REGULATION OF CELLULAR SIGNALLING PATHWAYS BY ADENOVIRUS

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ABSTRACT

It is well established that adenoviruses inactivate the host cell DNA damage response to enhance viral DNA replication. This is achieved, in part, by the ability of viral E1B55K and E4ORF6 proteins to hijack host cell cullin-containing E3 ubiquitin ligase complexes and target key cellular proteins such as Mre11, p53 and DNA ligase IV for degradation via the ubiquitin-proteasome pathway. To assess the generality of this viral response, studies were undertaken using a panel of representative serotypes from adenovirus species A to E to determine how they interact with the host cell DNA damage response. Notably, serotypes from species B and D were unable to promote the degradation and/or relocalization of Mre11 and p53, although DNA ligase IV degradation was fundamentally conserved. Furthermore, species B and D serotypes induced the sustained overexpression of transcriptionally inactive p53, and induced both ATM and ATR kinase activity. As these events would typically be viewed as detrimental to virus survival, these data suggest that different adenovirus serotypes have evolved novel strategies in order to subvert the cellular DNA damage response during infection.

Adenoviruses have long been utilised as useful tools for identifying, and characterizing, the function of fundamental cellular proteins such as tumour suppressors and those involved in the DNA damage response. Therefore, studies were also carried out to identify novel Ad12E1B54K-interacting proteins through mass spectrometric analysis, and subsequently to examine the functional significance of these interactions. Several putative novel Ad12E1B54K-interacting proteins were identified using this approach, one being the transcriptional intermediary factor 1γ (TIF1γ), a transcriptional regulator that has recently been identified as a tumour suppressor. Further studies determined that TIF1γ was relocalized

to nuclear tracks in an E4ORF3-dependent manner early during adenovirus infection, and was subsequently degraded in a ubiquitin-mediated proteasome-dependent manner. Uniquely, TIF1γ degradation was shown to be E1B55K/E4ORF6-independent and E4ORF3-dependent. Data presented in this thesis also suggest that E4ORF3 does not utilise host cell cullin-based E3 ubiquitin ligases in order to promote TIF1γ degradation. The ability of E4ORF3 to target cellular substrates for degradation represents a novel way in which adenoviruses are able to target cellular substrates. Significantly, TIF1γ degradation was conserved during infection with Ad serotypes from species A to C which may highlight its importance for productive viral infection. It also appears that TIF1γ may have an as yet unidentified role in the DNA damage response since it was also found to interact with components of the ATR kinase pathway.

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“A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales.”
Marie Curie

“In science one tries to tell people, in such a way as to be understood by everyone, something that no one ever knew before. But in poetry, it's the exact opposite.”
Humphry Davy

“I have had my results for a long time: but I do not yet know how I am to arrive at them.”
Karl Friedrich Gauss

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CHAPTER 1

INTRODUCTION

1.1 ADENOVIRUS

1.1.1 DNA tumour viruses

Tumours arise when cells proliferate in an uncontrolled manner and eventually present malignant characteristics, such as their ability to invade surrounding tissues and metastasis. It has been a century since pioneering studies were carried out that discovered an avian retrovirus, Rous sarcoma virus (RSV), which was capable of inducing sarcomas in chickens; however it took many years to comprehend the significance of this discovery (Rous, 1911). Since this time, a plethora of DNA viruses from the families *Adenoviridae*, *Herpesviridae*, *Papillomaviridae* and *Polyomaviridae* have also been shown to induce tumourigenesis in humans or other animals, and in doing so have proven to be invaluable tools in the understanding of tumour biology (Javier and Butel, 2008). Although only a small proportion of virally-infected humans develop cancer, current estimates indicate that approximately 20% of human cancers worldwide (1.9 million new cases in the year 2002) are attributable to infectious agents; including the human immunodeficiency virus type 1 (HIV-1), human papillomavirus (HPV), human T-cell lymphotrophic virus 1 (HTLV-1), hepatitis B and C viruses, the Epstein-Barr virus (EBV) and the Kaposi's sarcoma-associated herpesvirus (KSHV). Of this 20%, a small proportion is also estimated to be caused by persistent infections of bacteria or parasites (Parkin, 2006).

Neoplastic transformation of cells by DNA viruses is a multistep process that can occur many years after the initial viral infection. The resulting cells display characteristic morphological and growth changes such as loss of contact inhibition, reduced adhesion, reduced serum requirements, disruption of the cytoskeleton and immortalization (Hanahan and Weinberg, 2000). These modifications are transmitted in a heritable manner, from parental cells to daughter cells, to form a transformed population which eventually develops into a tumour.

The basic mechanism for transformation often involves viral DNA integration into the cellular genome where it either permanently expresses early oncogenic viral genes, or causes the activation of adjacent cellular oncogenes to disrupt the normal cellular gene expression and signal transduction pathways. This is assisted by genetic, immunological and environmental factors, and the ability of most DNA tumour viruses to suppress the negative control on cell replication caused by cellular tumour suppressors such as p53 and the retinoblastoma gene product, pRB (Ludlow and Skuse, 1995, Hanahan and Weinberg, 2000). Indeed, as p53 and pRB are the most frequently altered regulatory pathways in human cancer, it is no surprise that DNA tumour viruses have been utilised experimentally to delineate the critical molecular events that occur during tumourigenesis (Berk, 2005).

Over the past 40 years tumour virologists have utilised DNA tumour viruses to study the molecular basis of cell growth. These studies have lead to revolutionary breakthroughs in our understanding of cancer, cell cycle regulation, transcription and DNA replication (Javier and Butel, 2008). Perhaps two of the most landmark discoveries have been the identification of p53 from simian virus 40 (SV40)-transformed murine cells, and the function of pRb through association with the adenovirus (Ad) early region 1A (E1A) gene product (Lane and Crawford, 1979, Linzer and Levine, 1979, Whyte *et al*., 1988).

1.1.2 Identification and classification

Adenoviruses were first discovered in 1953 by a postdoctoral fellow, and his colleagues, while working on a project at the National Institutes of Health (Bethesda, Maryland) hoping to isolate the "virus of the common cold" (Rowe *et al.*, 1953). These researchers cultured cells isolated from the adenoid and tonsil tissue of infected children, and noted that the cells

were rounded and clumped suggesting that they were infected by a virus; hence the family name *Adenoviridae* ("adeno" comes from the Greek word for gland). The next important finding came in 1962 when researchers discovered, for the first time, a human virus capable of inducing oncogenesis, albeit under experimental conditions (Trentin *et al*., 1962). This was Ad serotype 12 (Ad12) and it was capable of inducing "fast-growing malignant tumours at the site of intrapulmonary injection into newborn hamsters" (Trentin *et al*., 1962).

The *Adenoviridae* family is comprised of five serologically distinct genera depending on the host species: *Mastadenoviridae*, *Atadenovirus*, *Aviadenoviridae*, *Siadenoviridae* and the recently proposed *Ichtadenovirus* which infects certain species of fish (Carstens, 2010). Mammalian species of Ad fall into the *Mastadenovirus* genus, with at least 55 known human serotypes classified and subdivided into seven species (A to G) largely based on their ability to agglutinate erythrocytes (Eiz and Pring-Akerblom, 1997). Patterns of other viral properties are also observed within, and between, species including their oncogenic potential in rodents, the percentage of DNA homology and G+C content, and their cellular tropism (Table 1.1).

Since their discovery, adenoviruses have been isolated from every species of mammal, bird and amphibian studied, and appear to be highly host specific. Their oncogenic potential, however, is limited to mammals such as hamsters and rats, and *in vitro* all Ad serotypes tested to date have the capacity to transform human and rodent primary cells (McBride and Wiener, 1964, Russell, 2009). Indeed, sheared non-oncogenic Ad5E1 DNA and oncogenic Ad12E1 cDNA have the ability to transform human embryonic kidney (HEK), and human embryonic retinoblast (HER) cells, respectively (Graham *et al*., 1977, Byrd *et al*., 1982). In Ad-induced tumour cells and Ad-transformed cells, partial fragments of viral DNA occasionally become integrated into the host's cellular genome supposedly via a haphazard

Table 1.1. Classification of human adenoviruses. Serotypes used in this study are highlighted in red.

Species	Serotype	Oncogenicity in rodents	Transformation in tissue culture	% of G-C in DNA
A	**12**, 18, 31	High	+	48-49
B1	**3**, **7** , 16, 21, 50	Moderate	+	50-52
B2	**11**, 14, 34, 35, 55	Moderate	+	
C	1, 2, **5**, 6	Low or none	+	57-59
D	8, **9**, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-49, 51, 53, 54	Low or none	+	57-61
E	**4**	Low or none	+	57-59
F	40, 41	Not reported	+	51
G	52	Not reported	?	55

process at a random site; however there are also many claims in the literature that contest this view (Sambrook *et al*., 1980, Hilger-Eversheim and Doerfler, 1997, Doerfler, 2009).

Approximately one-third of human Ad serotypes are also known to cause a broad spectrum of diseases relating to respiratory, gastrointestinal and conjunctival syndromes in the young, elderly and immunocompromised (Hierholzer, 1992). Fewer serotypes have also been increasingly implicated in severe disorders such as hepatitis, viral meningitis, pneumonia and disseminated infection in AIDS and other immunocompromised patients (Hierholzer, 1992, Sarantis *et al*., 2004).

1.1.3 Structure and genome

Ad virions are composed of icosahedral capsids which are approximately 70 nm in diameter, comprised of 87% protein and 13% DNA, and have no membrane or lipid layer (Rux and Burnett, 2004). The unique structure of this capsid is formed of 240 homotrimeric hexons and 12 penton bases, each with a protruding fibre (Russell, 2009). Variation among fibre proteins accounts for the differing haemagglutinating properties of serotypes, and also mediates binding to cell surface receptors such as the coxsackie and adenovirus receptor (CAR; Zhang and Bergelson, 2005). Several minor capsid (IIIa, VI, VIII and IX) and core proteins (V, VII, X and the terminal protein; TP) are also expressed to provide stability for the mature virion and viral DNA (Russell, 2000, Vellinga *et al*., 2005).

Adenoviruses have linear genomes of double-stranded DNA (dsDNA) between 26 and 45 kilobase (kb) pairs making it the largest known non-enveloped virus. Sequence analysis has revealed that within Ad species genome homology ranges from 70-95%, while across species sequence homology is only 5-20% which reflects their distinct biology (Green *et al*. 1979).

Ad4 (species E) shows strong homology to several serotypes from species B, while serotypes from species A and F are highly related and the most divergent from those of other species (Bailey and Mautner, 1994, Davison *et al*., 2003). All adenoviruses also contain serotype-specific inverted terminal repeats (ITRs) of 100-140 base pairs (bp) which flank the 5' and 3' genome ends and encode origins of replication (Rauschhuber *et al*., 2011). A 55 kilodalton (kDa) TP is also covalently joined to the 5' end of each ITR to act as a primer for DNA replication.

1.1.4 Ad replication cycle

Once Ad DNA enters human cells it becomes uncoated and released into the nucleus where it may cause non-replicative latent infection, for example in the T-lymphocyte population of tonsils, or lytic infection (Neumann *et al*., 1987, Zhang *et al*., 2010). Lytic infectious cycles can occur in permissive human epithelial cells, such as those used throughout this study, which permit viral replication, progeny virus production, virion release and cell death (Zhang *et al*., 2010). The entire cycle takes approximately 20-24 hours in cultured HeLa cells but proceeds much slower in host organisms, and is divided into early and late stages of gene transcription, separated by the onset of viral DNA replication (Russell, 2000, Russell, 2009). Gene transcription is a tightly orchestrated process with the genome divided into eight transcriptional units which are similarly organized in all serotypes: five early (E1A, E1B, E2, E3 and E4), two intermediate (IX and IVa2), and one major late which produces five families of mRNA transcripts (L1-L5; Fig 1.1). A basic summary of the functions of these Ad gene products can be found in Table 1.2, but in brief, early region 2 (E2) is essential for viral DNA replication, and early region 3 (E3) encodes the adenovirus death protein (ADP) to facilitate cytolysis and virion release late in the infectious cycle, and is required for immune evasion by

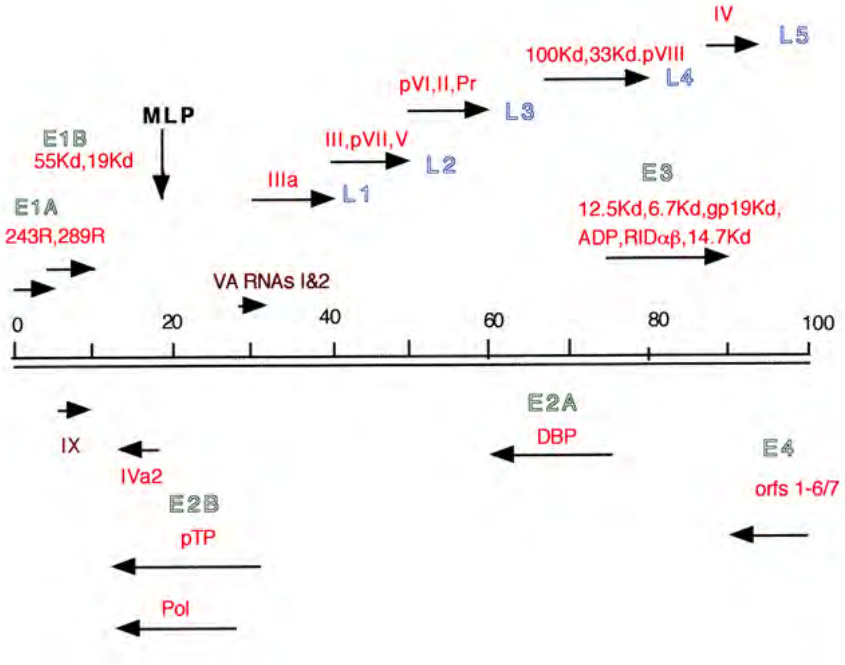


Fig 1.1. Diagrammatic representation of the adenoviral transcription map.

The location and direction of the major early and late transcription units are shown. The early transcripts are outlined in green, and the late transcripts are outlined in blue. Arrows indicate the direction of transcription (adapted from Russell, 2000).

Table 1.2. Basic functions of adenovirus gene products.

Transcription unit	Function of gene product
E1A	transcription, transformation, induces S-phase entry
E1B	transformation, blocks apoptosis
E2A	encodes DNA binding protein (DBP)
E2B	DNA polymerase
E3	immune modulation
E4	transcriptional regulation, mRNA transport, DNA replication, host cell shut off
IX & IVa2	transcriptional activation of the major late promoter (MLP) to regulate the early to late switch, synthesis of structural proteins
late	synthesis of structural proteins

affecting cell surface receptor signalling (Tollefson *et al*., 1996, Liu *et al*., 2003, Fessler *et al*., 2004). More recently a 24 kDa late protein has also been identified as the U exon protein (UXP) which is predicted to have a function relevant to Ad DNA replication or RNA transcription (Tollefson *et al*., 2007). E1A, early region 1B (E1B) and early region 4 (E4) are the only early proteins expressed by all genera of *Adenoviridae* and their specific roles are discussed further below.

The expression of early viral genes relies on E1A which is a powerful transcriptional activator capable of amplifying its own expression, and that of other early proteins which drive viral DNA replication and late gene expression. Typically 6-8 hours post-infection, once the host cell is primed, Ad DNA replication proceeds from either end of the linear genome using viral proteins transcribed from the E2 region. These include the DNA binding protein (DBP) and DNA polymerase (AdPol) which are encoded by the E2A and E2B proteins, respectively, and pTP which is the TP precursor (van der Vliet and Levine 1973, de Jong *et al*., 2003, Liu *et al*., 2003).

