011). "Pharmacological activation of a novel p53-dependent S-phase checkpoint involving CHK-1." Cell Death & Disease **2**: e160.

Alderton, G. K., L. Galbiati, et al. (2006). "Regulation of mitotic entry by microcephalin and its overlap with ATR signalling." Nature Cell Biology **8**(7): 725-733.

Alderton, G. K., H. Joenje, et al. (2004). "Seckel syndrome exhibits cellular features demonstrating defects in the ATR-signalling pathway." Human Molecular Genetics **13**(24): 3127-3138.

Araujo, F. D., T. H. Stracker, et al. (2005). "Adenovirus type 5 E4orf3 protein targets the Mre11 complex to cytoplasmic aggresomes." Journal of Virology **79**(17): 11382-11391.

Babiss, L. E., H. S. Ginsberg, et al. (1985). "Adenovirus E1B proteins are required for accumulation of late viral mRNA and for effects on cellular mRNA translation and transport." Molecular and Cellular Biology **5**(10): 2552-2558.

Bagchi, S., P. Raychaudhuri, et al. (1990). "Adenovirus E1A proteins can dissociate heteromeric complexes involving the E2F transcription factor: a novel mechanism for E1A trans-activation." Cell **62**(4): 659-669.

Baker, A., K. J. Rohleder, et al. (2007). "Adenovirus E4 34k and E1b 55k oncoproteins target host DNA ligase IV for proteasomal degradation." Journal of Virology **81**(13): 7034-7040.

Bakkenist, C. J. and M. B. Kastan (2003). "DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation." Nature **421**(6922): 499-506.

Bakkenist, C. J. and M. B. Kastan (2004). "Initiating cellular stress responses." Cell **118**(1): 9-17.

Ball, H. L., M. R. Ehrhardt, et al. (2007). "Function of a conserved checkpoint recruitment domain in ATRIP proteins." Molecular and Cellular Biology **27**(9): 3367-3377.

Barker, D. D. and A. J. Berk (1987). "Adenovirus proteins from both E1B reading frames are required for transformation of rodent cells by viral infection and DNA transfection." Virology **156**(1): 107-121.

Barlow, C., S. Hirotsune, et al. (1996). "Atm-deficient mice: a paradigm of ataxia telangiectasia." Cell **86**(1): 159-171.

Bartek, J., C. Lukas, et al. (2004). "Checking on DNA damage in S phase." Nature reviews. Molecular Cell Biology **5**(10): 792-804.

Bartek, J. and J. Lukas (2001). "Mammalian G1- and S-phase checkpoints in response to DNA damage." Current Opinion in Cell Biology **13**(6): 738-747.

Bartek, J. and N. Mailand (2006). "TOPping up ATR activity." Cell **124**(5): 888-890.

Bartkova, J., Z. Horejsi, et al. (2005). "DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis." Nature **434**(7035): 864-870.

Bekker-Jensen, S., J. Rendtlew Danielsen, et al. (2010). "HERC2 coordinates ubiquitin-dependent assembly of DNA repair factors on damaged chromosomes." Nature Cell Biology **12**(1): 80-86; sup pp 81-12.

Ben-Israel, H. and T. Kleinberger (2002). "Adenovirus and cell cycle control." Frontiers in Bioscience : A Journal and Virtual Library **7**: d1369-1395.

Benna, C., P. Scannapieco, et al. (2000). "A second timeless gene in Drosophila shares greater sequence similarity with mammalian tim." Current Biology : CB **10**(14): R512-513.

Berget, S. M., C. Moore, et al. (1977). "Spliced segments at the 5' terminus of adenovirus 2 late mRNA." Proceedings of the National Academy of Sciences of the United States of America **74**(8): 3171-3175.

Berk, A. J. (2005). "Recent lessons in gene expression, cell cycle control, and cell biology from adenovirus." Oncogene **24**(52): 7673-7685.

Bischoff, J. R., D. H. Kirn, et al. (1996). "An adenovirus mutant that replicates selectively in p53-deficient human tumor cells." Science **274**(5286): 373-376.

Blackford, A. N., R. K. Bruton, et al. (2008). "A role for E1B-AP5 in ATR signaling pathways during adenovirus infection." Journal of Virology **82**(15): 7640-7652.

Blackford, A. N. and R. J. Grand (2009). "Adenovirus E1B 55-kilodalton protein: multiple roles in viral infection and cell transformation." Journal of Virology **83**(9): 4000-4012.

Blackford, A. N., R. N. Patel, et al. (2010). "Adenovirus 12 E4orf6 inhibits ATR activation by promoting TOPBP1 degradation." Proceedings of the National Academy of Sciences of the United States of America **107**(27): 12251-12256.

Blanchette, P., C. Y. Cheng, et al. (2004). "Both BC-box motifs of adenovirus protein E4orf6 are required to efficiently assemble an E3 ligase complex that degrades p53." Molecular and Cellular Biology **24**(21): 9619-9629.

Blanchette, P., K. Kindsmuller, et al. (2008). "Control of mRNA export by adenovirus E4orf6 and E1B55K proteins during productive infection requires E4orf6 ubiquitin ligase activity." Journal of Virology **82**(6): 2642-2651.

Blaut, M. A., N. V. Bogdanova, et al. (2010). "TOPBP1 missense variant Arg309Cys and breast cancer in a German hospital-based case-control study." Journal of Negative Results in Biomedicine **9**: 9.

Bogoyevitch, M. A., Y. Y. Yeap, et al. (2012). "WD40-repeat protein 62 is a JNK-phosphorylated spindle pole protein required for spindle maintenance and timely mitotic progression." Journal of Cell Science.

Boichuk, S., L. Hu, et al. (2010). "Multiple DNA damage signaling and repair pathways deregulated by simian virus 40 large T antigen." Journal of Virology **84**(16): 8007-8020.

Bond, J., E. Roberts, et al. (2002). "ASPM is a major determinant of cerebral cortical size." Nature Genetics **32**(2): 316-320.

Bond, J., E. Roberts, et al. (2005). "A centrosomal mechanism involving CDK5RAP2 and CENPJ controls brain size." Nature Genetics **37**(4): 353-355.

Bondesson, M., K. Ohman, et al. (1996). "Adenovirus E4 open reading frame 4 protein autoregulates E4 transcription by inhibiting E1A transactivation of the E4 promoter." Journal of Virology **70**(6): 3844-3851.

Boner, W., E. R. Taylor, et al. (2002). "A Functional interaction between the human papillomavirus 16 transcription/replication factor E2 and the DNA damage response protein TopBP1." The Journal of Biological Chemistry **277**(25): 22297-22303.

Boulanger, P. A. and G. E. Blair (1991). "Expression and interactions of human adenovirus oncoproteins." The Biochemical Journal **275 (Pt 2)**: 281-299.

Boyd, J. M., T. Subramanian, et al. (1993). "A region in the C-terminus of adenovirus 2/5 E1a protein is required for association with a cellular phosphoprotein and important for the negative modulation of T24-ras mediated transformation, tumorigenesis and metastasis." The EMBO Journal **12**(2): 469-478.

Boyer, J. L. and G. Ketner (2000). "Genetic analysis of a potential zinc-binding domain of the adenovirus E4 34k protein." The Journal of Biological Chemistry **275**(20): 14969-14978.

Branton, P. E. and D. E. Roopchand (2001). "The role of adenovirus E4orf4 protein in viral replication and cell killing." Oncogene **20**(54): 7855-7865.

Braunstein, S., M. L. Badura, et al. (2009). "Regulation of protein synthesis by ionizing radiation." Molecular and Cellular Biology **29**(21): 5645-5656.

Brenner, S. and R. W. Horne (1959). "A negative staining method for high resolution electron microscopy of viruses." Biochimica et Biophysica Acta **34**: 103-110.

Brooks, C. L. and W. Gu (2003). "Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation." Current Opinion in Cell Biology **15**(2): 164-171.

Brown, C. J., S. Lain, et al. (2009). "Awakening guardian angels: drugging the p53 pathway." Nature Reviews. Cancer **9**(12): 862-873.

Brown, E. J. and D. Baltimore (2000). "ATR disruption leads to chromosomal fragmentation and early embryonic lethality." Genes & Development **14**(4): 397-402.

Busby, E. C., D. F. Leistritz, et al. (2000). "The radiosensitizing agent 7-hydroxystaurosporine (UCN-01) inhibits the DNA damage checkpoint kinase hChk1." Cancer Research **60**(8): 2108-2112.

Byrd, P., K. W. Brown, et al. (1982). "Malignant transformation of human embryo retinoblasts by cloned adenovirus 12 DNA." Nature **298**(5869): 69-71.

Byrd, P. J., R. J. Grand, et al. (1988). "Host range mutants of adenovirus type 12 E1 defective for lytic infection, transformation, and oncogenicity." Virology **163**(1): 155-165.