In short, DNA replication is initiated by a novel protein-priming mechanism in which AdPol catalyzes a covalent linkage between the 5'-terminal nucleotide dCMP (deoxycytidine monophosphate) and a pTP serine residue. This dCMP/pTP complex then acts as a primer for nascent strand DNA synthesis via a strand displacement mechanism in the presence of AdPol and DBP. The cellular transcription factors, nuclear factor I (NF-I) and nuclear factor III (NF-III) promote replication by binding to AdPol and pTP, respectively, enhancing their affinity for viral DNA sequences (Nagata *et al*., 1982, de Jong *et al*., 2003, Liu *et al*., 2003). Although DBP acts to promote ATP-independent elongation, a further cellular protein nuclear factor II (NF-II) which is type I DNA topoisomerase, is also required for complete elongation by relaxing supercoiled DNA (Nagata *et al*., 1983). Finally, during virion

maturation pTP is cleaved via an intermediate TP (iTP) at two sites by a virally encoded 23 kDa protease. This yields a stable TP which is important for DNA-nuclear matrix association and protection from DNA unwinding and exonuclease activity (Dunsworth-Browne *et al*., 1980, Stillman *et al*., 1981, Schaack *et al*., 1990). Succeeding viral DNA replication, transcription of the intermediate and late genes is initiated from the major late promoter (MLP) to generate structural proteins required for the synthesis and assembly of mature virion particles.

In order to preserve viral DNA replication and obtain the maximal production of virions, adenoviruses must alter the host's cellular environment to control gene expression, the cell cycle and apoptosis. The approaches used to accomplish this are detailed later in this Chapter with particular attention on the circumvention of the cellular DNA damage response (DDR).

1.1.5 E1A

E1A is the first Ad mRNA to be transcribed and is detected approximately 1 hour post-infection. The E1A region encodes two major RNA products, 12S and 13S which in Ad5 correspond to 243 and 289 amino acid residue products, respectively (Boulanger and Blair, 1991). These gene products contain three highly conserved regions (CR1, CR2 and CR4), and due to alternative RNA splicing the 13S protein also contains an extra conserved region CR3 (Gallimore and Turnell, 2001). 12S and 13S have the capacity to interact with in excess of 30 cellular proteins, and in doing so both sabotages normal cell cycle control and induces viral DNA synthesis. Some of these proteins are illustrated in the linear representation of 13S E1A seen in Figure 1.2.

Crucially, E1A regulates the transcriptional activation and repression of several early viral and cellular genes. And although this protein is not a DNA-binding transcription factor itself, it instead acts indirectly to transactivate gene expression by interacting with a number of cellular proteins that function as transcription factors, co-activators, co-repressors, nucleosomal remodelling factors and general transcriptional machinery (Gallimore and Turnell, 2001). The best characterized mechanism by which E1A transactivates gene expression is through the E2F family of transcription factors. In this instance, CR1 and CR2 regions act together to bind and deregulate the transcriptional repressor functions of the pRB tumour suppressor gene product (and the related pocket proteins p107 and p130), displacing it from E2F responsive promoters. Along with remodelling of the surrounding chromatin, this "freeing" of E2F activates transcription from a variety of genes containing E2F-binding sites in their promoters, such as the Ad E2 early promoter (Bagchi *et al*., 1990, Ikeda and Nevins, 1993, Ghosh and Harter, 2003). Transcription of the cellular genes CDK2, cyclin E and cyclin A are also activated in this manner which leads to quiescent cells being driven into S-phase, circumvention of normal cell growth restrictions and priming of the cell for viral DNA replication (Grand *et al*., 1998).

E1A CR1 also directs cell cycle progression through simultaneously binding to the homologous histone acetyltransferases (HATs) CBP (CREB binding protein) and p300 (Howe *et al*., 1990, Pelka *et al*., 2009). CBP and p300 are transcriptional co-activators important for the transcriptional regulation of cell cycle mediators, such as p53 and nuclear factor-kappa B (NF-κB), through the acetylation of core histone proteins (Turnell and Mymryk, 2006). E1A has also recently been shown to inhibit the E3 ubiquitin ligase SCF^{Fbw7} (a Skp/cullin/F-box complex that contains Fbw7 [F-box and WD repeat domain-containing 7] as the F-box protein) which is likely to induce unregulated proliferation during Ad infection,

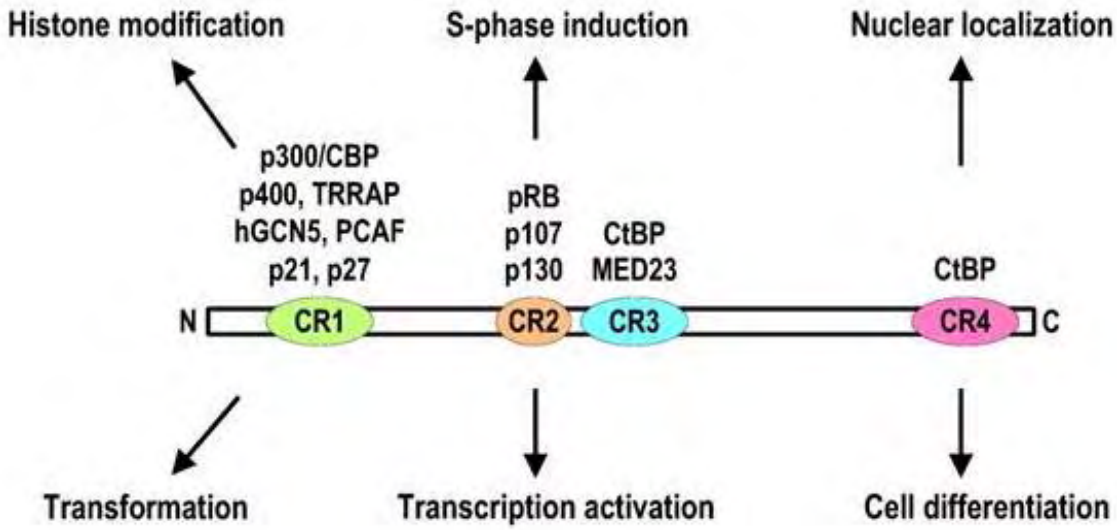


Fig 1.2. Linear representation of Ad5 13S E1A and its biological functions

The four conserved regions of E1A (CR1-CR4) are represented by the coloured oblongs, above which are listed their principle cellular binding partners. The cellular roles of these distinct regions are also illustrated (adapted from Zheng, 2010).

since the ubiquitin ligase properties of this complex are involved in cell growth, survival and differentiation (Isobe *et al*., 2009).

The E1A domains responsible for binding to pRB and CBP/p300 have also been implicated in stabilizing the tumour suppressor protein p53 and promoting p53-dependent apoptosis (Dabbas and White, 1993, Querido *et al*., 1997, Samuelson and Lowe, 1997). Furthermore, E1A can stabilize p53 indirectly through the tumour suppressor protein $p19^{ARF}$, in a manner distinct from its DNA damage-induced regulation (de Stanchina *et al*., 1998). Even though stabilizing an important pro-apoptotic protein such as p53 may appear deleterious for a replicating virus, the expression of other downstream viral proteins such as E1B and E4 counteract this action, and one study suggests that functional p53 can even promote virus replication and late virus protein expression (Royds *et al*., 2006).

Interestingly, the same activities of E1A that deregulate cell cycle progression are also required for oncogenic transformation by the virus. Indeed, the ability of E1A to immortalize primary cells *in vitro* is through the binding of cellular proteins such as pRB, p53 and p300/CBP. However the cooperation of other oncogenes, such as E1B or activated mutant $p21^{ras}$ expression, are required for full transformation and tumourigenesis in rodents (Sarnow *et al*., 1982a, Frisch and Mymryk., 2002). E4ORF6 (early region 4, open reading frame 6) has also been shown to interact with, and regulate, the transcriptional functions of p53 in an E1B55K-independent manner to enhance E1A-mediated transformation of primary baby rat kidney cells (Dobner *et al*., 1996, Nevels *et al*., 1997).

1.1.6 E1B

The E1B gene encodes at least five different polypeptides, including E1B19K and E1B55K (compared to the Ad5 protein, the Ad12 protein lacks several amino acid residues and is referred to as E1B54K), which are made from the same transcript but use alternative reading frames and hence have no identical regions (Takayesu *et al*., 1994, Sieber and Dobner, 2007). The principle roles for these proteins are the inhibition of apoptosis and cooperation with E1A to promote transformation. A linear representation of Ad5E1B55K can be found in Figure 1.3.

To divert cells away from the pro-apoptotic pathway induced by E1A, E1B55K has evolved a number of mechanisms which, along with E4 proteins, act to counteract p53 stabilization in the early stages of viral infection. In transformed cells E1B55K actively binds to p53-responsive promoters in order to represses its transcriptional activity, while during active Ad infection p53 activity is additionally counteracted by E1B55K E3 SUMO1 ligase activity which stimulates the conjugation of the small ubiquitin-like modifier (SUMO) to p53 (Yew and Berk, 1994, Muller and Dobner, 2008, Pennella *et al*., 2010). This SUMO modification maximally inhibits p53 function by initially tethering it into discrete nuclear tracks to decrease its mobility, and then by facilitating its nuclear export and ultimately its degradation (Pennella *et al*., 2010). The proteasomal degradation of p53 is regulated by interactions between E1B55K and E4ORF6 which associate with cellular proteins to form an E3 ubiquitin ligase complex. This is achieved by E4ORF6 acting as an adapter protein via two BC boxes which hijack cellular cullin-based E3 ubiquitin ligase complexes to ubiquitylate p53, targeting it to the 26S proteasome for proteolytic degradation (Steegenga *et al*., 1998, Querido *et al*., 2001, Blanchette *et al*., 2004, Luo *et al*., 2007). While E4ORF6 can bind p53 independently, in this instance substrate specificity is managed through E1B55K which

directly interacts with p53 and ensures its recruitment to the ligase complex (Blanchette *et al.*, 2004). The establishment of virally-mediated E3 ubiquitin ligase complexes and their importance during Ad infection will be discussed in more detail below.

Work carried out using Ad5E1B55K-deleted viruses also discovered that E1B55K has important roles during the late stages of Ad infection. Indeed, E1B55K is required for the nucleocytoplasmic transport and translocation of late viral mRNAs via the CRM1 (chromosome region maintenance 1) export pathway, and for the inhibition of host cell mRNA transport and translation (Babiss *et al.*, 1985, Kindsmüller *et al.*, 2007, Woo and Berk, 2007). These functions are associated with E1B55K's RNA-transporter functions such as an N-terminal nuclear export signal (NES), a carboxy-terminal (C-terminal) nuclear localization signal (NLS) and a ribonucleoprotein (RNP) RNA-binding motif (Horridge and Leppard 1998, Krätzer *et al.*, 2000).

E1B19K is a functional homolog of the cellular BCL-2 (pro-apoptotic B-cell lymphoma-2) family anti-apoptotic MCL-1 (myeloid cell leukaemia-1) protein, and acts to inhibit p53-independent apoptosis. E1B19K achieves this by mimicking MCL-1 and inhibiting the co-oligomerization of two other BCL-2 family members, BAX (BCL-2-associated X) and BAK (BCL-2-antagonist killer). This inhibition prevents the formation of pores in the mitochondrial membrane, cytochrome c release, caspase activation and consequently apoptosis (Cuconati *et al.*, 2002).

1.1.7 E4 region

Alternatively spliced E4 mRNAs potentially encode seven open reading frames (ORFs) of leftward-oriented genes (*ORF1*, *ORF2*, *ORF3*, *ORF3/4*, *ORF4*, *ORF6* and *ORF6/7*) all of

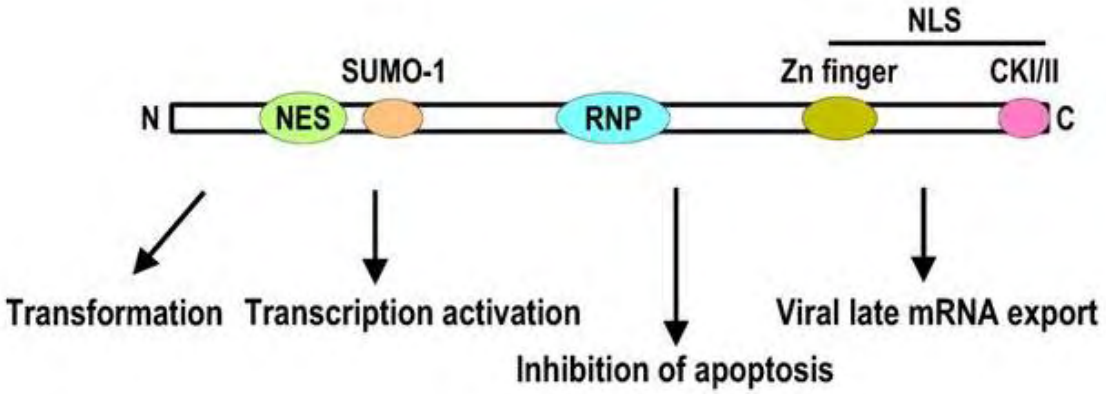


Fig 1.3. Linear representation of Ad5E1B55K and its biological functions

Five different Ad5E1B55K motifs are represented by the coloured oblongs, below which can be found their principle biological functions. CKI/II, casein kinase I/II phosphorylation site; NES, nuclear export signal; NLS, nuclear localization signal; RNP, ribonucleoprotein motif; Zn finger, putative C_2H_2 zinc finger (adapted from Zheng, 2010).

which, bar *E4ORF3/4*, have been demonstrated to be present in infected cells (Cutt *et al*., 1987, Thomas *et al*., 2001). The E4 region encompasses a diverse range of vital functions including transcriptional regulation, mRNA transport, DNA replication, virus particle assembly and host cell shutoff, all of which are reflected by the defective phenotype of viruses where the entire E4 region is deleted (Halbert *et al*., 1985, Weiden and Ginsberg, 1994, Tauber and Dobner, 2001). Mutational analysis has discovered that alterations within individual ORFs only have nominal effects suggesting that they are dispensable for lytic growth, however, mutants lacking both E4ORF3 and E4ORF6 were seriously restricted for DNA replication and growth suggesting a functional redundancy between these proteins (Halbert *et al*., 1985). Indeed, E4ORF3 and E4ORF6 have now been shown to be functionally redundant during lytic growth, in that they can compensate at varying degrees for each other's defects (Bridge and Ketner, 1989, Huang and Hearing, 1989). Despite this functional redundancy, E4ORF3 and E4ORF6 act by very distinct mechanisms to aid viral growth and replication.

The *E4ORF6* gene encodes a highly conserved protein which contains a NLS and NES, along with an amphipathic arginine-rich α-helical nuclear retention signal (NRS) that is believed to target E1B55K/E4ORF6 complexes to the nucleus (Orlando and Ornelles, 1999). In addition, it also contains two BC boxes which indirectly interact with E1B55K via cellular E3 ubiquitin ligase complexes, and a central cysteine-rich region which forms a functional zinc-binding domain that has also been found to mediate E1B55K interactions (Boyer and Ketner, 2000, Blanchette *et al*., 2008). E4ORF6 is also known to directly bind to the C-terminal regulatory domain of p53 and block p53-mediated transcriptional activation. This is achieved by inhibiting the interaction between the N-terminal activation domain of p53, and the transcriptional initiation complex of certain transcriptional targets (Dobner *et al*., 2006).