Campaner, S., P. Kaldis, et al. (2005). "Sil phosphorylation in a Pin1 binding domain affects the duration of the spindle checkpoint." Molecular and Cellular Biology **25**(15): 6660-6672.

Carson, C. T., N. I. Orazio, et al. (2009). "Mislocalization of the MRN complex prevents ATR signaling during adenovirus infection." EMBO Journal **28**(6): 652-662.

Carson, C. T., R. A. Schwartz, et al. (2003). "The Mre11 complex is required for ATM activation and the G2/M checkpoint." The EMBO Journal **22**(24): 6610-6620.

Carvalho, T., J. S. Seeler, et al. (1995). "Targeting of adenovirus E1A and E4-ORF3 proteins to nuclear matrix-associated PML bodies." The Journal of Cell Biology **131**(1): 45-56.

Cescutti, R., S. Negrini, et al. (2010). "TopBP1 functions with 53BP1 in the G1 DNA damage checkpoint." The EMBO Journal **29**(21): 3723-3732.

Chapman, J. R. and S. P. Jackson (2008). "Phospho-dependent interactions between NBS1 and MDC1 mediate chromatin retention of the MRN complex at sites of DNA damage." EMBO Reports **9**(8): 795-801.

Chen, M. S., C. E. Ryan, et al. (2003). "Chk1 kinase negatively regulates mitotic function of Cdc25A phosphatase through 14-3-3 binding." Molecular and Cellular Biology **23**(21): 7488-7497.

Chen, Z., Z. Xiao, et al. (2006). "Selective Chk1 inhibitors differentially sensitize p53-deficient cancer cells to cancer therapeutics." International Journal of Cancer. Journal International du Cancer **119**(12): 2784-2794.

Cheng, C. Y., P. Blanchette, et al. (2007). "The adenovirus E4orf6 E3 ubiquitin ligase complex assembles in a novel fashion." Virology **364**(1): 36-44.

Cheng, C. Y., T. Gilson, et al. (2011). "The E4orf6/E1B55K E3 ubiquitin ligase complexes of human adenoviruses exhibit heterogeneity in composition and substrate specificity." Journal of Virology **85**(2): 765-775.

Cherubini, G., T. Petouchoff, et al. (2006). "E1B55K-deleted adenovirus (ONYX-015) overrides G1/S and G2/M checkpoints and causes mitotic catastrophe and endoreduplication in p53-proficient normal cells." Cell Cycle **5**(19): 2244-2252.

Chini, C. C. and J. Chen (2003). "Human claspin is required for replication checkpoint control." The Journal of Biological Chemistry **278**(32): 30057-30062.

Chou, D. M. and S. J. Elledge (2006). "Tipin and Timeless form a mutually protective complex required for genotoxic stress resistance and checkpoint function." Proceedings of the National Academy of Sciences of the United States of America **103**(48): 18143-18147.

Chow, L. T., R. E. Gelinas, et al. (1977). "An amazing sequence arrangement at the 5' ends of adenovirus 2 messenger RNA." Cell **12**(1): 1-8.

Cliby, W. A., C. J. Roberts, et al. (1998). "Overexpression of a kinase-inactive ATR protein causes sensitivity to DNA-damaging agents and defects in cell cycle checkpoints." The EMBO Journal **17**(1): 159-169.

Corona-Rivera, J. R., E. Corona-Rivera, et al. (2001). "Report and review of the fetal brain disruption sequence." European journal of pediatrics **160**(11): 664-667.

Cortez, D., S. Guntuku, et al. (2001). "ATR and ATRIP: partners in checkpoint signaling." Science **294**(5547): 1713-1716.

Cory, S., D. C. Huang, et al. (2003). "The Bcl-2 family: roles in cell survival and oncogenesis." Oncogene **22**(53): 8590-8607.

Costanzo, V., K. Robertson, et al. (2000). "Reconstitution of an ATM-dependent checkpoint that inhibits chromosomal DNA replication following DNA damage." Molecular Cell **6**(3): 649-659.

Cuadrado, M., B. Martinez-Pastor, et al. (2006). "ATM regulates ATR chromatin loading in response to DNA double-strand breaks." The Journal of Experimental Medicine **203**(2): 297-303.

Cuconati, A., C. Mukherjee, et al. (2003). "DNA damage response and MCL-1 destruction initiate apoptosis in adenovirus-infected cells." Genes & Development **17**(23): 2922-2932.

Cuconati, A. and E. White (2002). "Viral homologs of BCL-2: role of apoptosis in the regulation of virus infection." Genes & Development **16**(19): 2465-2478.

Cutt, J. R., T. Shenk, et al. (1987). "Analysis of adenovirus early region 4-encoded polypeptides synthesized in productively infected cells." Journal of Virology **61**(2): 543-552.

Dallaire, F., P. Blanchette, et al. (2009). "Identification of integrin alpha3 as a new substrate of the adenovirus E4orf6/E1B 55-kilodalton E3 ubiquitin ligase complex." Journal of Virology **83**(11): 5329-5338.

Davison, A. J., M. Benko, et al. (2003). "Genetic content and evolution of adenoviruses." The Journal of General Virology **84**(Pt 11): 2895-2908.

de Jong, R. N. and P. C. van der Vliet (1999). "Mechanism of DNA replication in eukaryotic cells: cellular host factors stimulating adenovirus DNA replication." Gene **236**(1): 1-12.

de Jong, R. N., P. C. van der Vliet, et al. (2003). "Adenovirus DNA replication: protein priming, jumping back and the role of the DNA binding protein DBP." Current Topics in Microbiology and Immunology **272**: 187-211.

DeCaprio, J. A., J. W. Ludlow, et al. (1988). "SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene." Cell **54**(2): 275-283.

Delacroix, S., J. M. Wagner, et al. (2007). "The Rad9-Hus1-Rad1 (9-1-1) clamp activates checkpoint signaling via TopBP1." Genes & Development **21**(12): 1472-1477.

Dheekollu, J. and P. M. Lieberman (2011). "The replisome pausing factor Timeless is required for episomal maintenance of latent Epstein-Barr virus." Journal of Virology **85**(12): 5853-5863.

Diamandopoulos, G. T. (1996). "Cancer: an historical perspective." Anticancer Research **16**(4A): 1595-1602.

Dobner, T., N. Horikoshi, et al. (1996). "Blockage by adenovirus E4orf6 of transcriptional activation by the p53 tumor suppressor." Science **272**(5267): 1470-1473.

Donaldson, M. M., W. Boner, et al. (2007). "TopBP1 regulates human papillomavirus type 16 E2 interaction with chromatin." Journal of Virology **81**(8): 4338-4342.

Dornreiter, I., L. F. Erdile, et al. (1992). "Interaction of DNA polymerase alpha-primase with cellular replication protein A and SV40 T antigen." The EMBO Journal **11**(2): 769-776.

Dosch, T., F. Horn, et al. (2001). "The adenovirus type 5 E1B-55K oncoprotein actively shuttles in virus-infected cells, whereas transport of E4orf6 is mediated by a CRM1-independent mechanism." Journal of Virology **75**(12): 5677-5683.

Doucas, V., A. M. Ishov, et al. (1996). "Adenovirus replication is coupled with the dynamic properties of the PML nuclear structure." Genes & Development **10**(2): 196-207.

Downey, J. F., D. T. Rowe, et al. (1983). "Mapping of a 14,000-dalton antigen to early region 4 of the human adenovirus 5 genome." Journal of Virology **45**(2): 514-523.

Duda, D. M., L. A. Borg, et al. (2008). "Structural insights into NEDD8 activation of cullin-RING ligases: conformational control of conjugation." Cell **134**(6): 995-1006.

Dudgeon, J. A. (1971). "Cytomegalovirus infection." Archives of Disease in Childhood **46**(249): 581-583.

Dunsworth-Browne, M., R. E. Schell, et al. (1980). "Adenovirus terminal protein protects single stranded DNA from digestion by a cellular exonuclease." Nucleic Acids Research **8**(3): 543-554.

Dyson, N., P. M. Howley, et al. (1989). "The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product." Science **243**(4893): 934-937.

Echavarria, M. (2008). "Adenoviruses in immunocompromised hosts." Clinical Microbiology Reviews **21**(4): 704-715.

Eliyahu, D., A. Raz, et al. (1984). "Participation of p53 cellular tumour antigen in transformation of normal embryonic cells." Nature **312**(5995): 646-649.

Endter, C., J. Kzhyshkowska, et al. (2001). "SUMO-1 modification required for transformation by adenovirus type 5 early region 1B 55-kDa oncoprotein." Proceedings of the National Academy of Sciences of the United States of America **98**(20): 11312-11317.

Epstein, L. G. and H. A. Gelbard (1999). "HIV-1-induced neuronal injury in the developing brain." Journal of Leukocyte Biology **65**(4): 453-457.