As discussed above, E4ORF6 cooperates with E1B55K to provoke the degradation of p53 (Steegenga *et al*., 1998, Querido *et al*., 2001, Blanchette *et al*., 2004). However, parallel to this inactivation another highly conserved E4 gene product, E4ORF3, which is tightly associated with the nuclear matrix, also acts to restructure both the nucleus and the cytoplasm of infected cells by redistributing specific cellular proteins. Markedly is the promyelocytic leukemia (PML) protein which forms discrete elongated tracks that eventually surround sites of viral replication (Carvalho *et al*., 1995, Konig *et al*., 1999). Disruption of these nuclear PML oncogenic domains (PODs) has been attributed to a direct interaction between E4ORF3 and a 40 amino acid residue sequence within the specific PML isoform PMLII (Hoppe *et al*., 2006, Leppard *et al*., 2009). The function of this reorganization is undefined, however, evidence suggests that it facilitates the immobilization of cellular proteins and the ability of E4ORF3 to epigenetically silence target genes (Pennella *et al*., 2010, Soria *et al*., 2010).

The relocalization and inactivation of p53 into these nuclear tracks is also promoted by E4ORF3. Indeed, E4ORF3 has recently been found to induce heterochromatin formation through trimethylation of histone H3 at lysine residue 9 (H3K9me3) at p53 target promoters to prevent p53-DNA binding (Soria *et al*., 2010). This silencing of p53-activated transcription is independent of E1B55K/E4ORF6-mediated degradation, and synonymous with p53 stabilization in response to DNA damage (Lakin and Jackson, 1999, Soria *et al*., 2010). The role of E4ORF3 in reorganizing other cellular and viral proteins will be discussed in more detail below.

Besides targeting cellular substrates during infection E4ORF6, and possibly E4ORF3, also cooperate with E1B55K to increase late viral protein production by facilitating the nuclear export and cytoplasmic accumulation of late viral mRNAs, and blocking cellular mRNA nuclear export (Halbert *et al*., 1985, Shepard and Ornelles 2004, Woo and Berk, 2007). The

ability of E4ORF6 to interact with cellular E3 ubiquitin ligase complexes is necessary for these late functions since mutant viruses lacking E4ORF6 BC boxes are defective for viral growth and viral mRNA nuclear export (Corbin-Lickfett and Bridge 2003, Blanchette *et al*., 2008). This suggests the E3 ubiquitin ligase activity of E4ORF6 may have an as yet unidentified role in mRNA transport and stability. Furthermore, a recent study has demonstrated that E4ORF3 also inactivates components of cytoplasmic mRNA processing bodies by relocalizing them into cytoplasmic aggresomes, presumably to aid late viral mRNA accumulation (Greer *et al*., 2011).

E4ORF4 is a non-essential protein for Ad infection, although it has been shown to negatively regulate E1A transactivation of the viral E4 and E2 promoters and have an inhibitory role during Ad replication (Bridge *et al*., 1993, Bondesson *et al*., 1996, Mannervik *et al*., 1999). Interestingly, when expressed alone E4ORF4 has the capacity to induce p53-independent apoptosis in human cancer cells, but not in normal cells, via an interaction with the Bα subunit of the protein phosphatase 2A (PP2A) complex (Marcellus *et al*., 2000, Shtrichman *et al*., 2000). PP2A regulates the CDC25A (cell division cycle 25A) protein in G_2/M cell cycle checkpoint activation, therefore its interaction with E4ORF4 is proposed to initiate G_2 arrest (Li *et al*., 2009). Consequently, E4ORF4 has great therapeutic potential as a high frequency of human tumours are p53-deficient and resistant to conventional treatments which are reliant on p53-dependent apoptosis (Shtrichman *et al*., 2000, Branton and Roopchand, 2001).

The E4ORF6/7 fusion protein is also involved in apoptosis and the transactivation of the E2F family of transcription factors alongside E1A, while E4ORF1 and E4ORF2 have unknown functions in the late stages of the lytic life cycle and show oncogenic properties (O'Connor and Hearing, 2000, Tauber and Dobner, 2001, Thomas *et al*., 2001, Weitzman, 2005, Seandel *et al*., 2008, Thomas *et al*., 2009). Indeed, E4ORF1 is the primary oncogenic determinant for

the Ad9-mediated development of mammary tumours in rats (Thomas *et al*., 2001, Glaunsinger *et al*., 2001).

1.1.8 Clinical importance of studying adenoviruses

Since their discovery nearly 60 years ago, adenoviruses have been, and continue to be, utilised as model systems to study fundamental aspects of molecular and cellular biology such as tumourigenesis, cell cycle control and the cellular response to DNA damage (Berk, 2005, Weitzman and Ornelles, 2005). Indeed, due to its broad range of properties and transformation potential, E1A is the most widely studied viral oncogene and is responsible for the identification and/or characterization of numerous cellular proteins including pRB, E2F, CtBP (C-terminal-binding protein) and p300 (Yee and Branton *et al*., 1985, Kovesdi *et al*., 1986, Whyte *et al*., 1988, Boyd *et al*., 1993). The fundamental nature and function of the infamous tumour suppressor p53 was also revealed via studies on DNA tumour viruses such as Ad (Russell, 2000). Historically, alternative splicing was also first described by scientists studying Ad2 where one pre-mRNA molecule was shown to be spliced and processed to result in a variety of mature mRNA molecules with different arrangements of exons (Berget *et al*., 1977, Chow *et al*., 1977).

As discussed above adenoviruses represent the first known family of human viruses capable of causing cancer, and have widely been shown to induce tumours in rodents, yet despite its abundance in other human diseases, adenoviruses are rarely associated with human cancer (Trentin *et al*., 1962, Hierholzer, 1992, Sarantis *et al*., 2004). Recent data does indicate however, that Ad serotypes from species B and D may possess transforming potential towards humans, as real-time quantitative PCR (RQ-PCR) and *in situ* hybridization assays

have detected Ad DNA at a high frequency in paediatric brain tumours (Kosulin *et al*., 2007). During tumour development in rodents it is widely accepted that early Ad gene products, E1A and E1B, cooperate to mediate cellular transformation via a "hit-and-run" mechanism since viral DNA sequences are undetectable in the resulting tumour cell (Nevels *et al*., 2001). While the majority of Ad infections are self-limited by the normal immune response and there is currently no specific therapy or treatment, several drugs including cidofovir and ribavirin are being developed which may have the potential to treat infected patients (Miyamura *et al*., 2000, Neofytos *et al*., 2007).

Oncolytic viruses such as Ad also have the potential to be exploited as therapeutic tools. One such example is the Ad5E1B55K-deficient *dl*1520 virus which has been shown to preferentially infect and lyse cancer cells; however its efficacy in clinical trials of cancer patients has been conflicting (Heise *et al*., 1999, Khuri *et al*., 2000, Cherubini *et al*., 2006). The majority of clinical Ad research however has been concerned with its implication as an experimental vector for gene therapy, cancer therapy and recombinant vaccine development. Indeed, adenoviruses have been utilised as a vehicle for gene delivery since the early 1990s due to their ability to infect a wide variety of cells, remain relatively stable *in vitro* and be easily genetically manipulated. Currently, adenoviruses are the most commonly used gene-delivery vehicle representing nearly 25% of all vectors used in gene therapy clinical trials, however their effectiveness is yet to be established (Robinson *et al*., 2011).

There are undoubtedly more lessons to be derived from studying the natural biology of Ad infection that will contribute to our understanding of cellular biology and the molecular basis of human disease, and may help develop clinically approved therapeutic drugs. In the current study, further insights are provided into Ad virology from a range of serotypes, and through

Ad-host interactions the molecular function of potentially novel cellular mechanisms are explored.

1.2 THE DNA DAMAGE RESPONSE

1.2.1 DNA damage and cancer

Genome integrity is under constant challenge from endogenous and environmental sources. To cope with these assaults cells have evolved a series of biochemical pathways to detect, and repair, damaged DNA. However, when these processes are compromised, errantly-repaired DNA or unrepaired DNA can lead to genomic instability and the development of cancer. The two main cytotoxic lesions that threaten genome integrity are DNA double-strand (DSBs) and single-strand breaks (SSBs). Generally, DSBs are generated through exposure of cells to ionising radiation (IR) while SSBs are caused by ultraviolet (UV) radiation, however, there is a large amount of crosstalk between the pathways involved in signalling these lesions. In addition, errors in DNA replication, stalled or collapsed replication forks and the production of reactive oxygen intermediates can also elicit DDR reactions during normal S phase progression (Cha and Kleckner, 2002, Petermann and Caldecott, 2006).

Defects in DDR pathways contribute to neurodegenerative disorders, immune deficiencies, cardiovascular disease and other inheritable human diseases. Notably, individuals with germline mutations in their DDR genes are characterized by sensitivity to DNA damaging agents, and are commonly predisposed to tumour development (Gorgoulis *et al*., 2005). These genomic instability syndromes include, but are not limited to, ataxia telangiectasia (A-T), ataxia telangiectasia-like disorder (ATLD), Fanconi anaemia (FA), Li-Fraumeni syndrome, LIG4 syndrome (DNA ligase IV), Nijmegen breakage syndrome (NBS), NBS-like

syndrome, RIDDLE (radiosensitivity, immunodeficiency, dysmorphic features and learning difficulties) syndrome and Seckel syndrome (Jackson and Bartek, 2009).

Current cancer treatments such as radiotherapy and alkylating drugs exploit the DDR by inducing toxic lesions in cells. Many of these strategies however pose adverse side effects and are often non-specific. Therefore, it is not surprising that specific DNA-damaging agents are being developed and trialled in combination with traditional therapies, and are at the forefront of the latest cancer therapy research. Although the therapeutic outcome of this strategy remains to be determined, several DNA-damaging drugs have proven to be highly effective for the induction of cell cycle arrest and cell death, and have increased the efficacy of current treatments for breast and ovarian cancer in clinical trials (Helleday *et al*., 2008). One promising example are inhibitors that target the poly(ADP-ribose) polymerase (PARP) which is a scaffold protein that recruits other proteins necessary for DNA repair. Studies have successfully demonstrated that irrespective of p53 status PARP inhibitors preferentially kill neoplastic cells to reduce harmful off-target effects, and can induce human tumour regression in combination with chemotherapy (Weil and Chen, 2011). This is due to the fact that tumour cells, because of their frequency of replication and genomic susceptibility, have increased frequency of mutations which can be exploited by drugs such as PARP inhibitors.

1.2.2 p53

Often described as the 'guardian of the genome', the p53 protein is mutated in over 50% of human cancers and regulates an array of cellular processes including DNA repair, cell cycle arrest and apoptosis, as demonstrated in Figure 1.4 (Lane, 1992, Vogelstein *et al*., 2000, Whibley *et al*., 2009). The *p53* gene was first described in 1979 by six independent

researchers who believed it to be an oncogene, however it later became clear that *p53* was in fact a tumour suppressor gene (Vogelstein *et al*., 2000). Tumourigenesis can inactivate p53 by several mechanisms including DNA mutations (e.g. lung, brain and colon tumours), viral infection (e.g. cervical and liver tumours and lymphoma) and mislocalization from the nucleus to the cytoplasm (e.g. breast tumours and neuroblastoma). In unstressed cells p53 remains inactive by its key negative regulator MDM2 (murine double minute 2) via two mechanisms. Firstly by directly binding to the N-terminal transactivation domain of p53 to inhibit its function as a transcription factor, and secondly by functioning as an E3 ubiquitin ligase to target p53 for proteasomal degradation (Oliner *et al*., 1993, Wu *et al*., 1993, Haupt *et al*., 1997, Fang *et al*., 2000). Furthermore, p53 transcriptionally activates MDM2 through a p53 response element causing the expression levels of p53 and MDM2 to be regulated through an autoregulatory negative feedback loop (Wu *et al*., 1993, Fang *et al*., 2000). MDM2 itself is often described as an oncogene as it is overexpressed in more than forty different types of malignancies, and although the basis of this oncogenicity remains unclear it is speculated that MDM2 multiplication stimulates excessive p53 degradation (Leach *et al*., 1993, Rayburn *et al*., 2005).

Of the various pathways that activate the p53 network, the molecular mechanisms behind p53 activation in response to DSBs are perhaps the most comprehensively understood and will be discussed in more detail later in this Chapter. In short, upon DNA damage p53 accumulates to induce cell cycle arrest allowing time for the DNA to repair. If this repair fails p53 can also trigger cell suicide by apoptosis to prevent the lesion being passed on to daughter cells. Besides activation by the DDR (often via phosphorylation), Figure 1.4 demonstrates that a plethora of other proteins from other pathways can also regulate p53 function via post-

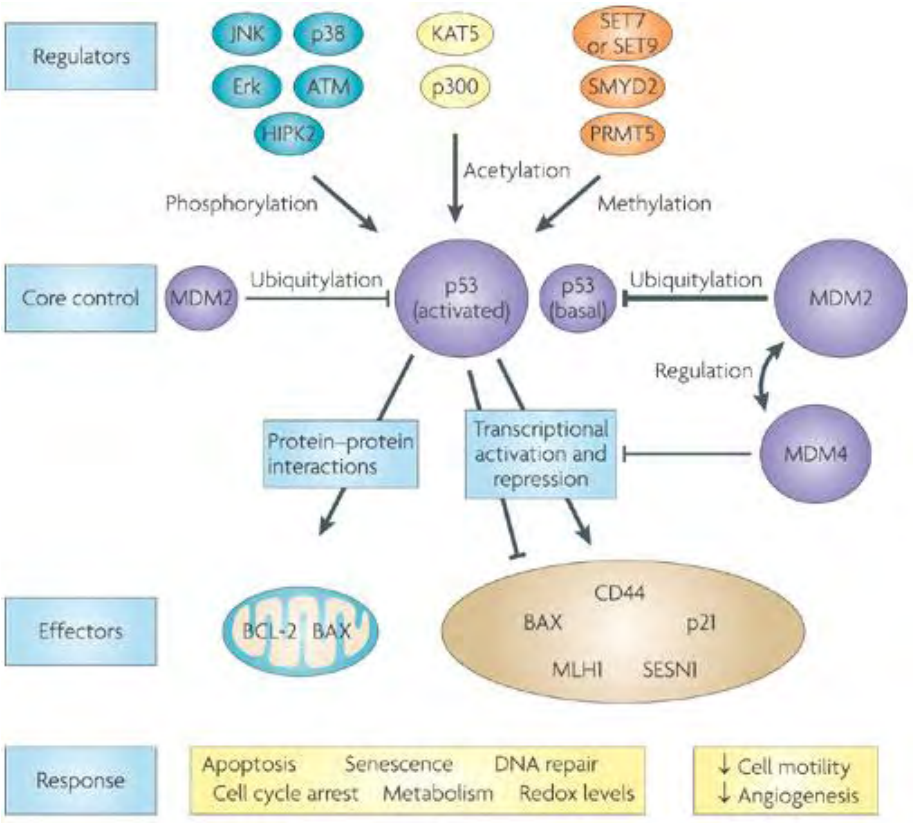


Fig 1.4. The p53 pathway

This illustration highlights the complexity of p53 regulation and its importance in maintaining genome integrity. p53 function is regulated by at least 50 known enzymes (only some of which are listed above) which affect its interaction with MDM2. As a transcription factor, p53 can activate or repress genes involved in DNA repair, cell cycle arrest, apoptosis and senescence, while through protein-protein interactions it can also induce apoptosis via the mitochondrial pathway. All of the responses regulated by p53 are fundamental for normal cell growth and development, however, if compromised they can lead to tumourigenesis (adapted from Whibley *et al*., 2009).

translational modifications, such as acetylation and methylation, which disrupt interactions with MDM2 and generally result in the stabilization of p53 (Whibley *et al*., 2009). p53 exerts its function as a tumour suppressor in two ways. Firstly, via physical interactions with members of the BCL-2 family, p53 has the ability to promote apoptosis by inducing the release of cytochrome c from the mitochondria (Oda *et al*., 2000, Mihara *et al*., 2003). Secondly, its role as a transcription factor allows p53 to bind to p53 response elements within a broad spectrum of target genes in order to active or repress their expression. Of particular importance to this study is p21 which is activated by p53 in response to DNA damage, and is a potent regulator of cell cycle progression through interactions with cyclin-CDK (cyclin-dependent kinase) complexes (Harper *et al*., 1993).

1.2.3 Cell cycle checkpoints

The cellular response to DNA damage involves a series of proteins classed as sensors, mediators, transducers and effectors which form an integrated network to rapidly detect and signal DNA lesions (Fig 1.5). Within seconds of DNA damage induction, many of these proteins dynamically accumulate on chromatin domains flanking the lesion in focal PML-containing POD structures termed foci. These nuclear structures reflect local chromatin expansion, histone modification and the assembly of diverse proteins within the vicinity (Kruhlak *et al*., 2006).

The detection of DNA damage initiates pathways that affect cell cycle control, DNA repair and transcription, and if the damage is beyond repair, apoptosis. Integral components of the DDR are the distinct checkpoint pathways which act as safeguards to arrest cells at specific stages throughout the cell cycle to allow the cell time to carry out the appropriate DNA repair

functions. This temporary pause is lifted upon completion of the repair and cells can continue an error-free cycle.

Transitions between cell cycle phases are tightly controlled, thus activated checkpoints in damaged cells can block S phase entry (G_1/S checkpoint), delay S phase progression (intra-S phase checkpoint) or prevent mitotic entry (G_2/M phase checkpoint). A simplified illustration of the mammalian cell cycle and the mechanisms behind its regulation is shown in Figure 1.6. Recent evidence has suggested there is a lack of a DNA damage checkpoint during mitosis, as although early response events are triggered, full DDR activation does not occur until cells enter G_1 and the DNA is less condensed, and presumably more accessible (Giunta *et al*., 2010).

Ataxia telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) proteins, which belong to the phosphatidylinositol 3-kinase-like kinase (PIKK) family, are key upstream players in checkpoint pathways (Sancar *et al*., 2004, You *et al*., 2005). Mutation of these genes result in rare genetic human conditions (A-T and Seckel syndrome), however while the *ATM* gene is non-essential, *ATR*-null mice are inviable suggesting that this kinase is essential for cell and organism survival (Brown *et al*., 2000, Liu *et al*., 2000). ATM responds primarily to DSBs, while ATR responds to stalled DNA replication forks and ssDNA (single-stranded DNA) generated by DNA damaging agents (Bakkenist and Kastan, 2004). However, there is also evidence of some overlap and cross-talk between these two pathways *in vivo* (Matsuoka *et al*., 2000, Zhao and Piwnica-Worms, 2001, Gatei *et al*., 2003). Indeed, ATR also responds to DSBs in an ATM-dependent manner and ATM has been shown to be activated through ATR signalling during UV and hydroxyurea (HU) treatment (Adams *et al*., 2006, Jazayeri *et al*., 2006, Stiff *et al*., 2006). Furthermore, overexpression of ATR complements the radioresistant DNA synthesis defect in cells lacking ATM (Cliby *et al*., 1998).

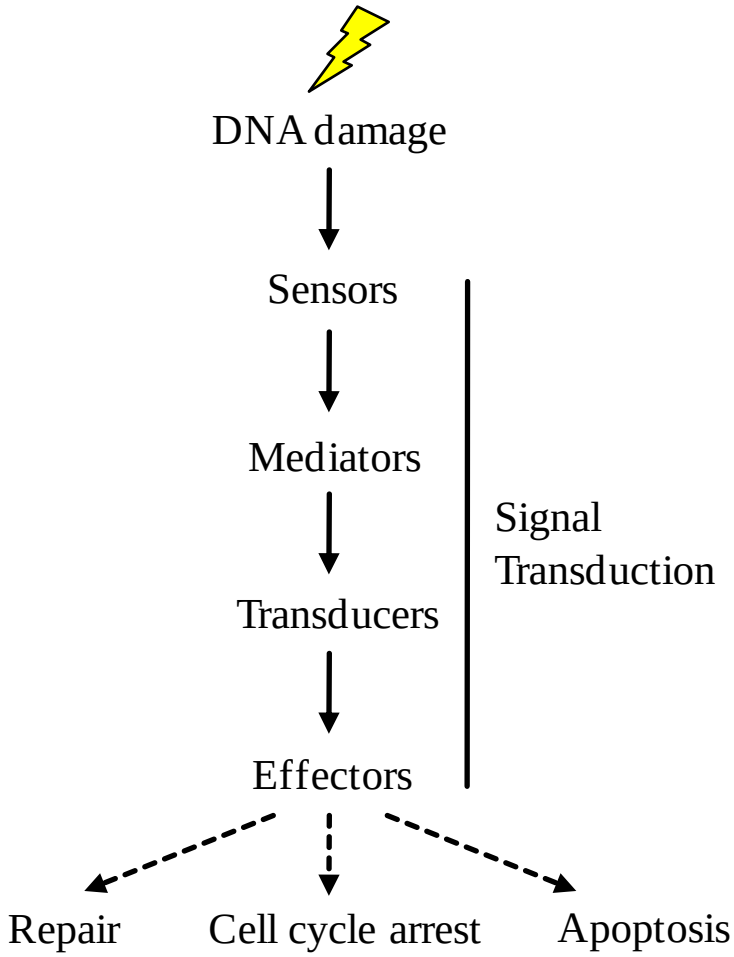


Fig 1.5. DNA damage signal transduction

DNA damage is signalled by a cascade involving sensors, mediators, transducers and effectors. Initially DNA damage is recognized by sensor proteins which activate the signalling cascade. The recruitment of transducers, with the help of mediators, amplifies the signal to suppress effector proteins. This triggers cell cycle arrest at specific phases, DNA repair or apoptosis if the damage is too severe.

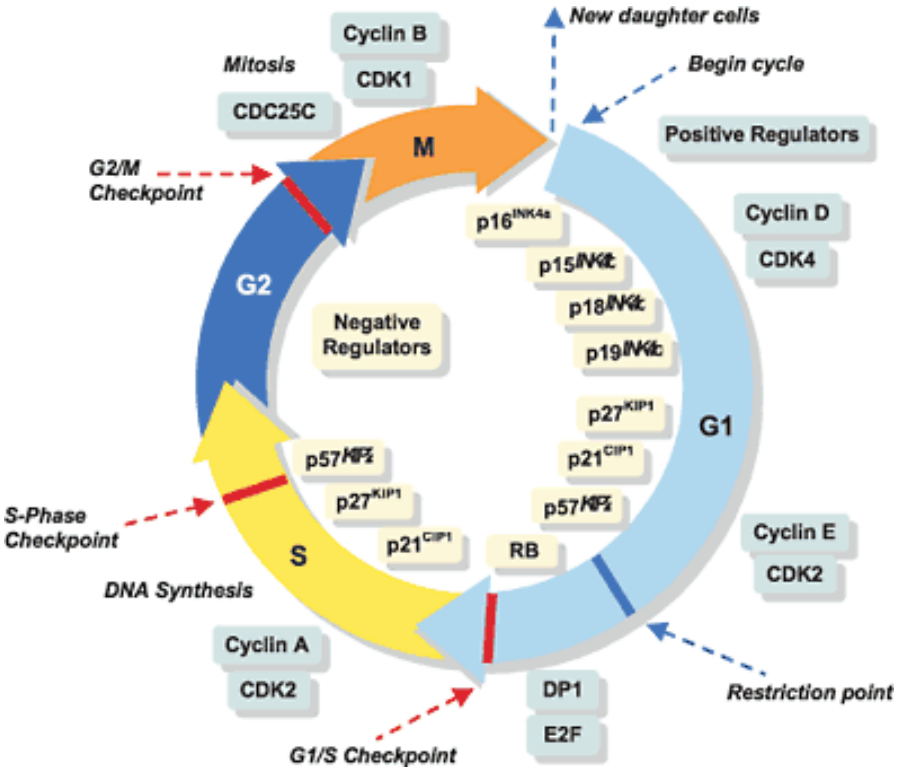


Fig 1.6. The mammalian cell cycle

The four distinct phases of the cell cycle (G_1, S, G_2 and M) which encompass DNA synthesis and mitotic cell division are shown above. Cell cycle progression is positively regulated by the activity of cyclins and CDKs, shown in blue, and negatively regulated by proteins shown in pink. Red arrows indicate the locations of cell cycle checkpoints while the blue arrow signifies the restriction point where cells are irreversibly committed to the next cell division (adapted from Kong *et al.*, 2003).

1.2.4 Activation of the ATM kinase

The hierarchical assembly of proteins at sites of DSBs is highly ordered, rapid (reaching maximal accumulation within a few minutes) and dependent on a string of phosphorylation and ubiquitin mediated events (Fig 1.7). The ATM protein kinase has a central function in communicating the detection of DSBs by phosphorylating key players in various branches of the DDR network. In unirradiated cells, ATM exists as an inactive dimer where its kinase domain is bound to an internal domain of a neighbouring ATM molecule containing serine 1981. Upon the induction of DNA damage, the kinase domain of one ATM molecule phosphorylates the serine 1981 residue of an interacting ATM molecule which leads to the dissociation of active ATM monomers (Bakkenist and Kastan, 2003).

A number of proteins that contain BRCT (BRCA1 C-terminal) domains are involved in the efficient activation and relocalization of ATM in response to DNA damage, including Nbs1 and the mediator of DNA damage checkpoint 1 (MDC1) protein. Specifically, via its interaction with the C-terminal region of Nbs1 (a member of the Mre11-Rad50-Nbs1, or MRN, complex), ATM recruitment is dependent on MDC1 (Desai-Mehta *et al*., 2001, Uziel *et al*., 2003, You *et al*., 2005, Chapman and Jackson, 2008, Melander *et al*., 2008, Spycher *et al*., 2008). Indeed, recent data has shown that association between the Nbs1-FHA (forkhead-associated) domain and the MDC1-SDT (Ser-Asp-Thr) repeat region constitutively phosphorylated by casein kinase 2 (CK2), is required for the relocalization of the MDC1-MRN-ATM complex (Chapman and Jackson, 2008, Melander *et al*., 2008, Spycher *et al*., 2008). The MDC1-MRN-ATM complex is tethered to damaged chromatin via interactions between tandem MDC1-BRCT repeat regions and the ATM-phosphorylated form of a variant histone H2A (histone 2A), H2AX (γH2AX). H2AX phosphorylation is one of the earliest events in DSB signalling and γH2AX serves as a major marker for damaged chromatin by

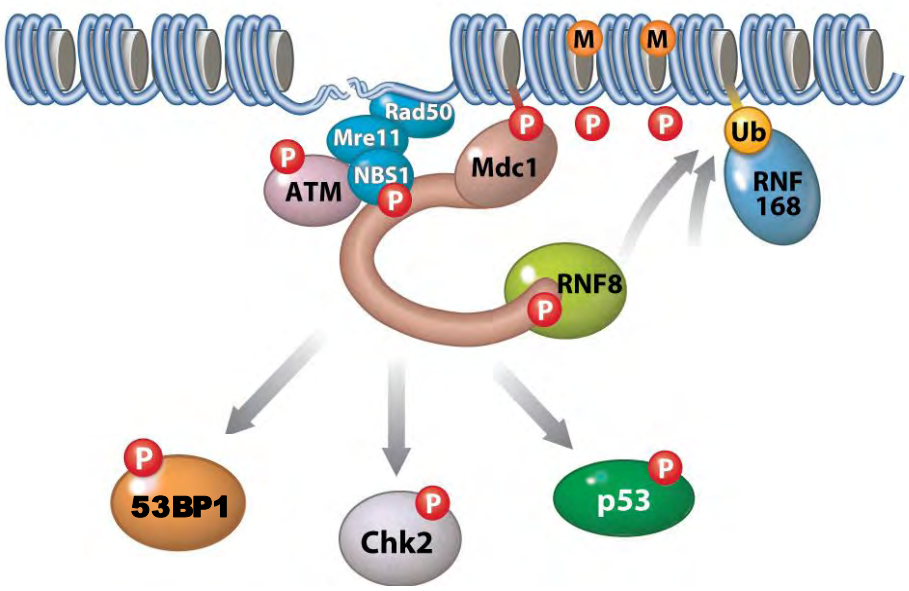


Fig 1.7. The cellular response to DSBs

DSBs, generated from IR or collapsed DNA replication forks, are sensed by the MRN complex which induces ATM kinase activation. ATM phosphorylates H2AX and, along with MRN, recruits and activates a series of mediator proteins (i.e. MDC1) and the transducer kinase Chk2. RNF8 and RNF168 act to amplify this signal by ubiquitylating γH2AX allowing further recruitment. The transduced signal subsequently activates a number of downstream effector proteins such as p53 and 53BP1 to mediate checkpoint signalling, DSB processing and DNA repair. P, phosphate; M, methyl; Ub, ubiquitin (Weitzman *et al*., 2010).

flanking DNA breaks (Rogakou *et al.*, 1999, Lee *et al.*, 2005, Stucki *et al.*, 2005). Equally, the de-phosphorylation of γH2AX is important for attenuating the checkpoint signal to allow sister chromatid separation and the cell cycle to resume after DNA repair (Chowdhury *et al.*, 2005).

Along with Nbs1 and MDC1, a number of other proteins that contain BRCT domains are also involved in the efficient activation of ATM. Two such proteins are BRCA1 (breast cancer susceptibility gene 1) and 53BP1 (p53-binding protein 1) which, as well as being phosphorylated by ATM at sites of DNA damage, act as co-activator or mediator proteins to facilitate ATM function (Xu *et al.*, 2002, Lee *et al.*, 2010).

Once recruited and activated, ATM phosphorylates a number of transducer and effector substrates which triggers a signalling cascade and results in checkpoint activation (Bakkenist and Kastan, 2004). One crucial target for the ATM kinase is the checkpoint kinase 2 (Chk2) protein which is phosphorylated on threonine residue 68 at sites of DNA damage (Matsuoka *et al.*, 2000). Chk2 is a central effector kinase responsible for the activation of cell cycle checkpoints and apoptosis by stabilizing p53 (Hirao *et al.*, 2000).

1.2.5 Activation of the ATR kinase

In human cells, ATR exists in a stable heterodimeric complex along with the regulatory protein ATR-interacting protein (ATRIP) which is often referred to as an obligate ATR subunit as there is no difference in phenotype from the loss of these genes (Cortez *et al.*, 2001). In contrast to ATM, the ATR-ATRIP complex is not post-translationally modified following DNA damage, nor does it have any increased activity in kinase assays (Bakkenist and Kastan, 2004). This complex binds indirectly to DNA lesions or sites of stalled

replication via an interaction between ATRIP and replication protein A (RPA) which is a heterotrimeric ssDNA-binding complex (Fig 1.8; Wold, 1997, Zou and Elledge, 2003). In this regard, ssDNA can directly trigger checkpoint activation as it is generated both at stalled replication forks and by 5' to 3' resection of DSBs (presumably by the MRN complex) which leaves long 3'-ended tails (Garvik *et al*., 1995). Although RPA recruits ATR to ssDNA it is not sufficient for ATR activation. This is dependent on the Rad9-Hus1-Rad1 (9-1-1 or RHR) complex which functions as a sliding checkpoint clamp and is topologically linked to DNA by the Rad17-RFC2-5 (RSR) "clamp loader" complex (Zou *et al*., 2001, Ellison and Stillman, 2003, Majka and Burgers, 2007). This "loading" is driven by ATP and is independent of ATR function or localization (Zou *et al*., 2001, Ellison and Stillman, 2003). The Rad17 protein is also an important substrate for ATR kinase activity. This phosphorylation requires Hus1 which suggests that the 9-1-1 complex enables ATR to target its substrates (Zou *et al*., 2001).