Erez, A., A. Castiel, et al. (2007). "The SIL gene is essential for mitotic entry and survival of cancer cells." Cancer Research **67**(9): 4022-4027.

Erez, A., M. Chaussepied, et al. (2008). "The mitotic checkpoint gene, SIL is regulated by E2F1." International Journal of Cancer. Journal International du Cancer **123**(7): 1721-1725.

Erez, A., M. Perelman, et al. (2004). "Sil overexpression in lung cancer characterizes tumors with increased mitotic activity." Oncogene **23**(31): 5371-5377.

Evans, J. D. and P. Hearing (2005). "Relocalization of the Mre11-Rad50-Nbs1 complex by the adenovirus E4 ORF3 protein is required for viral replication." Journal of Virology **79**(10): 6207-6215.

Falck, J., J. Coates, et al. (2005). "Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage." Nature **434**(7033): 605-611.

Falck, J., N. Mailand, et al. (2001). "The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis." Nature **410**(6830): 842-847.

Fallaux, F. J., O. Kranenburg, et al. (1996). "Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors." Human Gene Therapy **7**(2): 215-222.

Finlay, C. A., P. W. Hinds, et al. (1989). "The p53 proto-oncogene can act as a suppressor of transformation." Cell **57**(7): 1083-1093.

Fish, J. L., Y. Kosodo, et al. (2006). "Aspm specifically maintains symmetric proliferative divisions of neuroepithelial cells." Proceedings of the National Academy of Sciences of the United States of America **103**(27): 10438-10443.

Fong, K. W., Y. K. Choi, et al. (2008). "CDK5RAP2 is a pericentriolar protein that functions in centrosomal attachment of the gamma-tubulin ring complex." Molecular Biology of the Cell **19**(1): 115-125.

Forrester, N. A., R. N. Patel, et al. (2012). "Adenovirus E4orf3 targets transcriptional intermediary factor 1gamma for proteasome-dependent degradation during infection." Journal of Virology **86**(6): 3167-3179.

Forrester, N. A., G. G. Sedgwick, et al. (2011). "Serotype-specific inactivation of the cellular DNA damage response during adenovirus infection." Journal of Virology **85**(5): 2201-2211.

Frisch, S. M. and J. S. Mymryk (2002). "Adenovirus-5 E1A: paradox and paradigm." Nature reviews. Molecular Cell Biology **3**(6): 441-452.

Gallimore, P. H., P. J. Byrd, et al. (1985). "Properties of rat cells transformed by DNA plasmids containing adenovirus type 12 E1 DNA or specific fragments of the E1 region: comparison of transforming frequencies." Cancer Research **45**(6): 2670-2680.

Gallimore, P. H. and A. S. Turnell (2001). "Adenovirus E1A: remodelling the host cell, a life or death experience." Oncogene **20**(54): 7824-7835.

Garcia-Mata, R., Y. S. Gao, et al. (2002). "Hassles with taking out the garbage: aggravating aggresomes." Traffic **3**(6): 388-396.

Garcia, V., K. Furuya, et al. (2005). "Identification and functional analysis of TopBP1 and its homologs." DNA Repair (Amst) **4**(11): 1227-1239.

Gastaldello, S., S. Hildebrand, et al. (2010). "A deneddylase encoded by Epstein-Barr virus promotes viral DNA replication by regulating the activity of cullin-RING ligases." Nature Cell Biology **12**(4): 351-361.

Gatei, M., K. Sloper, et al. (2003). "Ataxia-telangiectasia-mutated (ATM) and NBS1-dependent phosphorylation of Chk1 on Ser-317 in response to ionizing radiation." The Journal of Biological Chemistry **278**(17): 14806-14811.

Gonzalez, R., W. Huang, et al. (2006). "Adenovirus E1B 55-kilodalton protein is required for both regulation of mRNA export and efficient entry into the late phase of infection in normal human fibroblasts." Journal of Virology **80**(2): 964-974.

Goodrum, F. D., T. Shenk, et al. (1996). "Adenovirus early region 4 34-kilodalton protein directs the nuclear localization of the early region 1B 55-kilodalton protein in primate cells." Journal of Virology **70**(9): 6323-6335.

Gotter, A. L. (2003). "Tipin, a novel timeless-interacting protein, is developmentally co-expressed with timeless and disrupts its self-association." Journal of Molecular Biology **331**(1): 167-176.

Gotter, A. L., T. Manganaro, et al. (2000). "A time-less function for mouse timeless." Nature Neuroscience **3**(8): 755-756.

Gotter, A. L., C. Suppa, et al. (2007). "Mammalian Timeless and Tipin are evolutionarily conserved replication fork-associated factors." Journal of Molecular Biology **366**(1): 36-52.

Graham, F. L., J. Smiley, et al. (1977). "Characteristics of a human cell line transformed by DNA from human adenovirus type 5." The Journal of General Virology **36**(1): 59-74.

Grand, R. J., M. L. Grant, et al. (1994). "Enhanced expression of p53 in human cells infected with mutant adenoviruses." Virology **203**(2): 229-240.

Grand, R. J., A. P. Ibrahim, et al. (1998). "Human cells arrest in S phase in response to adenovirus 12 E1A." Virology **244**(2): 330-342.

Grand, R. J., J. Parkhill, et al. (1999). "Definition of a major p53 binding site on Ad2E1B58K protein and a possible nuclear localization signal on the Ad12E1B54K protein." Oncogene **18**(4): 955-965.

Graser, S., Y. D. Stierhof, et al. (2007). "Cep68 and Cep215 (Cdk5rap2) are required for centrosome cohesion." Journal of Cell Science **120**(Pt 24): 4321-4331.

Gravel, S., J. R. Chapman, et al. (2008). "DNA helicases Sgs1 and BLM promote DNA double-strand break resection." Genes & Development **22**(20): 2767-2772.

Griffith, E., S. Walker, et al. (2008). "Mutations in pericentrin cause Seckel syndrome with defective ATR-dependent DNA damage signaling." Nature Genetics **40**(2): 232-236.

Halbert, D. N., J. R. Cutt, et al. (1985). "Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff." Journal of Virology **56**(1): 250-257.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.

Harada, J. N., A. Shevchenko, et al. (2002). "Analysis of the Adenovirus E1B-55K-Anchored Proteome Reveals Its Link to Ubiquitination Machinery." Journal of Virology **76**(18): 9194-9206.

Harper, J. W., G. R. Adami, et al. (1993). "The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases." Cell **75**(4): 805-816.

Heise, C. C., A. M. Williams, et al. (1999). "Intravenous administration of ONYX-015, a selectively replicating adenovirus, induces antitumoral efficacy." Cancer Research **59**(11): 2623-2628.

Hershko, A., H. Heller, et al. (1983). "Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown." The Journal of Biological Chemistry **258**(13): 8206-8214.

Hirao, A., A. Cheung, et al. (2002). "Chk2 is a tumor suppressor that regulates apoptosis in both an ataxia telangiectasia mutated (ATM)-dependent and an ATM-independent manner." Molecular and Cellular Biology **22**(18): 6521-6532.

Hirao, A., Y. Y. Kong, et al. (2000). "DNA damage-induced activation of p53 by the checkpoint kinase Chk2." Science **287**(5459): 1824-1827.

Hoege, C., B. Pfander, et al. (2002). "RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO." Nature **419**(6903): 135-141.

Hoeller, D., C. M. Hecker, et al. (2006). "Ubiquitin and ubiquitin-like proteins in cancer pathogenesis." Nature reviews. Cancer **6**(10): 776-788.

Hoppe, A., S. J. Beech, et al. (2006). "Interaction of the adenovirus type 5 E4 Orf3 protein with promyelocytic leukemia protein isoform II is required for ND10 disruption." Journal of Virology **80**(6): 3042-3049.

Horne, R. W., S. Brenner, et al. (1959). "The icosahedral form of an adenovirus." J. Mol. Biol(1): 84-86.

Horridge, J. J. and K. N. Leppard (1998). "RNA-binding activity of the E1B 55-kilodalton protein from human adenovirus type 5." Journal of Virology **72**(11): 9374-9379.

Houtgraaf, J. H., J. Versmissen, et al. (2006). "A concise review of DNA damage checkpoints and repair in mammalian cells." Cardiovascular Revascularization Medicine : Including Molecular Interventions **7**(3): 165-172.

Howe, J. A. and S. T. Bayley (1992). "Effects of Ad5 E1A mutant viruses on the cell cycle in relation to the binding of cellular proteins including the retinoblastoma protein and cyclin A." Virology **186**(1): 15-24.

Howe, J. A., J. S. Mymryk, et al. (1990). "Retinoblastoma growth suppressor and a 300-kDa protein appear to regulate cellular DNA synthesis." Proceedings of the National Academy of Sciences of the United States of America **87**(15): 5883-5887.