Two other mediator proteins, Claspin and TOPBP1 (topoisomerase [DNA] II binding protein 1), are also required for ATR to phosphorylate its key downstream target, checkpoint kinase 1 (Chk1; Kumagai *et al*., 2005, Liu *et al*., 2006, You *et al*., 2006). TOPBP1 binds directly to the phosphorylated C-terminus of Rad9 and interacts with ATR via its co-activator protein ATRIP, while Claspin (activated by an as yet unidentified kinase) binds directly to the kinase domain of Chk1 to mediate its recruitment to ATR in a process dependent on TIMELESS and TIPIN (TIMELESS-interacting protein) association (Jeong *et al*., 2003, Chou and Elledge, 2006, Liu *et al*., 2006, Mordes *et al*., 2008).

Chk1 is phosphorylated by ATR on serine residues 317 and 345 and has diverse roles in activating DNA damage, DNA replication and mitotic spindle checkpoints (Liu *et al*., 2000, Zhao and Piwnica-Worms, 2001, Dai and Grant, 2010). *Chk1*, like *ATR*, has been shown to be an essential gene in mice, and as discussed below has vital roles in cell cycle regulation in

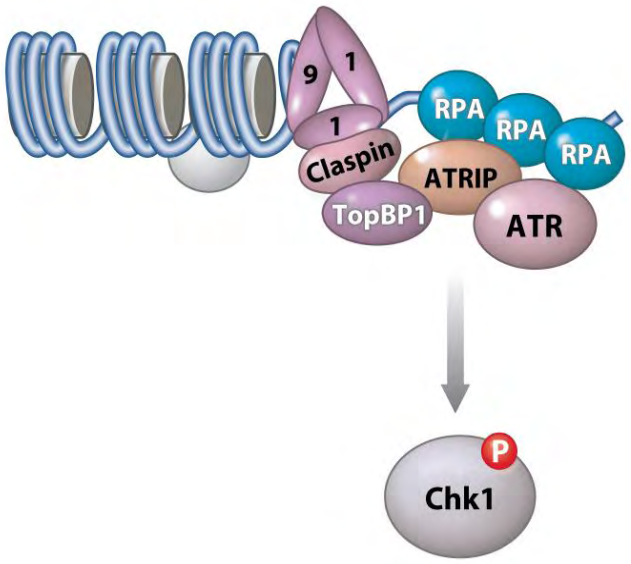


Fig 1.8. The cellular response to ssDNA

ssDNA generated from UV radiation, stalled DNA replication forks or processed DSBs becomes coated in RPA which recruits the ATR-ATRIP complex. The 9-1-1 complex is independently recruited to ssDNA along with claspin and TOPBP1, which together activate ATR-ATRIP kinase activity enabling it to phosphorylate the downstream transducer protein Chk1. Chk1 subsequently activates effector proteins to trigger cell cycle arrest and maintain replication fork stability. P; phosphate (Weitzman *et al*., 2010).

response to checkpoint activation (Liu *et al*., 2000).

1.2.6 The MRN complex

Amongst the first proteins to be recruited to DNA damage foci is the MRN complex which contains three members Mre11, Rad50 and Nbs1. Chromatin immunoprecipitation (ChIP) analysis used to examine protein recruitment at sites of DSBs discovered that Mre11 binding is rapid and is distributed within 10 kb of the break, while being most concentrated in the first 2 kb (Shroff *et al*., 2004). This is a crucial event in the DSB response, highlighted by the fact that functional MRN is required for efficient ATM kinase activation (Uziel *et al*., 2003, You *et al*., 2005).

In addition to being an early sensor of DSBs, MRN is often considered to be a keystone complex as it connects multiple cellular pathways including DNA damage checkpoint signalling, DNA repair and telomere maintenance (Williams *et al*., 2007). Given its diverse involvement in the DDR, it is no surprise that this complex is often targeted during viral infection and a major focus of this study will concentrate on MRN complex regulation by adenoviruses. The three members of the MRN complex each contribute in different ways to its function and have very distinct properties as reflected by their domain organization (Fig 1.9). Inherited mutations in *Mre11*, *Nbs1* and *Rad50* genes result in ATLD, NBS and NBS-like chromosome instability syndromes, respectively, which although are clinically distinct all exhibit DNA damage checkpoint defects (Taalman *et al*., 1983, Stewart *et al*., 1999, Waltes *et al*., 2009).

Mre11 is a highly conserved nuclease and phosphoesterase protein. Specifically, it harbours dsDNA 3′-to-5′ exonuclease, ssDNA endonuclease, DNA annealing, and unwinding abilities

(Paull and Gellert, 1998, Trujillo *et al*., 1998, Assenmacher and Hopfner, 2004, Williams *et al*., 2008). The Mre11 protein also confers the MRN complexes' ability to bind DNA through two C-terminal DNA-binding domains (DBD; de Jager *et al*., 2001). Two Mre11 and two Rad50 molecules make up the "core" heterotetrameric fraction of the complex which has versatile DNA-binding capabilities.

Rad50 is composed of N- and C-terminal nucleotide binding motifs which dimerize to form a bipartite ATP-binding cassette (ABC)-ATPase domain and consequenctly bind and unwind dsDNA termini (Hopfner *et al*., 2000). The central portion of the protein contains a zinc hook (CxxC) motif separating a coiled-coil domain to form two flexible "arms" which protrude away from the DNA to mediate interactions between DNA termini (de Jager *et al*., 2001, Hopfner *et al*., 2002). Currently, structural and functional data suggest a model whereby Mre11 facilitates short-range tethering of DNA ends, and the long coiled-coil Rad50 "arms" enables long-range synapsis of DNA to prevent chromosome separation (Hopfner *et al*., 2002, Williams *et al*., 2008).

While the core MRN complex possesses DNA binding abilities, its final member, Nbs1 which gives an overall stoichiometry of $Mre11_2Rad50_2Nbs1_2$, is essential for communicating the presence of DNA breaks to the rest of the checkpoint machinery. Nbs1 is eukaryotic specific and directly binds Mre11 and ATM through distinct motifs within its C-terminal region, an interaction required for their recruitment to DNA lesions (Desai-Mehta *et al*., 2001, You *et al*., 2005). Nbs1 itself is also a downstream substrate for ATM-mediated phosphorylation which is important for ensuring appropriate checkpoint activation in response to DNA damage (Lim *et al*., 2000, Wu *et al*., 2000). As mentioned, Nbs1 also has

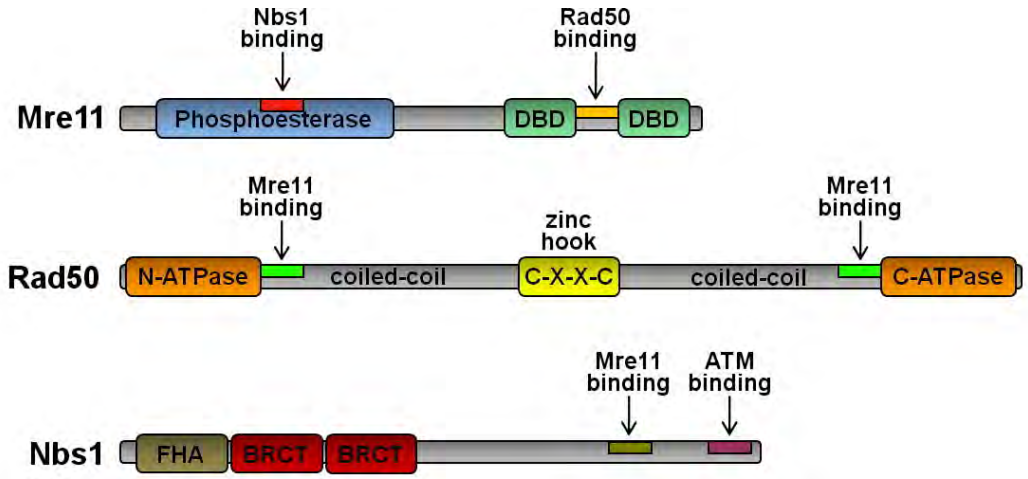


Fig 1.9. Linear representation of Mre11, Rad50, and Nbs1 proteins

Large coloured boxes indicate catalytic domains within Mre11, Rad50 and Nbs1 while arrows and small coloured boxes indicate the location of known binding motifs (Lamarche *et al*., 2010).

N-terminal FHA and BRCT domains which mediate the phospho-dependent interactions necessary for recruiting repair and checkpoint proteins to DNA damage foci (Lukas *et al*., 2004, Chapman and Jackson, 2008, Lloyd *et al*., 2009).

1.2.7 Mediator proteins

Mediator proteins are a class of proximal checkpoint regulators that play important roles in amplifying, and maintaining, the cellular responses which are signalled by ATM and ATR. This group of proteins includes 53BP1, BRCA1, TOPBP1 and MDC1, all of which contain BRCT protein-protein interaction domains (Schultz *et al*., 2000, Yamane *et al*., 2002, Stewart *et al*., 2003). All of these mediators are phosphorylated by ATM or ATR and act by simultaneously associating with DNA damage sensors such as γH2AX, transducers such as Chk1 and Chk2, and effectors such as p53 to confer signal transduction specificity at certain phases of the cell cycle (Zhang *et al*., 1998, Schultz *et al*., 2000, Lukas *et al*., 2004, Stucki *et al*., 2005). Other mediator proteins include SMC1 (structural maintenance of chromosomes 1) and Claspin, the latter of which, along with its requirements for ATR activation, is also involved in DNA replication and sensing of replication forks (Yazdi *et al*., 2002, Liu *et al*., 2006, Yoo *et al*., 2006).

Mediator proteins often have multiple roles in the DDR. In addition to activating ATR kinase activity in response to ssDNA, experiments carried out in *Xenopus laevis* discovered that in response to dsDNA ends TOPBP1 is phosphorylated by ATM to activate ATR in an RPA-independent manner (Yoo *et al*., 2007). Moreover, as well as localizing the MRN complex, MDC1 is also required to recruit the ubiquitin ligase RNF8 (RING-finger 8) to sites of DNA damage (Huen *et al*., 2007, Mailand *et al*., 2007). RNF8 mono-ubiquitylates histones H2A

and H2AX, and through this higher order chromatin restructuring allows for a second wave of protein accumulation including 53BP1 and BRCA1 (Mailand *et al*., 2007). These ubiquitylation modifications are also augmented by another ligase, RNF168 (RING-finger 168), which recognizes ubiquitylated histones and amplifies the signal by generating lysine 63-linked poly-ubiquitin chains (Doil *et al*., 2009, Stewart *et al*., 2009). Recently another E3 ubiquitin ligase, HERC2 (HECT domain and RLD 2), was also found to promote H2AX poly-ubiquitylation at sites of DNA damage. HERC2 forms a complex with RNF8 to mediate its interaction with the E2 enzyme Ubc13 (ubiquitin-conjugating enzyme 13) and to maintain levels of RNF168 (Bekker-Jensen *et al*., 2010). It has also been recently suggested that chromatin relaxation occurs in two stages, beginning with γH2AX poly-ubiquitylation and the recruitment of sensor proteins, and followed by ubiquitylation of another histone H2B (Moyal *et al*., 2011). ATM-dependent mono-ubiquitylation of H2B by the RNF20-RNF40 E3 ubiquitin ligase heterodimer is thought to facilitate the recruitment of proteins necessary for timely DSB repair (Moyal *et al*., 2011).

1.2.8 Transducer and effector proteins

The mechanisms and molecular components involved in checkpoint activation are specific to the cell cycle phase and nature of the lesion (Sancar *et al*., 2004, Zierhut and Diffley, 2008). While many of the initial signalling events and sensor and transducer proteins are common to all pathways, the effector components of these pathways which actively inhibit phase transition give checkpoints their unique properties (Sancar *et al*., 2004).

The two key transducers of the DDR are the serine/threonine protein kinases Chk1 and Chk2, which are substrate specific and function in different pathways. DSB signals are transduced

via ATM-dependent phosphorylation of Chk2, and UV-induced signals are transduced via ATR-dependent phosphorylation of Chk1, although as mentioned there is also evidence of some overlap and cross-talk between the two pathways (Matsuoka *et al*., 2000, Zhao and Piwnica-Worms, 2001, Gatei *et al*., 2003). Upon activation, these transducer kinases become dissociated from chromatin in order to facilitate signal transmission to multiple soluble effector proteins throughout the cell, such as p53 and the CDC25A phosphotyrosine phosphatase which are essential regulators of cell cycle progression (Chen *et al*., 2003, Li and Stern, 2005, Smits *et al*., 2006, Boutros *et al*., 2007). As mentioned above, the specificity of DDR pathways between phases of the cell cycle manifests in the distinctive recruitment of these effector proteins, a few of which are discussed below. ATM pathways are activated rapidly in response to DNA damage, irrespective of the cell cycle phase, whereas ATR is activated more slowly and predominantly only in S or G_2 phase cells (Cimprich and Cortez, 2008).

The G_1/S checkpoint activates two pathways to prevent DNA damaged cells from entering S phase by inhibiting the initiation of replication. Initially, activated transducer kinases phosphorylate CDC25A targeting it for proteolytic degradation (Falck *et al*., 2001). This lack of active CDC25A results in the accumulation of phosphorylated CDK2 complexes, which are unable to facilitate the loading of CDC45 onto the replication origins necessary for DNA replication induction (Costanzo *et al*., 2000). The second series of events is less rapid and required for a sustained G_1/S arrest. In this pathway the critical effector protein p53 is phosphorylated and activated by ATM, both directly and indirectly, allowing it to transactivate a number of target proteins (Canman *et al*., 1998). One such protein is the CDK inhibitor $p21^{WAF1/Cip1}$ which binds CDK proteins to inhibit cyclin E-CDK2 and cyclin D-CDK4 complex formation to obstruct S phase entry (Harper *et al*., 1993, Bunz *et al*., 1998,

Lavin and Jackson, 1999). In the case of cyclin D-CDK4 dissociation, this is achieved as the inactivated complex fails to phosphorylate Rb an event required to release it from E2F to initiate transcription (Lin *et al*., 2001). Both of these pathways are primarily mediated by Chk2, however basal Chk1 activity is required for constitutive CDC25A turnover in unperturbed cells (Dai and Grant, 2010).

The intra-S phase checkpoint is activated by DNA damage encountered during S phase, or by unrepaired DNA that evaded the G_1/S checkpoint and caused a block in replication (Paulovich and Hartwell, 1995). The activation of this checkpoint is mediated by two p53-independent pathways, one of which is synonymous to the CDC25A-CDK2 pathway described for the G_1/S phase checkpoint, and one involving SMC1 and the Fanconi anaemia complementation group D2 (FANCD2) protein (Falck *et al*., 2001, Xiao *et al*., 2003, Pichierri and Rosselli, 2004). In the second pathway, Nbs1- and BRCA1-dependent phosphorylation of SMC1 and FANCD2 leads to replication inhibition supposedly through SMC1 phosphorylation repressing sister chromatid cohesion; the mechanism behind FANCD2 repression remains unclear however is thought to involve BRCA1 (Michaelis *et al*., 1997, Lim *et al*., 2000, Kim *et al*., 2002, Nakanishi *et al*., 2002, Xu *et al*., 2002, Pichierri and Rosselli, 2004). Both of these pathways are p53-independent, however recent evidence suggests that p53 may be involved in a novel p53-dependent intra-S phase checkpoint involving Chk1 which acts to prevent DNA synthesis (Attardi *et al*., 2004, Ahmed *et al*., 2011).