Huang, M. M. and P. Hearing (1989). "Adenovirus early region 4 encodes two gene products with redundant effects in lytic infection." Journal of Virology **63**(6): 2605-2615.

Huang, T. T. and A. D. D'Andrea (2006). "Regulation of DNA repair by ubiquitylation." Nature Reviews. Molecular Cell Biology **7**(5): 323-334.

Huang, T. T., S. M. Nijman, et al. (2006). "Regulation of monoubiquitinated PCNA by DUB autocleavage." Nature Cell Biology **8**(4): 339-347.

Huen, M. S., R. Grant, et al. (2007). "RNF8 transduces the DNA-damage signal via histone ubiquitylation and checkpoint protein assembly." Cell **131**(5): 901-914.

Hughes, H. E. and M. Miskin (1986). "Congenital microcephaly due to vascular disruption: in utero documentation." Pediatrics **78**(1): 85-87.

Huh, K., X. Zhou, et al. (2007). "Human papillomavirus type 16 E7 oncoprotein associates with the cullin 2 ubiquitin ligase complex, which contributes to degradation of the retinoblastoma tumor suppressor." Journal of Virology **81**(18): 9737-9747.

Hung, L. Y., H. L. Chen, et al. (2004). "Identification of a novel microtubule-destabilizing motif in CPAP that binds to tubulin heterodimers and inhibits microtubule assembly." Molecular Biology of the Cell **15**(6): 2697-2706.

Isobe, T., T. Hattori, et al. (2009). "Adenovirus E1A inhibits SCF(Fbw7) ubiquitin ligase." The Journal of Biological Chemistry **284**(41): 27766-27779.

Jackson, A. P., H. Eastwood, et al. (2002). "Identification of microcephalin, a protein implicated in determining the size of the human brain." American Journal of Human Genetics **71**(1): 136-142.

Jackson, A. P., D. P. McHale, et al. (1998). "Primary autosomal recessive microcephaly (MCPH1) maps to chromosome 8p22-pter." American Journal of Human Genetics **63**(2): 541-546.

Jackson, S. P. and J. Bartek (2009). "The DNA-damage response in human biology and disease." Nature **461**(7267): 1071-1078.

Javier, R. T. (1994). "Adenovirus type 9 E4 open reading frame 1 encodes a transforming protein required for the production of mammary tumors in rats." Journal of Virology **68**(6): 3917-3924.

Javier, R. T. and J. S. Butel (2008). "The history of tumor virology." Cancer Research **68**(19): 7693-7706.

Jayaram, S., T. Gilson, et al. (2008). "E1B 55k-independent dissociation of the DNA ligase IV/XRCC4 complex by E4 34k during adenovirus infection." Virology **382**(2): 163-170.

Jazayeri, A., J. Falck, et al. (2006). "ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks." Nature Cell Biology **8**(1): 37-45.

Jeffers, L. J., B. J. Coull, et al. (2008). "Distinct BRCT domains in Mcph1/Brit1 mediate ionizing radiation-induced focus formation and centrosomal localization." Oncogene **27**(1): 139-144.

Jeon, Y., E. Ko, et al. (2011). "TopBP1 deficiency causes an early embryonic lethality and induces cellular senescence in primary cells." The Journal of Biological Chemistry **286**(7): 5414-5422.

Jeon, Y., K. Y. Lee, et al. (2007). "Human TopBP1 participates in cyclin E/CDK2 activation and preinitiation complex assembly during G1/S transition." The Journal of Biological Chemistry **282**(20): 14882-14890.

Jochemsen, H., G. S. Daniels, et al. (1982). "Identification of adenovirus-type 12 gene products involved in transformation and oncogenesis." Virology **122**(1): 15-28.

Kaindl, A. M., S. Passemard, et al. (2010). "Many roads lead to primary autosomal recessive microcephaly." Progress in Neurobiology **90**(3): 363-383.

Kamura, T., K. Maenaka, et al. (2004). "VHL-box and SOCS-box domains determine binding specificity for Cul2-Rbx1 and Cul5-Rbx2 modules of ubiquitin ligases." Genes & Development **18**(24): 3055-3065.

Karen, K. A., P. J. Hoey, et al. (2009). "Temporal regulation of the Mre11-Rad50-Nbs1 complex during adenovirus infection." J Virol **83**(9): 4565-4573.

Karppinen, S. M., H. Erkko, et al. (2006). "Identification of a common polymorphism in the TopBP1 gene associated with hereditary susceptibility to breast and ovarian cancer." European Journal of Cancer **42**(15): 2647-2652.

Kasai, K., S. Inaguma, et al. (2008). "SCL/TAL1 interrupting locus derepresses GLI1 from the negative control of suppressor-of-fused in pancreatic cancer cell." Cancer Research **68**(19): 7723-7729.

Kastan, M. B. and J. Bartek (2004). "Cell-cycle checkpoints and cancer." Nature **432**(7015): 316-323.

Kemp, M. G., Z. Akan, et al. (2010). "Tipin-replication protein A interaction mediates Chk1 phosphorylation by ATR in response to genotoxic stress." The Journal of Biological Chemistry **285**(22): 16562-16571.

Khuri, F. R., J. Nemunaitis, et al. (2000). "a controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer." Nature Medicine **6**(8): 879-885.

Kim, J. E., S. A. McAvoy, et al. (2005). "Human TopBP1 ensures genome integrity during normal S phase." Molecular and Cellular Biology **25**(24): 10907-10915.

Kim, J. E., K. Minter-Dykhouse, et al. (2006). "Signaling networks controlled by the MRN complex and MDC1 during early DNA damage responses." Mol Carcinog **45**(6): 403-408.

Kim, S. M., A. Kumagai, et al. (2005). "Phosphorylation of Chk1 by ATM- and Rad3-related (ATR) in Xenopus egg extracts requires binding of ATRIP to ATR but not the stable DNA-binding or coiled-coil domains of ATRIP." The Journal of Biological Chemistry **280**(46): 38355-38364.

Kim, S. T., B. Xu, et al. (2002). "Involvement of the cohesin protein, Smc1, in Atm-dependent and independent responses to DNA damage." Genes & Development **16**(5): 560-570.

Kindsmuller, K., P. Groitl, et al. (2007). "Intranuclear targeting and nuclear export of the adenovirus E1B-55K protein are regulated by SUMO1 conjugation." Proceedings of the National Academy of Sciences of the United States of America **104**(16): 6684-6689.

Kleinberger, T. (2000). "Induction of apoptosis by adenovirus E4orf4 protein." Apoptosis : An International Journal on Programmed Cell Death **5**(3): 211-215.

Kleinberger, T. and T. Shenk (1993). "Adenovirus E4orf4 protein binds to protein phosphatase 2A, and the complex down regulates E1A-enhanced junB transcription." Journal of Virology **67**(12): 7556-7560.

Koike, N., A. Hida, et al. (1998). "Identification of the mammalian homologues of the Drosophila timeless gene, Timeless1." FEBS letters **441**(3): 427-431.

Kondratov, R. V. and M. P. Antoch (2007). "Circadian proteins in the regulation of cell cycle and genotoxic stress responses." Trends in Cell Biology **17**(7): 311-317.

Kornitzer, D., R. Sharf, et al. (2001). "Adenovirus E4orf4 protein induces PP2A-dependent growth arrest in Saccharomyces cerevisiae and interacts with the anaphase-promoting complex/cyclosome." The Journal of Cell Biology **154**(2): 331-344.

Kosulin, K., C. Haberler, et al. (2007). "Investigation of adenovirus occurrence in pediatric tumor entities." Journal of Virology **81**(14): 7629-7635.

Kovesdi, I., R. Reichel, et al. (1986). "Identification of a cellular transcription factor involved in E1A trans-activation." Cell **45**(2): 219-228.

Kumagai, A. and W. G. Dunphy (2000). "Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in Xenopus egg extracts." Molecular Cell **6**(4): 839-849.

Kumagai, A., J. Lee, et al. (2006). "TopBP1 activates the ATR-ATRIP complex." Cell **124**(5): 943-955.

Kumagai, A., A. Shevchenko, et al. (2010). "Treslin collaborates with TopBP1 in triggering the initiation of DNA replication." Cell **140**(3): 349-359.

Kumar, A., S. C. Girimaji, et al. (2009). "Mutations in STIL, encoding a pericentriolar and centrosomal protein, cause primary microcephaly." American Journal of Human Genetics **84**(2): 286-290.

Kummar, S., A. Chen, et al. (2012). "Advances in using PARP inhibitors to treat cancer." BMC Medicine **10**: 25.

Lakdawala, S. S., R. A. Schwartz, et al. (2008). "Differential requirements of the C terminus of Nbs1 in suppressing adenovirus DNA replication and promoting concatemer formation." Journal of Virology **82**(17): 8362-8372.

Lakin, N. D. and S. P. Jackson (1999). "Regulation of p53 in response to DNA damage." Oncogene **18**(53): 7644-7655.