The function of an activated G_2/M checkpoint is to prevent cells from undergoing mitotic division in the presence of DNA damage. For this to occur, two parallel pathways are again induced both of which require ATM and ATR kinase activity; ATM- and MRN complex-dependent DSB resection generates ssDNA to recruit ATR (Xu *et al*., 2002, Carson *et al*.,

2003, Jazayeri *et al*., 2006). The ultimate target of these pathways is the cyclin B-CDK1 complex which, when dephosphorylated by CDC25A phosphatase, promotes progression into mitosis (Coleman and Dunphy, 1994). Initially, Chk1/Chk2-dependent phosphorylation inactivates CDC25A to ensure that cyclin B-CDK1 complexes remain phosphorylated and cells arrest at the G_2/M transition (Peng *et al*., 1997, Sanchez *et al*., 1997). The subsequent non-essential pathway involves p53 which, through transcriptional induction of CDK complex inhibitors such as p21, promotes the maintenance of checkpoint arrest (Lavin and Jackson, 1999, Taylor and Stark, 2001).

1.2.9 DNA Repair

The repair mechanisms signalled by checkpoints to fix DNA damage are also lesion- and cell cycle phase-specific, and involve distinct sequences of catalytic events mediated by multiple proteins (Sonoda *et al*., 2006). Indeed, failure to coordinate DNA repair with cell cycle progression can lead to genomic instability, cell death and cancer (Branzei and Foiani, 2008).

In mammalian cells SSBs are restored through base excision repair (BER), a mechanism which is initiated by DNA glycosylases and that uses the sister strand as a template to direct repair (Sancar *et al*., 2004). DSBs are predominantly repaired via an illegitimate process called non-homologous end-joining (NHEJ), where the two ends of a DSB are ligated together without the need for sequence homology between the ends (Sargent *et al*., 1997). In brief, NHEJ involves detection of DSBs by a DNA-dependent protein kinase (DNA-PK) complex consisting of the Ku70/Ku80 heterodimer and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), processing of DNA end termini by MRN, Artemis and various DNA polymerases, and finally ligation via the XRCC4 (X-ray repair complementing

defective repair in Chinese hamster cells 4)/DNA ligase IV complex (Gottlieb and Jackson, 1993, Lee *et al*., 1997, Weterings and Gent, 2004, Collis *et al*., 2005).

While NHEJ can repair DNA at every stage of the cell cycle, accurate homologous recombination (HR) requires a sister chromatid to serve as a template to copy DNA sequences and is therefore restricted to S or G_2 phase (Sargent *et al*., 1997, Sonoda *et al*., 2006, Branzei and Foiani, 2008). HR is a multi-step process and although several HR sub-pathways exist, their initiation is dependent on the MRN complex which functions together with the C-terminal binding protein interacting protein (CtIP) to generate ssDNA by the 5'-3' resection of DSBs (Limbo *et al*., 2007). HR also requires RecQ helicase family members and a Rad51 nucleoprotein filament, to facilitate the unwinding of DNA, and catalyse the transfer of DNA via a holliday junction which forms between the two sister chromatids, respectively (Yu *et al*., 2001, Hu *et al*., 2007).

1.2.10 The role of ubiquitin

Protein ubiquitylation is a post-translational modification that modifies protein function, and in doing so regulates many critical cellular pathways including cell cycle progression, cellular differentiation, apoptosis and DNA repair (Welchman *et al*., 2005). Ubiquitin itself is a small highly conserved polypeptide that is "ubiquitously" expressed in eukaryotic organisms and targets an exponentially growing list of cellular proteins including tumour suppressors, growth modulators and transcriptional activators (Ciehanover *et al*., 1978, Wilkinson *et al*., 1980, Weissman, 2001).

While ubiquitin modifications encompass vast functional diversities, one major role is the targeting of proteins, including misfolded proteins, for degradation by a multi-subunit ATP-

dependent protease termed the proteasome (Thrower *et al*., 2000, Glickman and Ciechanover, 2002, Xie, 2010).

Ubiquitylation, as illustrated in Figure 1.10 is a multistep process resulting in the covalent attachment of ubiquitin onto target proteins, and involves the activity of at least three types of enzyme: the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme and the E3 ubiquitin ligase (Hershko *et al*., 1983, Welchman *et al*., 2005). Initially, free ubiquitin is activated by ATP-dependent adenylation allowing thioester bonds to form between the activated ubiquitin and the active site of the E1 enzyme. A *trans*-thiolation reaction then conjugates the modified ubiquitin onto the E2 enzyme which is able to associate with an E3 enzyme; which may or may not already have the substrate bound (Weissman, 2001). The final step in this reaction is the transfer of ubiquitin onto the amino group of the substrate (often a lysine residue) via an isopeptide bond in a process that depends on the E3 enzyme.

E3 ubiquitin ligase enzymes are highly diverse and are divided into two main classes that are characterized by the presence of either a HECT (homologous to the E6-AP carboxyl terminus) or RING (really interesting new gene) domain (Al-Hakim *et al*., 2010). For HECT E3 ubiquitin ligases, ubiquitin is first transferred onto the HECT domain before being passed onto the substrate, while RING E3 ubiquitin ligases act as scaffold proteins and facilitate the transfer of ubiquitin directly from the E2 to the substrate (Al-Hakim *et al*., 2010). It should also be noted that ubiquitylation is a dynamic and reversible process, and numerous de-ubiquitylating (DUBs) enzymes also exist in the cell to catalyse the hydrolysis of ubiquitin-substrate bonds (Wilkinson, 2000).

The fate of ubiquitin-tagged proteins is dependent on the type of linkage. For example, a single protein can be modified on more than one lysine residue and this can be either by a single ubiquitin molecule (mono-ubiquitylation) or a chain of ubiquitin molecules linked via

lysine residues (poly-ubiquitylation). There are seven lysine residues within ubiquitin that can potentially be linked to form poly-ubiquitin chains, each of which assigns a different function. Chains comprising of four or more K48-linked ubiquitin molecules target the conjugated substrate to the 26S proteasome for degradation, while amongst other processes, K63-linkages are vital for the regulation of DNA repair (Spence *et al*., 1995, Thrower *et al*., 2000). Indeed, in conjunction with phosphorylation, the importance of ubiquitylation in the cellular DDR is become increasingly evident with a growing number of E3 ubiquitin ligases being identified including RNF8, RNF186 and HERC2 which promote the sustained recruitment of downstream checkpoint and repair proteins at sites of damage (Huen *et al*., 2007, Mailand *et al*., 2007, Stewart *et al*., 2009, Bekker-Jensen *et al*., 2010).

Several multi-subunit E3 ubiquitin ligases also exist in eukaryotic cells, including the SCF^{Fbw7} (Skp/cullin/F-box) and the APC/C (anaphase promoting complex/cyclosome) complexes which control cell cycle checkpoints by targeting cyclins and other proteins for degradation (Busino *et al*., 2003, Peschiaroli *et al*., 2006, Peters, 2006). These complexes are characterized by the presence of a catalytic RING finger protein, an adaptor protein, a substrate recognition protein and a cullin (or cullin-like) scaffold subunit (Zheng *et al*., 2002, Peters, 2006). In addition to checkpoint regulation, cullin-based E3 ubiquitin ligase complexes have also been found to facilitate the degradation of viral substrates. The nature of this regulation with regards to Ad infection will be discussed later in this Chapter.

Although less significant to the current study, it should also be noted that in addition to phosphorylation and ubiquitylation, other post-translational modifications such as acetylation, methylation, SUMOylation and neddylation are also utilised to regulate protein function in the DDR (Oberle and Blattner, 2010).

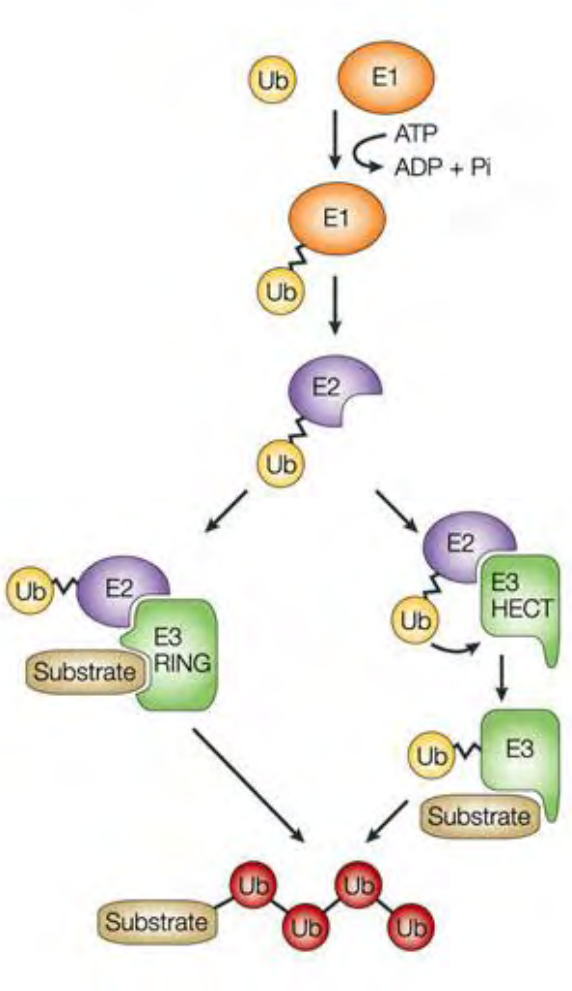


Fig 1.10. The ubiquitin system

Ubiquitylation is a process whereby ubiquitin is covalently attached to target proteins by a hierarchical set of three enzymes. The E1 enzyme catalyses the ATP-dependent activation of ubiquitin and transfers it to the E2 ubiquitin conjugating enzyme. This complex then associates with the E3 ubiquitin ligase enzyme which attaches ubiquitin to a lysine residue in the target protein via one of two mechanisms. RING domain E3 ubiquitin ligases directly transfer ubiquitin from the E2 to its substrate, while the active-site of HECT domain E3 ubiquitin ligases must first associate with ubiquitin before transferring it to the substrate. Ub, ubiquitin (adapted from Di Fiore *et al*., 2003).

1.3 REGULATION OF THE CELLULAR DNA DAMAGE RESPONSE DURING ADENOVIRUS INFECTION

1.3.1 Concatemerization of Ad genomes

Adenoviruses have been known to affect many cellular processes to ensure their own integrity, namely by evading the host cell's immune response and apoptotic programmes. In more recent history, research has shown that in order to ensure monomeric Ad genomes remain intact and replication efficient, they must also circumvent the host cell's DDR. It is suggested that upon entry of Ad linear dsDNA into cells the terminal portions of this "foreign" DNA are recognised by the DDR as DSBs; at late times of infection the cell can be presented with as many as 100 000 of these Ad dsDNA ends (Weitzman and Ornelles, 2005). If unsuppressed, this recognition would lead to a cascade of signalling events and the recruitment of sensor, mediator, transducer and effector proteins necessary to elicit a DDR and process or repair the DNA 'break'. Therefore, adenoviruses have developed to interact with the cellular DDR apparatus in order to over-ride this obstacle and establish a productive infection.

The first indication of this came from observations of Ad5 mutant viruses harbouring deletions in the E4 region (*dl*366 and *dl*808). Infections with these viruses resulted in the formation of large concatemers of viral DNA as visualized by pulsed field gel electrophoresis (PFGE; Weiden and Ginsberg, 1994). Concatemers are covalently linked monomers of DNA which are joined by no specific orientation i.e. junctions can consist of head-to-head, head-to-tail or tail-to-tail joints (Weiden and Ginsberg, 1994). Since ITRs containing the viral origins of DNA replication are lost in this linkage process, concatemers are often described as dead-end molecules unable to replicate. Furthermore, this cumulative effect results in Ad DNA being too large to be packaged within the viral capsid (Bett *et al*., 1993).

As direct ligation of viral genomes, and the presence of large deletions at these junctions, during E4 mutant virus infection implied that the DDR and NHEJ pathways are targeted by proteins encoded by the E4 region, later studies addressed the role of specific DNA repair factors in the formation of Ad concatemers. In 2002, Stracker *et al*. carried out important studies using an Ad5 mutant virus, *dl*1004, which lacked the entire E4 region. *dl*1004 was defective for DNA replication and late protein synthesis, and upon DNA analysis by PFGE it was clear that this was due to the formation of viral concatemers (Stracker *et al*., 2002). The authors then went on to investigate the importance of the core components of the NHEJ pathway on viral concatemer formation using mutant cell lines that fail to express Mre11 (ATLD), DNA-PKcs (MO59J) and DNA ligase IV (180BRM). Significantly, they found that these mutant cell lines were capable of rescuing the concatemer phenotype observed during *dl*1004 infection, suggesting that Mre11, DNA-PKcs and DNA ligase IV are important for concatemer formation and likely to be inactivated during wild-type (*wt*) Ad infection (Stracker *et al*., 2002). In contrast, the *dl*1004 mutant phenotype was not rescued in A-T cells which lack a functional ATM protein, suggesting that ATM is not required for concatemerization (Stracker *et al*., 2002). Continuing studies from the same laboratory also determined that the MRN complex is required for ATM and ATR activation in response to DNA damage, since *dl*1004 infection activated cellular DDR pathways, while *wt* Ad infection did not (Carson *et al*., 2003).

E4 mutant viruses are defective for DNA replication and late protein synthesis, but until 2008 it was unclear if this was indeed a consequence of DDR signalling and concatemer formation. Using the E1B55K R240A mutant protein and H354 insertion mutant protein, which both induce DNA ligase IV degradation and prevent concatemer formation, it was found that only R240A (which degrades the MRN complex) promoted Ad replication (Lakdawala *et al*.,

2008). In addition, Ad concatemer formation was prevented by treating A-T cells with caffeine to inhibit ATM and ATR signalling, although the defective replication of *dl*1004 was not rescued. This suggested that DDR pathway activation and concatemer formation does not inhibit viral DNA replication, and rather it is a direct consequence of MRN activity (Lakdawala *et al*., 2008).

1.3.2 Adenoviral proteins target cellular proteins for degradation

As alluded to above, Ad oncoproteins target cellular substrates for destruction via the ubiquitin-dependent proteasome-mediated pathway. Ubiquitylation (as previously described; Fig 1.10) is an enzymatic process whereby the small, highly conserved regulatory protein ubiquitin is conjugated to a substrate through covalent linkage of a lysine residue (Ciehanover *et al*., 1978, Wilkinson *et al*., 1980). This often leads to substrate degradation via the 26S proteasome which is a major protease complex composed of one 20S core cylinder particle, and two regulatory cap-shaped 19S particles (Wilkinson *et al*., 2000, Xie, 2010). The 26S proteasome is involved in the ATP- and ubiquitin-dependent degradation of regulatory and abnormal proteins within the cell, and has important roles in cell cycle regulation and cellular immune and stress responses (Xie, 2010).

The current study focuses on the impact Ad infection has on RING E3 ubiquitin ligases, however there is some evidence to suggest that penton base proteins of adenoviruses can interact with HECT E3 ubiquitin ligases, presumably to modulate viral internalization (Galinier *et al*., 2002). One class of multi-subunit RING E3 ubiquitin ligases are the cullin-based E3 ubiquitin ligases (CRLs; Zheng *et al*., 2002, Peters, 2006). CUL1 was the first cullin protein to be identified in 1996 and since then six further members have been

discovered (CUL2, 3, 4A, 4B, 5 and 7), along with two proteins which contain a cullin homology domain (APC2 and PARC/CUL9; Kipreos *et al*., 1996, Yu *et al*., 1998, Petroski and Deshaies, 2005, Skaar *et al*., 2007). To date Ad oncoproteins are known to influence CUL1-, CUL2- and CUL5-based E3 ubiquitin ligases, as well as the APC/C which is another multi-subunit RING E3 ubiquitin ligase (Querido *et al*., 2001, Turnell *et al*., 2005, Isobe *et al*., 2009). Indeed, via its ability to bind p300/CBP, E1A interferes with the activity of the APC/C in order to regulate mitotic progression and/or promote genomic instability and cellular transformation (Turnell *et al*., 2005). Ad E1A can also attenuate the activity of the SCF^{Fbw7} complex by directly binding to its CUL1 component. This complex is usually involved in cell cycle control and signal transduction, thus presumably it is deregulated by Ad to aid unrestrained proliferation (Isobe *et al*., 2009).