Lane, D. P. (1992). "Cancer. p53, guardian of the genome." Nature **358**(6381): 15-16.

Lane, D. P. and L. V. Crawford (1979). "T antigen is bound to a host protein in SV40-transformed cells." Nature **278**(5701): 261-263.

Latonen, L. and M. Laiho (2005). "Cellular UV damage responses--functions of tumor suppressor p53." Biochimica et Biophysica Acta **1755**(2): 71-89.

Leach, F. S., T. Tokino, et al. (1993). "p53 Mutation and MDM2 amplification in human soft tissue sarcomas." Cancer Research **53**(10 Suppl): 2231-2234.

Lee, J., A. Kumagai, et al. (2007). "The Rad9-Hus1-Rad1 checkpoint clamp regulates interaction of TopBP1 with ATR." The Journal of Biological Chemistry **282**(38): 28036-28044.

Leman, A. R., C. Noguchi, et al. (2010). "Human Timeless and Tipin stabilize replication forks and facilitate sister-chromatid cohesion." Journal of Cell Science **123**(Pt 5): 660-670.

Leppard, K. N. and R. D. Everett (1999). "The adenovirus type 5 E1b 55K and E4 Orf3 proteins associate in infected cells and affect ND10 components." The Journal of General Virology **80 (Pt 4)**: 997-1008.

Levine, A. J. (1988). "Oncogenes of DNA tumor viruses." Cancer Research **48**(3): 493-496.

Li, H., R. Baskaran, et al. (2008). "Chk2 is required for HSV-1 ICP0-mediated G2/M arrest and enhancement of virus growth." Virology **375**(1): 13-23.

Li, J. and D. F. Stern (2005). "DNA damage regulates Chk2 association with chromatin." The Journal of Biological Chemistry **280**(45): 37948-37956.

Lilley, C. E., C. T. Carson, et al. (2005). "DNA repair proteins affect the lifecycle of herpes simplex virus 1." Proceedings of the National Academy of Sciences of the United States of America **102**(16): 5844-5849.

Lilley, C. E., M. S. Chaurushiya, et al. (2010). "A viral E3 ligase targets RNF8 and RNF168 to control histone ubiquitination and DNA damage responses." The EMBO Journal **29**(5): 943-955.

Lilley, C. E., R. A. Schwartz, et al. (2007). "Using or abusing: viruses and the cellular DNA damage response." Trends in Microbiology **15**(3): 119-126.

Lin, W. C., F. T. Lin, et al. (2001). "Selective induction of E2F1 in response to DNA damage, mediated by ATM-dependent phosphorylation." Genes & Development **15**(14): 1833-1844.

Linzer, D. I. and A. J. Levine (1979). "Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells." Cell **17**(1): 43-52.

Liu, H., J. H. Naismith, et al. (2003). "Adenovirus DNA replication." Current Topics in Microbiology and Immunology **272**: 131-164.

Liu, K., N. Bellam, et al. (2009). "Regulation of p53 by TopBP1: a potential mechanism for p53 inactivation in cancer." Molecular and Cellular Biology **29**(10): 2673-2693.

Liu, K., Y. Luo, et al. (2004). "TopBP1 recruits Brg1/Brm to repress E2F1-induced apoptosis, a novel pRb-independent and E2F1-specific control for cell survival." Genes & Development **18**(6): 673-686.

Liu, K., J. C. Paik, et al. (2006). "Regulation of TopBP1 oligomerization by Akt/PKB for cell survival." The EMBO Journal **25**(20): 4795-4807.

Liu, Q., S. Guntuku, et al. (2000). "Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint." Genes & Development **14**(12): 1448-1459.

Liu, S., B. Shiotani, et al. (2011). "ATR autophosphorylation as a molecular switch for checkpoint activation." Molecular Cell **43**(2): 192-202.

Liu, Y., A. Shevchenko, et al. (2005). "Adenovirus exploits the cellular aggresome response to accelerate inactivation of the MRN complex." Journal of Virology **79**(22): 14004-14016.

Lobrich, M. and P. A. Jeggo (2007). "The impact of a negligent G2/M checkpoint on genomic instability and cancer induction." Nature Reviews. Cancer **7**(11): 861-869.

Lombard, D. B. and L. Guarente (2000). "Nijmegen breakage syndrome disease protein and Mre11 at PML nuclear bodies and meiotic telomeres." Cancer Research **60**(9): 2331-2334.

Mackey, J. K., W. S. Wold, et al. (1979). "Transforming region of group A, B, and C adenoviruses: DNA homology studies with twenty-nine human adenovirus serotypes." Journal of Virology **29**(3): 1056-1064.

Mailand, N., S. Bekker-Jensen, et al. (2007). "RNF8 ubiquitylates histones at DNA double-strand breaks and promotes assembly of repair proteins." Cell **131**(5): 887-900.

Mailand, N., A. V. Podtelejnikov, et al. (2002). "Regulation of G(2)/M events by Cdc25A through phosphorylation-dependent modulation of its stability." The EMBO Journal **21**(21): 5911-5920.

Mannervik, M., S. Fan, et al. (1999). "Adenovirus E4 open reading frame 4-induced dephosphorylation inhibits E1A activation of the E2 promoter and E2F-1-mediated transactivation independently of the retinoblastoma tumor suppressor protein." Virology **256**(2): 313-321.

Marcellus, R. C., H. Chan, et al. (2000). "Induction of p53-independent apoptosis by the adenovirus E4orf4 protein requires binding to the Balpha subunit of protein phosphatase 2A." Journal of Virology **74**(17): 7869-7877.

Mathias, P., M. Galleno, et al. (1998). "Interactions of soluble recombinant integrin alphav beta5 with human adenoviruses." Journal of Virology **72**(11): 8669-8675.

Matsuoka, S., B. A. Ballif, et al. (2007). "ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage." Science **316**(5828): 1160-1166.

Matsuoka, S., G. Rotman, et al. (2000). "Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro." Proceedings of the National Academy of Sciences of the United States of America **97**(19): 10389-10394.

McBride, W. D. and A. Wiener (1964). "In Vitro Transformation of Hamster Kidney Cells by Human Adenovirus Type 12." Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine **115**: 870-874.

McGowan, C. H. and P. Russell (1993). "Human Wee1 kinase inhibits cell division by phosphorylating p34cdc2 exclusively on Tyr15." The EMBO Journal **12**(1): 75-85.

Meek, D. W. (2004). "The p53 response to DNA damage." DNA Repair **3**(8-9): 1049-1056.

Meier, O. and U. F. Greber (2003). "Adenovirus endocytosis." The Journal of Gene Medicine **5**(6): 451-462.

Melander, F., S. Bekker-Jensen, et al. (2008). "Phosphorylation of SDT repeats in the MDC1 N terminus triggers retention of NBS1 at the DNA damage-modified chromatin." The Journal of Cell Biology **181**(2): 213-226.

Melendy, T. and B. Stillman (1993). "An interaction between replication protein A and SV40 T antigen appears essential for primosome assembly during SV40 DNA replication." The Journal of Biological Chemistry **268**(5): 3389-3395.

Mochida, G. H. (2009). "Genetics and biology of microcephaly and lissencephaly." Seminars in Pediatric Neurology **16**(3): 120-126.

Mohni, K. N., C. M. Livingston, et al. (2010). "ATR and ATRIP are recruited to herpes simplex virus type 1 replication compartments even though ATR signaling is disabled." Journal of Virology **84**(23): 12152-12164.

Momand, J., G. P. Zambetti, et al. (1992). "The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation." Cell **69**(7): 1237-1245.

Moody, C. A. and L. A. Laimins (2009). "Human papillomaviruses activate the ATM DNA damage pathway for viral genome amplification upon differentiation." PLoS Pathogens **5**(10): e1000605.

Moore, M., N. Horikoshi, et al. (1996). "Oncogenic potential of the adenovirus E4orf6 protein." Proceedings of the National Academy of Sciences of the United States of America **93**(21): 11295-11301.

Mordes, D. A. and D. Cortez (2008). "Activation of ATR and related PIKKs." Cell Cycle **7**(18): 2809-2812.

Mordes, D. A., G. G. Glick, et al. (2008). "TopBP1 activates ATR through ATRIP and a PIKK regulatory domain." Genes & Development **22**(11): 1478-1489.

Muller, S. and T. Dobner (2008). "The adenovirus E1B-55K oncoprotein induces SUMO modification of p53." Cell cycle **7**(6): 754-758.

Nagata, K., R. A. Guggenheimer, et al. (1982). "Adenovirus DNA replication in vitro: identification of a host factor that stimulates synthesis of the preterminal protein-dCMP complex." Proceedings of the National Academy of Sciences of the United States of America **79**(21): 6438-6442.

Nagata, K., R. A. Guggenheimer, et al. (1983). "Adenovirus DNA replication in vitro: synthesis of full-length DNA with purified proteins." Proceedings of the National Academy of Sciences of the United States of America **80**(14): 4266-4270.

Nakanishi, K., T. Taniguchi, et al. (2002). "Interaction of FANCD2 and NBS1 in the DNA damage response." Nature Cell Biology **4**(12): 913-920.

Nam, E. A., R. Zhao, et al. (2011). "Thr-1989 phosphorylation is a marker of active ataxia telangiectasia-mutated and Rad3-related (ATR) kinase." The Journal of Biological Chemistry **286**(33): 28707-28714.

Nevels, M., T. Spruss, et al. (1999). "The adenovirus E4orf6 protein contributes to malignant transformation by antagonizing E1A-induced accumulation of the tumor suppressor protein p53." Oncogene **18**(1): 9-17.

Nevels, M., B. Tauber, et al. (2001). ""Hit-and-run" transformation by adenovirus oncogenes." Journal of Virology **75**(7): 3089-3094.

Nicholas, A. K., M. Khurshid, et al. (2010). "WDR62 is associated with the spindle pole and is mutated in human microcephaly." Nature Genetics **42**(11): 1010-1014.

Niida, H., Y. Katsuno, et al. (2007). "Specific role of Chk1 phosphorylations in cell survival and checkpoint activation." Molecular and Cellular Biology **27**(7): 2572-2581.

Niida, H. and M. Nakanishi (2006). "DNA damage checkpoints in mammals." Mutagenesis **21**(1): 3-9.

Nijman, S. M., T. T. Huang, et al. (2005). "The deubiquitinating enzyme USP1 regulates the Fanconi anemia pathway." Molecular Cell **17**(3): 331-339.

O'Connor, R. J. and P. Hearing (2000). "The E4-6/7 protein functionally compensates for the loss of E1A expression in adenovirus infection." Journal of Virology **74**(13): 5819-5824.

O'Driscoll, M., A. P. Jackson, et al. (2006). "Microcephalin: a causal link between impaired damage response signalling and microcephaly." Cell Cycle **5**(20): 2339-2344.

O'Driscoll, M., V. L. Ruiz-Perez, et al. (2003). "A splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome." Nature Genetics **33**(4): 497-501.

Oliner, J. D., J. A. Pietenpol, et al. (1993). "Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53." Nature **362**(6423): 857-860.

Opitz, J. M. and M. C. Holt (1990). "Microcephaly: general considerations and aids to nosology." Journal of Craniofacial Genetics and Developmental Biology **10**(2): 175-204.

Orazio, N. I., C. M. Naeger, et al. (2011). "The adenovirus E1b55K/E4orf6 complex induces degradation of the Bloom helicase during infection." Journal of Virology **85**(4): 1887-1892.

Orlando, J. S. and D. A. Ornelles (1999). "An arginine-faced amphipathic alpha helix is required for adenovirus type 5 e4orf6 protein function." Journal of Virology **73**(6): 4600-4610.

Parada, L. F., H. Land, et al. (1984). "Cooperation between gene encoding p53 tumour antigen and ras in cellular transformation." Nature **312**(5995): 649-651.

Parker, L. L. and H. Piwnica-Worms (1992). "Inactivation of the p34cdc2-cyclin B complex by the human WEE1 tyrosine kinase." Science **257**(5078): 1955-1957.

Parkin, D. M. (2006). "The global health burden of infection-associated cancers in the year 2002." International Journal of Cancer. Journal International du Cancer **118**(12): 3030-3044.

Pennella, M. A., Y. Liu, et al. (2010). "Adenovirus E1B 55-kilodalton protein is a p53-SUMO1 E3 ligase that represses p53 and stimulates its nuclear export through interactions with promyelocytic leukemia nuclear bodies." Journal of Virology **84**(23): 12210-12225.

Petermann, E. and K. W. Caldecott (2006). "Evidence that the ATR/Chk1 pathway maintains normal replication fork progression during unperturbed S phase." Cell Cycle **5**(19): 2203-2209.

Petroski, M. D. and R. J. Deshaies (2005). "Function and regulation of cullin-RING ubiquitin ligases." Nature reviews. Molecular Cell Biology **6**(1): 9-20.

Polo, S. E., A. N. Blackford, et al. (2012). "Regulation of DNA-end resection by hnRNPU-like proteins promotes DNA double-strand break signaling and repair." Molecular Cell **45**(4): 505-516.

Pruijn, G. J., W. van Driel, et al. (1986). "Nuclear factor III, a novel sequence-specific DNA-binding protein from HeLa cells stimulating adenovirus DNA replication." Nature **322**(6080): 656-659.

Querido, E., P. Blanchette, et al. (2001). "Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex." Genes & Development **15**(23): 3104-3117.

Querido, E., J. G. Teodoro, et al. (1997). "Accumulation of p53 induced by the adenovirus E1A protein requires regions involved in the stimulation of DNA synthesis." Journal of Virology **71**(5): 3526-3533.

Rai, R., H. Dai, et al. (2006). "BRIT1 regulates early DNA damage response, chromosomal integrity, and cancer." Cancer Cell **10**(2): 145-157.

Rai, R., A. Phadnis, et al. (2008). "Differential regulation of centrosome integrity by DNA damage response proteins." Cell Cycle **7**(14): 2225-2233.

Ramaswamy, S., K. N. Ross, et al. (2003). "A molecular signature of metastasis in primary solid tumors." Nature Genetics **33**(1): 49-54.

Rauschhuber, C., A. Wolf, et al. (2011). "Transcriptional activity of inverted terminal repeats of various human adenovirus serotypes." The Journal of General Virology **92**(Pt 3): 669-674.

Rayburn, E., R. Zhang, et al. (2005). "MDM2 and human malignancies: expression, clinical pathology, prognostic markers, and implications for chemotherapy." Current Cancer Drug Targets **5**(1): 27-41.

Rhodes, D. A., B. de Bono, et al. (2005). "Relationship between SPRY and B30.2 protein domains. Evolution of a component of immune defence?" Immunology **116**(4): 411-417.

Robinson, C. M., D. Seto, et al. (2011). "Molecular evolution of human species D adenoviruses." Infection, Genetics and Evolution : Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases **11**(6): 1208-1217.

Rodriguez, M. S., J. M. Desterro, et al. (2000). "Multiple C-terminal lysine residues target p53 for ubiquitin-proteasome-mediated degradation." Molecular and Cellular Biology **20**(22): 8458-8467.

Rogakou, E. P., C. Boon, et al. (1999). "Megabase chromatin domains involved in DNA double-strand breaks in vivo." The Journal of Cell Biology **146**(5): 905-916.

Roth, J., M. Dobbelstein, et al. (1998). "Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein." The EMBO Journal **17**(2): 554-564.

Rous, P. (1911). "A Sarcoma of the Fowl Transmissible by an Agent Separable from the Tumor Cells." The Journal of Experimental Medicine **13**(4): 397-411.

Rowe, W. P., R. J. Huebner, et al. (1953). "Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture." Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine **84**(3): 570-573.

Russell, W. C. (2000). "Update on adenovirus and its vectors." The Journal of General Virology **81**(Pt 11): 2573-2604.

Russell, W. C. (2009). "Adenoviruses: update on structure and function." The Journal of General Virology **90**(Pt 1): 1-20.

Saban, S. D., M. Silvestry, et al. (2006). "Visualization of alpha-helices in a 6-angstrom resolution cryoelectron microscopy structure of adenovirus allows refinement of capsid protein assignments." Journal of Virology **80**(24): 12049-12059.

Samuelson, A. V. and S. W. Lowe (1997). "Selective induction of p53 and chemosensitivity in RB-deficient cells by E1A mutants unable to bind the RB-related proteins." Proceedings of the National Academy of Sciences of the United States of America **94**(22): 12094-12099.

Sancar, A., L. A. Lindsey-Boltz, et al. (2004). "Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints." Annual Review of Biochemistry **73**: 39-85.

Sanchez, Y., C. Wong, et al. (1997). "Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25." Science **277**(5331): 1497-1501.

Sarantis, H., G. Johnson, et al. (2004). "Comprehensive detection and serotyping of human adenoviruses by PCR and sequencing." Journal of Clinical Microbiology **42**(9): 3963-3969.

Sarikas, A., T. Hartmann, et al. (2011). "The cullin protein family." Genome Biology **12**(4): 220.

Sarnow, P., P. Hearing, et al. (1982). "Identification and characterization of an immunologically conserved adenovirus early region 11,000 Mr protein and its association with the nuclear matrix." Journal of Molecular Biology **162**(3): 565-583.

Sarnow, P., Y. S. Ho, et al. (1982). "Adenovirus E1b-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells." Cell **28**(2): 387-394.

Sato, K., E. Sundaramoorthy, et al. (2012). "A DNA-Damage Selective Role for BRCA1 E3 Ligase in Claspin Ubiquitylation, CHK1 Activation, and DNA Repair." Current Biology : CB.