Of importance to the work described here is the effect Ad infection has on CUL2- and CUL5-based E3 ubiquitin ligases. Cellular complexes containing these scaffold proteins also include a heterodimeric Elongin BC adaptor complex which links CUL2 and CUL5 to the substrate recognition receptor boxes VHL (von Hippel-Lindau) and SOCS (suppressor of cytokine signalling), respectively (Kamura *et al*., 2004, Mahrour *et al*., 2008). Other members of the cullin family utilise different substrate receptors (Petroski and Deshaies, 2005)

The catalytic E3 ubiquitin ligase activity of the RING domain (often known as RBX1 or ROC1) is bound to a C-terminal cullin-homology domain in all CRLs, and is controlled by the covalent attachment of NEDD8 (neural precursor cell-expressed developmentally down-regulated 8) in a process known as neddylation; indeed, NEDD8 attachment is a prerequisite for the E3 ubiquitin ligase activity of these complexes (Petroski and Deshaies, 2005, Duda *et al*., 2008). NEDD8 is a member of the ubiquitin-like protein family, and as such its linkage is analogous to ubiquitylation involving its own E1, E2 and E3 enzymes (Pintard *et al*., 2003).

Furthermore, its conjugation is reversible and while NEDD8 attachment to a cullin protein is thought to induce CRL activity through conformational alterations, deneddylation has the reverse effect (Duda *et al.*, 2008).

The two major negative regulators of CRLs are CAND1 (cullin-associated and neddylation-dissociated) and the COP9 signalosome (CSN). The CSN possess isopeptidase metalloprotease activity to remove NEDD8 from cullin proteins causing CRL deactivation through entry into the CAND1 cycle (Lyapina *et al.*, 2001, Cope *et al.*, 2002). CAND1 binds only to CRLs lacking both neddylation and adaptors, and actively blocks cullin neddylation conjugation and adaptor protein binding sites (Min *et al.*, 2003). The purpose of this regulation and relationship to CSN regulation is uncertain, however it is likely to impact CRL complex stability and E3 ubiquitin ligase function (Bosu and Kipreos, 2008).

Adenoviruses were first found to interact with cullin members in 2001, when an Ad5E1B55K/E4ORF6 complex was discovered to essentially hijack CUL5- RBX1- and Elongin BC-containing E3 ubiquitin ligase complexes in order to ubiquitylate p53 (Querido *et al.*, 2001, Blanchette *et al.*, 2004). In this instance it was the viral protein E1B55K that formed the substrate recognition subunit rather than the cellular SOCS-box, while E4ORF6 directly bound the Elongin BC unit via one of its three BC box motifs (Blanchette *et al.*, 2004, Luo *et al.*, 2007). Other Ad serotypes have evolved different methods of inhibiting p53 and other cellular proteins. Indeed, our laboratory has previously shown that Ad12 utilises a CUL2-based CRL to induce the degradation of p53 (Blackford *et al.*, 2010).

Not surprisingly there are a growing number of cellular proteins (particularly those involved in the DDR) that are being identified as Ad E1B55K/E4ORF6 substrates. As described, Mre11 and DNA ligase IV inactivation is also driven by E1B55K/E4ORF6-dependent 26S proteasome degradation, however, the specific E3 ubiquitin ligases responsible for this

remains a matter of dispute in the literature (Stracker *et al*., 2002, Baker *et al*., 2007, Cheng *et al*., 2011; more detail to follow in Chapter 5). It is suggested that input viral DNA is sufficient to induce DDR pathways since MRN complex inactivation occurs prior to viral DNA accumulation (Karen *et al*., 2009). In addition, DNA ligase IV is also dissociated from the XRCC4 complex by E4ORF6 in an E1B55K-independent manner prior to degradation to prevent the NHEJ of viral genomes (Jayaram *et al*., 2008).

As mentioned, the potent ATR activating protein TOPBP1 is also degraded by Ad12 via CUL2-mediated ubiquitylation to circumvent ATR activation and inhibit Chk1 phosphorylation, interestingly however, this process is Ad12E1B54K-independent and wholly reliant on Ad12E4ORF6 (Blackford *et al*., 2010). This suggests that Ad12E4ORF6 has the capacity to independently behave as both a substrate recognition unit and as an adaptor linker protein (Blackford *et al*., 2010).

Another component of the DDR, Bloom helicase (BLM), is also degraded by Ad5 in an E1B55K/E4ORF6- and CUL5-dependent manner (Orazio *et al*., 2011). BLM is a RecQ DNA helicase and has been previously implicated in DNA end resection, however the functional relevance of this Ad-induced degradation remains unknown since HeLa-shBLM cells revealed that BLM is not responsible for concatemer formation (Gravel *et al*., 2008, Orazio *et al*., 2011).

The multifunctional death domain-associated protein (Daxx) has also recently been identified as an Ad5 substrate and is degraded by the 26S proteasome at late time points (Schreiner *et al*., 2010). In contrast to other Ad substrate degradation mechanisms, Ad5E4ORF6 is dispensable for Daxx depletion while Ad5E1B55K is suggested to establish a CUL5-based CRL through a highly conserved BC box motif. Ad5 infections in a Daxx knockdown cell line resulted in increased viral progeny and early viral protein production, suggesting that

Daxx may impact upon transcriptional repression during the early phase of infection (Schreiner *et al*., 2010).

In addition to intracellular proteins, the Ad5E1B55K/E4ORF6 CRL complex has also been shown to degrade the cell surface protein integrin alpha 3, presumably to either prevent re-infection or to promote the release and spread of progeny virions (Dallaire *et al*., 2009b). This degradation is almost absolute 16 hours post-infection (identical to Mre11) and was shown to be mediated by a CUL5-based CRL. Interestingly, integrin alpha 3 also acts as an alternative cellular receptor to facilitate internalization of Ad5 particles by endocytosis, and the onset of its degradation shows correlation with cell detachment from the extracelluar matrix (Salone *et al*., 2003, Dallaire *et al*., 2009b). During Ad5 infection, CUL5-based viral CRLs are also necessary for the stimulation of late viral protein synthesis by preventing dsRNA-dependent protein kinase (PKR) activity towards the translation factor eIF2α (eukaryotic initiation factor 2 alpha), and by stimulating viral nuclear mRNA export (Woo and Berk, 2007, Spurgeon and Ornelles, 2009).

Taken together the current literature suggests that different Ad serotypes have evolved varying mechanisms to degrade a broad spectrum of cellular proteins and to evade the host cell's DDR and NHEJ pathways.

1.3.3 E4ORF3 promotes the relocalization of cellular proteins

In addition to proteasomal degradation, cellular proteins are also inhibited by relocalization within the cell in order to negate DDR pathways. For instance, E4ORF3 has the capacity to restructure the nucleus and cytoplasm of infected cells and relocalize the tumour suppressors p53 and PML into nuclear track structures (Carvalho *et al*., 1995, Konig *et al*., 1999). The

basis of this remains elusive, however recent evidence suggests that immobilization of p53 in this manner prevents p53-DNA binding (Soria *et al*., 2010). Other proteins that associate with PODs and are degraded by adenoviruses, are similarly relocalized by E4ORF3 during Ad5 infection; however they have been described as separable events from PML reorganization (Konig *et al*., 1999, Araujo *et al*., 2005, Stracker *et al*., 2005). These proteins include the MRN complex which associates with PODs in response to IR, and Daxx whose transcriptional repression activity is thought to be inhibited by PML association (Li *et al*., 2000, Mirzoeva and Petrini, 2001, Stracker *et al*., 2005). The cellular localization of DNA ligase IV during Ad infection has yet to be determined.

MRN complex relocalization from the nucleoplasm into nuclear tracks by E4ORF3 is uncoupled from PML reorganization, and is thought to facilitate its degradation during Ad infection (Evans and Hearing, 2005, Stracker *et al*., 2005). Furthermore, in Ad5E1B55K transformed cells, and partially during Ad infection, Mre11 can also be found localized with E1B55K at centrosomes in cytoplasmic inclusion bodies called aggresomes (Araujo *et al*., 2005). Aggresomes are juxtanuclear establishing when aggregated proteins are transported by the motor protein, dynein, along microtubules towards the centrosome and can be formed by the individual expression of E4ORF3 or E1B55K (Garcia-Mata *et al*., 2002). These structures are scarce in normal cells but are believed to function as sites for rapid proteasomal degradation during times of cellular stress (such as viral infection, heat shock and chemical treatment) as they amass misfolded proteins and are enriched in components of the ubiquitin-proteasome pathway (Garcia-Mata *et al*., 2002). Studies have shown that 8 hours post-Ad5 infection Mre11 is immobilized into nuclear tracks, while 24 hours post-infection it is relocalized in an E4ORF3-dependent manner into E1B55K-containing aggresomes, which coincides with Mre11 degradation (Araujo *et al*., 2005, Liu *et al*., 2005). The direct binding

partner for E4ORF3 within the MRN complex has yet to be determined, although Nbs1 is dispensable for E4ORF3 to relocalize Rad50 and Mre11 (Araujo *et al*., 2005). The same scenario of relocalization also contributes to the inactivation of p53 during Ad5 infection, suggesting that adenoviruses exploit cellular aggresome formation in order to disrupt the restrictive effects of cellular DDR machinery on virus growth (Liu *et al*., 2005).

This process differs in cells infected with Ad4 and Ad12 viruses where, although Mre11 is still degraded, their E4ORF3 proteins lack the key isoleucine residue necessary for its ability to relocalize Mre11 into nuclear track structures. As a consequence, Mre11 does not localize to nuclear tracks but instead accumulates in nuclear viral replication centres (VRCs) where it could potentially cause problems for the virus by activating the DDR (Stracker *et al*., 2005, Carson *et al*., 2009). Therefore, to impede ATR signalling Ad12 has evolved to also induce the degradation of TOPBP1 (Blackford *et al*., 2010).

Relocalization into nuclear tracks during Ad infection is not the fate for all POD components. Indeed, TOPBP1 and BLM which associate with PODs in response to IR, are found at VRCs during Ad infection (Zhong *et al*., 1999, Xu *et al*., 2003, Stracker *et al*., 2005, Blackford *et al*., 2010). Although the significance of this relocalization is currently unknown, it is presumed that their residence at VRCs is not detrimental to viral DNA replication.

While the main objective of Ad is to ensure productive viral DNA replication by evading the cellular DDR, other key proteins of the pathway remain stable. For instance 53BP1, MDC1, BRCA1, ATRIP and the PIKK family members ATM, ATR and DNA-PK are not degraded by adenoviruses and reside in VRCs during Ad5 infection, albeit transiently in some cases (Carson *et al*., 2003, Mathew and Bridge, 2007). However, while H2AX is phosphorylated during Ad infection (suggesting some early DDR activation) the kinase activities of PIKKs are blocked through Mre11 and TOPBP1 degradation (Carson *et al*., 2003, Carson *et al*.,

2009). During Ad12, but not Ad5, infection ATR-dependent phosphorylation of RPA and Rad9 is also evident, however Chk1 phosphorylation is absent (Blackford *et al*., 2010). This further confirms that E4ORF6-induced TOPBP1 degradation prevents full ATR activation during Ad12 infection, while during Ad5 infection ATR activation is inhibited through combined Mre11 degradation and relocalization.

In addition to the ability of adenoviruses to circumvent the DDR to promote their own replication, E4ORF3 is also able to prevent ATR-dependent DNA damage signalling induced by non-viral sources (Carson *et al*., 2009). Indeed, in Ad5E4ORF3 transfected cells MDC1 is localized to sites of UV damage, while the MRN complex remains in nuclear tracks. This immobilization is sufficient to inhibit ATR-dependent events such as RPA phosphorylation (Carson *et al*., 2009).

1.3.4 Regulation of the cellular DNA damage response by other viruses

The role of DDR pathways during viral infection has received growing interest in recent years and is an evolving branch of cellular defence research. There are now a large number of viral proteins from an array of viruses that are known to interact with the DDR machinery, to either exploit or counter the effects of DDR pathways.

The circular HPV dsDNA virus is another example where cellular DDR pathways are deregulated during infection. This is achieved through the degradation of p53 and through the inhibition of the DNA single-strand break repair protein XRCC1 (Scheffner *et al*., 1993, Iftner *et al*., 2002). In contrast to Ad infection, epithelial differentiation by HPV has also been shown to activate the ATM pathway as ATM-dependent phosphorylation events are necessary to promote viral genome amplification (Moody and Laimins, 2009).

Interestingly, some viruses have also been shown to require, utilise and exploit DDR pathways to aid viral growth. The herpes simplex virus (HSV), for example, has evolved complex mechanisms to promote the efficient replication of its linear dsDNA genome, and its impact on the cellular DDR is multi-faceted. Indeed, DDR proteins such as ATM, ATR and 53BP1 are recruited to sites of HSV DNA replication possibly through ICP0, a viral E3 ubiquitin ligase protein. ICP0 has been shown to degrade the cellular histone ubiquitin ligases, RNF8 and RNF168, to promote mobilization of the repair proteins listed above (Lilley *et al*., 2010). The recruitment of these proteins to replication compartments benefits HSV, as ATM-dependent Chk2 phosphorylation and checkpoint activation is required for viral growth, and recent evidence has suggested that the presence of ATR/ATRIP may also promote viral gene expression and virus production (Li *et al*., 2008, Mohni *et al*., 2010). In contrast to this, other DDR proteins such as DNA-PK and Ku70 are targeted by ICP0 for proteasomal degradation, presumably to inhibit NHEJ (Parkinson *et al*., 1999).

Replication of SV40 is also dependent on the ability of the SV40 large tumour antigen (TAg) to exploit the DDR by promoting ATM and ATR kinase activity (Shi *et al*., 2005, Rohaly *et al*., 2010). DDR proteins, including the MRN complex and components of the Fanconi anemia pathway, are also recruited to VRCs where they are suspected to play an active role in SV40 DNA replication (Boichuk *et al*., 2010). Akin to Ad proteins, TAg has also been shown to induce the proteolysis of Mre11 at late times during infection, however its role in SV40 infection is unknown (Zhao *et al*., 2008). RNA viruses are also known to trigger DDR signalling pathways. Indeed, the HIV-1 accessory protein Vpr (viral protein R) induces G_2 arrest, and triggers the phosphorylation of repair proteins by activating the ATR kinase (Lai *et al*., 2005). This induction is thought to be mediated through the DNA damage-binding

protein 1 (DDB1)-CUL4A E3 ubiquitin ligase complex which promotes the degradation of unidentified cellular substrates (Belzile *et al*., 2010).

Together, these viruses serve as powerful tools for molecular biology purposes and have been integral in unravelling the complex nature of genome maintenance as they often induce signalling through the same cellular cascades. Furthermore, understanding the relationship between the DDR and viral infections has offered insights into viral pathology and may generate ideas for the development of antiviral drugs.