Savitsky, K., S. Sfez, et al. (1995). "The complete sequence of the coding region of the ATM gene reveals similarity to cell cycle regulators in different species." Human Molecular Genetics **4**(11): 2025-2032.

Schaack, J., W. Y. Ho, et al. (1990). "Adenovirus terminal protein mediates both nuclear matrix association and efficient transcription of adenovirus DNA." Genes & Development **4**(7): 1197-1208.

Schmidt, U., Y. Wollmann, et al. (2008). "Characterization of the interaction between the human DNA topoisomerase IIbeta-binding protein 1 (TopBP1) and the cell division cycle 45 (Cdc45) protein." The Biochemical Journal **409**(1): 169-177.

Schreiner, S., P. Wimmer, et al. (2010). "Proteasome-dependent degradation of Daxx by the viral E1B-55K protein in human adenovirus-infected cells." Journal of Virology **84**(14): 7029-7038.

Schultz, L. B., N. H. Chehab, et al. (2000). "p53 binding protein 1 (53BP1) is an early participant in the cellular response to DNA double-strand breaks." The Journal of Cell Biology **151**(7): 1381-1390.

Sehgal, A., J. L. Price, et al. (1994). "Loss of circadian behavioral rhythms and per RNA oscillations in the Drosophila mutant timeless." Science **263**(5153): 1603-1606.

Shen, J., W. Eyaid, et al. (2005). "ASPM mutations identified in patients with primary microcephaly and seizures." Journal of Medical Genetics **42**(9): 725-729.

Shi, Y., G. E. Dodson, et al. (2005). "Ataxia-telangiectasia-mutated (ATM) is a T-antigen kinase that controls SV40 viral replication in vivo." The Journal of Biological Chemistry **280**(48): 40195-40200.

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

Sieber, T. and T. Dobner (2007). "Adenovirus type 5 early region 1B 156R protein promotes cell transformation independently of repression of p53-stimulated transcription." Journal of Virology **81**(1): 95-105.

Smith, J. G., C. M. Wiethoff, et al. (2010). "Adenovirus." Current Topics in Microbiology and Immunology **343**: 195-224.

Smits, V. A., P. M. Reaper, et al. (2006). "Rapid PIKK-dependent release of Chk1 from chromatin promotes the DNA-damage checkpoint response." Current Biology : CB **16**(2): 150-159.

Soria, C., F. E. Estermann, et al. (2010). "Heterochromatin silencing of p53 target genes by a small viral protein." Nature **466**(7310): 1076-1081.

Spardy, N., K. Covella, et al. (2009). "Human papillomavirus 16 E7 oncoprotein attenuates DNA damage checkpoint control by increasing the proteolytic turnover of claspin." Cancer Research **69**(17): 7022-7029.

Spycher, C., E. S. Miller, et al. (2008). "Constitutive phosphorylation of MDC1 physically links the Mre11-RAD50-NBS1 complex to damaged chromatin." The Journal of Cell Biology **181**(2): 227-240.

Stewart, G. S., S. Panier, et al. (2009). "The RIDDLE syndrome protein mediates a ubiquitin-dependent signaling cascade at sites of DNA damage." Cell **136**(3): 420-434.

Stewart, G. S., B. Wang, et al. (2003). "MDC1 is a mediator of the mammalian DNA damage checkpoint." Nature **421**(6926): 961-966.

Stiff, T., S. A. Walker, et al. (2006). "ATR-dependent phosphorylation and activation of ATM in response to UV treatment or replication fork stalling." The EMBO Journal **25**(24): 5775-5782.

Stillman, B. W., J. B. Lewis, et al. (1981). "Identification of the gene and mRNA for the adenovirus terminal protein precursor." Cell **23**(2): 497-508.

Stracker, T. H., C. T. Carson, et al. (2002). "Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex." Nature **418**(6895): 348-352.

Stracker, T. H., D. V. Lee, et al. (2005). "Serotype-specific reorganization of the Mre11 complex by adenoviral E4orf3 proteins." Journal of Virology **79**(11): 6664-6673.

Takai, H., K. Tominaga, et al. (2000). "Aberrant cell cycle checkpoint function and early embryonic death in Chk1(-/-) mice." Genes & Development **14**(12): 1439-1447.

Tauber, B. and T. Dobner (2001). "Adenovirus early E4 genes in viral oncogenesis." Oncogene **20**(54): 7847-7854.

Tauber, B. and T. Dobner (2001). "Molecular regulation and biological function of adenovirus early genes: the E4 ORFs." Gene **278**(1-2): 1-23.

Taylor, W. R. and G. R. Stark (2001). "Regulation of the G2/M transition by p53." Oncogene **20**(15): 1803-1815.

Thomas, D. L., J. Schaack, et al. (2001). "Several E4 region functions influence mammary tumorigenesis by human adenovirus type 9." Journal of Virology **75**(2): 557-568.

Thrower, J. S., L. Hoffman, et al. (2000). "Recognition of the polyubiquitin proteolytic signal." The EMBO Journal **19**(1): 94-102.

Trentin, J. J., G. L. Van Hoosier, Jr., et al. (1968). "The oncogenicity of human adenoviruses in hamsters." Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine **127**(3): 683-689.

Trentin, J. J., Y. Yabe, et al. (1962). "The quest for human cancer viruses." Science **137**(3533): 835-841.

Trimborn, M., S. M. Bell, et al. (2004). "Mutations in microcephalin cause aberrant regulation of chromosome condensation." American journal of Human Genetics **75**(2): 261-266.

Trimborn, M., D. Schindler, et al. (2006). "Misregulated chromosome condensation in MCPH1 primary microcephaly is mediated by condensin II." Cell Cycle **5**(3): 322-326.

Trujillo, K. M., S. S. Yuan, et al. (1998). "Nuclease activities in a complex of human recombination and DNA repair factors Rad50, Mre11, and p95." The Journal of Biological Chemistry **273**(34): 21447-21450.

Turnell, A. S. and R. J. Grand (2012). "DNA viruses and the cellular DNA-damage response." The Journal of General Virology.

Turnell, A. S., R. J. Grand, et al. (1999). "The replicative capacities of large E1B-null group A and group C adenoviruses are independent of host cell p53 status." Journal of Virology **73**(3): 2074-2083.

Turnell, A. S., R. J. Grand, et al. (2000). "Regulation of the 26S proteasome by adenovirus E1A." The EMBO Journal **19**(17): 4759-4773.

Unsal-Kacmaz, K., P. D. Chastain, et al. (2007). "The human Tim/Tipin complex coordinates an Intra-S checkpoint response to UV that slows replication fork displacement." Molecular and Cellular Biology **27**(8): 3131-3142.

Unsal-Kacmaz, K., T. E. Mullen, et al. (2005). "Coupling of human circadian and cell cycles by the timeless protein." Molecular and Cellular Biology **25**(8): 3109-3116.

Uziel, T., Y. Lerenthal, et al. (2003). "Requirement of the MRN complex for ATM activation by DNA damage." The EMBO Journal **22**(20): 5612-5621.

Valentine, R. C. and H. G. Pereira (1965). "Antigens and structure of the adenovirus." Journal of Molecular Biology **13**(1): 13-20.

Van der Vliet, P. C. (1995). "Adenovirus DNA replication." Current Topics in Microbiology and Immunology **199 (Pt 2)**: 1-30.

van der Vliet, P. C. and A. J. Levine (1973). "DNA-binding proteins specific for cells infected by adenovirus." Nature: New Biology **246**(154): 170-174.

van der Voet, M., C. W. Berends, et al. (2009). "NuMA-related LIN-5, ASPM-1, calmodulin and dynein promote meiotic spindle rotation independently of cortical LIN-5/GPR/Galpha." Nature Cell Biology **11**(3): 269-277.

van Leeuwen, H. C., M. Rensen, et al. (1997). "The Oct-1 POU homeodomain stabilizes the adenovirus preinitiation complex via a direct interaction with the priming protein and is displaced when the replication fork passes." The Journal of Biological Chemistry **272**(6): 3398-3405.

Wasserman, T., K. Katsenelson, et al. (2010). "A novel c-Jun N-terminal kinase (JNK)-binding protein WDR62 is recruited to stress granules and mediates a nonclassical JNK activation." Molecular Biology of the Cell **21**(1): 117-130.

Weiden, M. D. and H. S. Ginsberg (1994). "Deletion of the E4 region of the genome produces adenovirus DNA concatemers." Proceedings of the National Academy of Sciences of the United States of America **91**(1): 153-157.

Weigel, S. and M. Dobbelstein (2000). "The nuclear export signal within the E4orf6 protein of adenovirus type 5 supports virus replication and cytoplasmic accumulation of viral mRNA." Journal of Virology **74**(2): 764-772.

Weissman, A. M. (2001). "Themes and variations on ubiquitylation." Nature reviews. Molecular Cell Biology **2**(3): 169-178.

Weitzman, M. D., C. T. Carson, et al. (2004). "Interactions of viruses with the cellular DNA repair machinery." DNA Repair **3**(8-9): 1165-1173.

Weitzman, M. D., C. E. Lilley, et al. (2010). "Genomes in conflict: maintaining genome integrity during virus infection." Annual Review of Microbiology **64**: 61-81.

Weitzman, M. D. and D. A. Ornelles (2005). "Inactivating intracellular antiviral responses during adenovirus infection." Oncogene **24**(52): 7686-7696.

Welchman, R. L., C. Gordon, et al. (2005). "Ubiquitin and ubiquitin-like proteins as multifunctional signals." Nature reviews. Molecular Cell Biology **6**(8): 599-609.

Werness, B. A., A. J. Levine, et al. (1990). "Association of human papillomavirus types 16 and 18 E6 proteins with p53." Science **248**(4951): 76-79.

Whibley, C., P. D. Pharoah, et al. (2009). "p53 polymorphisms: cancer implications." Nature Reviews. Cancer **9**(2): 95-107.

White, E. (2001). "Regulation of the cell cycle and apoptosis by the oncogenes of adenovirus." Oncogene **20**(54): 7836-7846.

Whyte, P., K. J. Buchkovich, et al. (1988). "Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product." Nature **334**(6178): 124-129.

Wilkinson, D. E. and S. K. Weller (2006). "Herpes simplex virus type I disrupts the ATR-dependent DNA-damage response during lytic infection." Journal of Cell Science **119**(Pt 13): 2695-2703.

Wilkinson, K. D. (2000). "Ubiquitination and deubiquitination: targeting of proteins for degradation by the proteasome." Seminars in Cell & Developmental Biology **11**(3): 141-148.

Wollnik, B. (2010). "A common mechanism for microcephaly." Nature Genetics **42**(11): 923-924.

Woo, J. L. and A. J. Berk (2007). "Adenovirus ubiquitin-protein ligase stimulates viral late mRNA nuclear export." Journal of Virology **81**(2): 575-587.

Wood, J. L., N. Singh, et al. (2007). "MCPH1 functions in an H2AX-dependent but MDC1-independent pathway in response to DNA damage." The Journal of Biological Chemistry **282**(48): 35416-35423.

Woods, C. G., J. Bond, et al. (2005). "Autosomal recessive primary microcephaly (MCPH): a review of clinical, molecular, and evolutionary findings." American Journal of Human Genetics **76**(5): 717-728.

Wright, J. A., K. S. Keegan, et al. (1998). "Protein kinase mutants of human ATR increase sensitivity to UV and ionizing radiation and abrogate cell cycle checkpoint control." Proceedings of the National Academy of Sciences of the United States of America **95**(13): 7445-7450.

Wu, E. and G. R. Nemerow (2004). "Virus yoga: the role of flexibility in virus host cell recognition." Trends in Microbiology **12**(4): 162-169.

Wu, X., J. H. Bayle, et al. (1993). "The p53-mdm-2 autoregulatory feedback loop." Genes & Development **7**(7A): 1126-1132.

Xiao, Z., J. Xue, et al. (2006). "Differential roles of checkpoint kinase 1, checkpoint kinase 2, and mitogen-activated protein kinase-activated protein kinase 2 in mediating DNA damage-induced cell cycle arrest: implications for cancer therapy." Molecular Cancer Therapeutics **5**(8): 1935-1943.

Xie, Y. (2010). "Structure, assembly and homeostatic regulation of the 26S proteasome." Journal of Molecular Cell Biology **2**(6): 308-317.

Xu, X., J. Lee, et al. (2004). "Microcephalin is a DNA damage response protein involved in regulation of CHK1 and BRCA1." The Journal of Biological Chemistry **279**(33): 34091-34094.

Yamane, K., J. Chen, et al. (2003). "Both DNA topoisomerase II-binding protein 1 and BRCA1 regulate the G2-M cell cycle checkpoint." Cancer Research **63**(12): 3049-3053.

Yamane, K., M. Kawabata, et al. (1997). "A DNA-topoisomerase-II-binding protein with eight repeating regions similar to DNA-repair enzymes and to a cell-cycle regulator." European Journal of Biochemistry / FEBS **250**(3): 794-799.

Yang, S. Z., F. T. Lin, et al. (2008). "MCPH1/BRIT1 cooperates with E2F1 in the activation of checkpoint, DNA repair and apoptosis." EMBO Reports **9**(9): 907-915.

Yazdi, P. T., Y. Wang, et al. (2002). "SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint." Genes & Development **16**(5): 571-582.

Yee, S. P. and P. E. Branton (1985). "Detection of cellular proteins associated with human adenovirus type 5 early region 1A polypeptides." Virology **147**(1): 142-153.

Yew, P. R. and A. J. Berk (1992). "Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein." Nature **357**(6373): 82-85.

Yew, P. R., X. Liu, et al. (1994). "Adenovirus E1B oncoprotein tethers a transcriptional repression domain to p53." Genes & Development **8**(2): 190-202.

Yondola, M. A. and P. Hearing (2007). "The adenovirus E4 ORF3 protein binds and reorganizes the TRIM family member transcriptional intermediary factor 1 alpha." Journal of Virology **81**(8): 4264-4271.

Yoo, H. Y., S. Y. Jeong, et al. (2006). "Site-specific phosphorylation of a checkpoint mediator protein controls its responses to different DNA structures." Genes & Development **20**(7): 772-783.

Yoo, H. Y., A. Kumagai, et al. (2007). "Ataxia-telangiectasia mutated (ATM)-dependent activation of ATR occurs through phosphorylation of TopBP1 by ATM." The Journal of Biological Chemistry **282**(24): 17501-17506.

Yoo, H. Y., A. Kumagai, et al. (2009). "The Mre11-Rad50-Nbs1 complex mediates activation of TopBP1 by ATM." Molecular Biology of the Cell **20**(9): 2351-2360.

Yoshizawa-Sugata, N. and H. Masai (2007). "Human Tim/Timeless-interacting protein, Tipin, is required for efficient progression of S phase and DNA replication checkpoint." The Journal of Biological Chemistry **282**(4): 2729-2740.

You, Z., C. Chahwan, et al. (2005). "ATM activation and its recruitment to damaged DNA require binding to the C terminus of Nbs1." Molecular and Cellular Biology **25**(13): 5363-5379.

Yu, T. W., G. H. Mochida, et al. (2010). "Mutations in WDR62, encoding a centrosome-associated protein, cause microcephaly with simplified gyri and abnormal cortical architecture." Nature Genetics **42**(11): 1015-1020.

Yu, X., S. Fu, et al. (2006). "BRCA1 ubiquitinates its phosphorylation-dependent binding partner CtIP." Genes & Development **20**(13): 1721-1726.

Yu, X., Y. Yu, et al. (2003). "Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif-Cul5-SCF complex." Science **302**(5647): 1056-1060.

Zantema, A., P. I. Schrier, et al. (1985). "Adenovirus serotype determines association and localization of the large E1B tumor antigen with cellular tumor antigen p53 in transformed cells." Molecular and Cellular Biology **5**(11): 3084-3091.

Zhang, D., K. Zaugg, et al. (2006). "A role for the deubiquitinating enzyme USP28 in control of the DNA-damage response." Cell **126**(3): 529-542.

Zhang, X., D. Liu, et al. (2009). "CDK5RAP2 is required for spindle checkpoint function." Cell Cycle **8**(8): 1206-1216.

Zhang, X., A. S. Turnell, et al. (2004). "The targeting of the proteasomal regulatory subunit S2 by adenovirus E1A causes inhibition of proteasomal activity and increased p53 expression." The Journal of Biological Chemistry **279**(24): 25122-25133.

Zhao, H. and H. Piwnica-Worms (2001). "ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1." Molecular and Cellular Biology **21**(13): 4129-4139.

Zhao, X., R. J. Madden-Fuentes, et al. (2008). "Ataxia telangiectasia-mutated damage-signaling kinase- and proteasome-dependent destruction of Mre11-Rad50-Nbs1

subunits in Simian virus 40-infected primate cells." Journal of Virology **82**(11): 5316-5328.

Zheng, Z. M. (2010). "Viral oncogenes, noncoding RNAs, and RNA splicing in human tumor viruses." International Journal of Biological Sciences **6**(7): 730-755.

Zou, L., D. Cortez, et al. (2002). "Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin." Genes & Development **16**(2): 198-208.

Zou, L. and S. J. Elledge (2003). "Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes." Science **300**(5625): 1542-1548.

Zou, L., D. Liu, et al. (2003). "Replication protein A-mediated recruitment and activation of Rad17 complexes." Proceedings of the National Academy of Sciences of the United States of America **100**(24): 13827-13832.