1.4 The TRIM family of proteins

1.4.1 TRIM proteins

Members of the TRIM (RBCC) family of proteins are characterized by an N-terminal tripartite motif which contains a RING finger moiety, one or two zinc-binding motifs named B-boxes, and a coiled-coil domain which is necessary for oligomerization and association with subcellular structures (Reymond *et al*., 2001, Meroni and Diez-Roux, 2005). The functional significance of B-box domains is poorly understood, however some evidence suggests that they may be important for the formation of cytoplasmic bodies and for higher-order oligomerization (Diaz-Griffero *et al*., 2009). To date there are 75 members in this multi-domain protein family and recent genomic analysis suggests that they can be divided into two groups based on domain structure, genome organization and evolutionary properties (Sardiello *et al*., 2008). Group 1 TRIM members contain a variety of C-terminal domains and are highly conserved, while group 2 members are less conserved and contain a C-terminal SPRY (splA/ryanodine receptor) domain (Sardiello *et al*., 2008).

A key feature of most TRIM proteins is their N-terminal RING domain implying a role for these proteins in ubiquitylation (Fig 1.10). Indeed, TRIM proteins have the potential to operate as single protein RING finger E3 ubiquitin ligases to mediate the direct transfer of ubiquitin from E2 enzymes to substrates. This has been demonstrated for several TRIM proteins where, through ubiquitylation and subsequent proteasomal degradation, they can mediate the expression of proteins involved in cell cycle regulation and cytokine signalling (Toniato *et al*., 2002, Urano *et al*., 2002). Although the role of TRIM protein C-terminal regions in ubiquitylation is unclear, data suggest that besides the RING finger, N-terminal B-box and coiled-coil domains may be responsible for substrate binding (Meroni and Diez-Roux, 2005).

TRIM proteins are involved in an array of biological processes ranging from apoptosis to transcriptional control and cell cycle regulation and as a consequence, when compromised, are also implicated in a variety of human pathologies including cancer (Meroni and Diez-Roux, 2005). Several TRIM proteins including TRIM1, TRIM5α, TRIM19 (PML), TRIM22 and TRIM30 are also implicated in the cellular response to viral infection where they often interfere with the virus replicative cycle (Nisole *et al*., 2005).

1.4.2 The TIF1 family

In addition to TRIM proteins being divided into two groups, they are also further divided into 11 highly homologous subfamilies (Carthagena *et al*., 2009). One such family is the transcriptional intermediary factor 1 (TIF1) family which contains four members in mammals: TIF1α (TRIM24), TIF1β (TRIM28), TIF1δ (TRIM66) and TIF1γ (TRIM33; Figure 1.11). Besides containing the N-terminal tripartite motif (TRIM/RBCC), TIF1 proteins

also harbour C-terminal plant homeobox (PHD) and bromodomains (BRD), and a unique TIF1 signature sequence (TSS) which is likely to participate in TIF1 dependent repression (Venturini *et al*., 1999). The presence of PHD and BRD motifs is characteristic of proteins known to function at the chromatin level. Indeed, previous studies have demonstrated that in TIF1α these domains mediate nucleosome interactions, possibly through hydrophobic bonds between PHD motifs and the alkyl groups of unmodified histones (Remboutsika *et al*., 2002, Slama and Geman, 2010, Tsai *et al*., 2010). PHD motifs are also highly reminiscent of RING fingers and while they have not been directly implicated in ubiquitylation, the PHD motif of TIF1β determines its activity as an E3 SUMO ligase and is required for SUMOylation of itself (auto-SUMOylation) and other substrates such as MDM2 (Wang *et al*., 2005, Ivanov *et al*., 2007, Zeng *et al*., 2008).

Akin to the domain homology witnessed between TIF1 family members, each of these proteins have demonstrated, or have the potential, to function as regulators of transcription (although the genes they regulate are somewhat variable). TIF1α, TIF1β and TIF1δ have all been shown to interact with, and phosphorylate, members of the heterochromatin protein 1 (HP1) family though PxVxL motifs to promote silencing on euchromatic genes (Nielsen *et al*., 1999, Khetchoumian *et al*., 2004). TIF1α and TIF1β also function as co-repressors when tethered to DNA thorough interactions with the widely distributed transcriptional silencing KRAB (Krüppel-associated box) domain of the Krüppel-like protein KOX1 (Friedman *et al*., 1996, Kim *et al*., 1996, Le Douarin *et al*., 1998). In addition, TIF1α has exclusively been found to mediate the transactivaton of a host of nuclear receptors including retinoid X (RXR), retinoic acid (RAR), vitamin D3 (VDR), oestrogen (ER) and progesterone (PR) receptors which are ligand-dependent transregulators that are capable of controlling gene expression

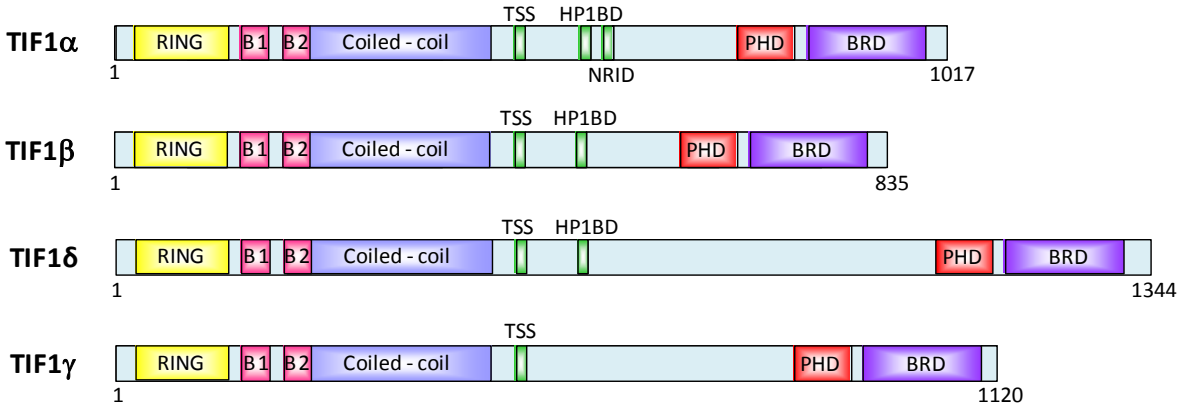


Fig 1.11. Linear representation of the TIF1 family of proteins

Coloured boxes correspond to the conserved domains of the TIF1 isoforms α, β, δ and γ: RING finger (yellow), B-boxes 1 and 2 (pink), coiled-coil (blue), TIF1 signature sequence (TSS), heterochromatin protein 1 (HP1) and nuclear receptor interaction (NRID) domains (green), plant homeobox domain (PHD; red) and bromodomain (BRD; purple).

(Le Douarin *et al*., 1996, Zhong *et al*., 1999). The clinical significance of TIF1 proteins is also highlighted by the fact that TIF1β and TIF1γ deficiencies in mice are embryonically lethal, and while TIF1α is not essential for embryogenesis it is a potent liver-specific tumour suppressor (Cammas *et al*., 2000, Khetchoumian *et al*., 2007, Kim and Kaartinen, 2008, Morsut *et al*., 2010). Furthermore, a recent study in mice discovered that besides TIF1α, hepatocellular carcinoma (HCC) formation is also promoted by the inactivation of TIF1β and TIF1γ, demonstrating that TIF1 proteins can also interact physically and functionally (Herquel *et al*., 2011). In contrast to its tumour suppressive properties, TIF1α is reportedly overexpressed in breast cancer where it aberrantly regulates ERα-mediated transcriptional activation leading to cellular proliferation and neoplasia in breast cells (Tsai *et al*., 2010).

1.4.3 TIF1γ

Low-stringency hybridization screens identified TIF1γ as the third member of the TIF1 family (Venturini *et al*., 1999). Although it was not implicated in nuclear receptor transactivation or found to associate with either KOX1 or the HP1 family, it nevertheless exhibited a strong silencing activity when tethered to a promoter (Venturini *et al*., 1999). Subsequent studies prompted by the Chernobyl disaster of 1986, discovered that thyroid papillary carcinomas (TPC) can be caused by defects in TIF1γ (Klugbauer and Rabes, 1999). More specifically, a chromosomal rearrangement occurs where the N-terminal portion of TIF1γ becomes fused to the tyrosine kinase domain of the *RET* (rearranged during transfection) proto-oncogene, forming a RET fused gene (RFG) which inappropriately activates tyrosine kinase activity and other protein functions (Klugbauer and Rabes, 1999). Recently, TIF1γ has also been identified as a tumour suppressor in the haematological malignancy chronic myelomonocytic leukemia (CMML) where it is downregulated by

hypermethylation of its gene promoter, and pancreatic ductal adenocarcinoma (PDAC) where it is thought to cooperate with the *KRAS* (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) oncogene (Vincent *et al.*, 2009, Aucagne *et al.*, 2011).

Studies into the transcriptional activities of TIF1γ have discovered that it can function as both a repressor and de-repressor of gene transcription. Indeed, through TRIM domain interactions, TIF1γ forms hetero-oligomers with TIF1α where it is hypothesised to partially relieve TIF1α-mediated repression of nuclear receptors (Peng *et al.*, 2002). TIF1γ also has an imperative role in the transforming growth factor beta (TGF-β) signalling pathway. TGF-β is a multi-functional cytokine that controls cellular proliferation, differentiation and homeostasis, and is often referred to as a tumour suppressor due to its antiproliferative properties during early tumour development and the fact that TGF-β pathway components are frequently mutationally inactivated in cancer (Derynck *et al.*, 2001, Tian *et al.*, 2011). In contrast however, a growing amount of evidence indicates that TGF-β can also promote processes that support tumour progression, such as angiogenesis and metastasis, suggesting a dual role for the cytokine (Pepper, 1997, Blobe *et al.*, 2000, Tian *et al.*, 2011).

Canonical TGF-β signalling is initiated by TGF-β binding, and activating, serine/threonine protein kinase receptors which leads to phosphorylation of the R-SMADS (small and mothers against decaplentaplegic-related), SMAD2 and SMAD3 (Massague *et al.*, 2005). Once activated, R-SMADS form heterodimeric complexes with the common mediator SMAD4 which then collectively enters the nucleus to regulate the expression of various genes (Massague *et al.*, 2005). TIF1γ has been shown to regulate this pathway by antagonizing the transcription of TGF-β responsive genes and there are two proposed mechanisms of how this is achieved. In the first model, Ectodermin (*Xenopus* homolog to TIF1γ) directly constrains SMAD4-dependent TGF-β signalling in embryonic development where it limits the

responsiveness of Nodal, a TGF-β superfamily ligand, by functioning as an E3 ubiquitin ligase to promote SMAD4 monoubiquitylation on lysine residue 519 (Dupont *et al*., 2005, Morsut *et al*., 2010). This modification disassembles R-SMAD-SMAD4 transcriptional complexes and promotes the relocalization of SMAD4 from the nucleus into the cytoplasm, where it is degraded by the proteasome (Dupont *et al*., 2005). There is also evidence to suggest that SMAD2 forms a trimeric complex with TIF1γ and SMAD4 to modulate this monoubiquitylation (Dupont *et al*., 2009).

In contrast, the second model found no evidence of TIF1γ-mediated SMAD4 ubiquitylation or degradation, and instead found that TIF1γ acts as a negative regulator of SMAD4 by competing with it to interact with activated R-SMADS (He *et al*., 2006). This association is proposed to stimulate erythroid differentiation of haematopoietic stem cells (HSC), as SMAD4 association inhibits their proliferation (He *et al*., 2006). It is of course possible that SMAD4 regulation by TIF1γ varies depending on the cellular context (i.e. embryonic or haematopoietic cells). In addition, the zebrafish *TIF1γ* homolog which is encoded by the *moonshine* (*mon*) gene is considered essential for embryonic and adult haematopoiesis as mutations in *mon* result in anaemia (Ransom *et al*., 1996, Ransom *et al*., 2004). This was recently attributed to TIF1γ/mon regulating the transcriptional elongation of erythroid genes by linking positive elongation factors to blood-specific transcription complexes (Bai *et al*., 2010).

1.4.4 TIF1α

As described above TIF1α possesses E3 ubiquitin ligase activity. This function is involved in p53 regulation where, together with the UbcH8 E2 ubiquitin conjugating enzyme, the RING

domain of TIF1α targets p53 for degradation and therefore acts as a negative regulator of p53-dependent pathways (Allton *et al*., 2009). This is further highlighted by the fact that siRNA mediated knockdown of TIF1α in human tumour-derived cells, and mutations of the *TIF1α* homolog, *bonus*, in *Drosophila*, cause unrestrained p53 activity and spontaneous apoptosis (Allton *et al*., 2009). Given the multiple roles of TIF1α, it is possible that this protein may lie at an intersection between signalling pathway networks regulated by p53 and/or nuclear receptors. Consequently, TIF1α has been suggested to be a therapeutic target to restore tumour suppression by p53, which is reflected by a recent finding that TIF1α overexpression in breast cancer is associated with poor prognosis and survival (Chambon *et al*., 2011).

TIF1β (also known as Krüppel-associated box [KRAB] domain-associated protein 1; KAP1) has also been shown to repress apoptotic pathways. Firstly by cooperating with MDM2, TIF1β promotes p53 ubiquitylation and degradation which stimulates p53–HDAC1 (histone deacetylase 1) interactions and p53 deacetylation (Wang *et al*., 2005). And secondly by inhibiting the activity of the E2F1 transcription factor, in a pRb-independent manner, TIF1β stimulates E2F1-HDAC1 interactions and E2F1 deacetylation (Wang *et al*., 2007).

1.4.4 Viral interactions with TRIM proteins

A growing number of TRIM proteins are believed to exhibit antiviral properties. The most documented of these proteins is PML (TRIM19) which has been shown to impair virus replication by sequestering viral proteins, inhibiting the synthesis of viral mRNA and inducing p53-dependent apoptosis. Thus, in addition to the aforementioned regulation by adenoviruses, at least 10 other DNA or RNA viruses have evolved various strategies to

abrogate the antiviral capacity of PML (Carvalho *et al.*, 1995, Geoffroy and Chelbi-Alix 2011).

The initial observation that PML is disrupted during virus infection came from Maul *et al*. in 1993. This paper reported that during HSV-1 infection, PML became redistributed in the nucleus by the immediate early gene 1 product, ICP0, which was later discovered to induce the proteasomal-degradation of PML via its function as an E3 ubiquitin ligase (Maul *et al*., 1993, Chelbi-Alix and de Thé, 1999). E6 and E7 HPV oncoproteins have also been reported to promote PML degradation during infection in order to prevent growth arrest and perturb transcriptional activation of p53 (Bischof *et al*., 2005, Louria-Hayon *et al*., 2009). Like adenoviruses, many other DNA viruses including the human cytomegalovirus (HCMV), KSHV, and EBV disorganize PML to evade cellular resistance mechanisms (Maul *et al*., 1993, Adamson and Kenney, 2001, Kang *et al*., 2006, Louria-Hayon *et al*., 2009). PML is also active against RNA virus replication. Indeed, the pre-integration complex (PIC) of HIV-1 triggers the rapid and transient, exportin-mediated relocalization of PML into the cytoplasm, however this mechanism remains controversial (Turelli *et al*., 2001).

Numerous other TRIM family members have also been discovered to exhibit antiviral activities. Indeed, one systematic screen of 55 TRIM proteins identified 20 which had antiviral activities towards HIV-1, murine leukemia virus (MLV) and avian leukosis virus (ALV) by affecting viral entry or release (Uchil *et al*., 2008). During HIV-1 infection, TRIM5α, TRIM11 and TRIM31 were found to restrict virus entry, TRIM22 and TRIM32 attenuated transcription of the HIV-1 long terminal repeat (LTR) transcriptional promoter, and TRIM25, TRIM31 and TRIM62 inhibited virus release from cells (Tissot and Mechti, 1995, Stremlau *et al*., 2004, Uchil *et al*., 2008). The antiviral properties of TRIM5α, the largest isoform of the TRIM5 gene, have received much interest in recent years in relation to

MLV and HIV-1 infection. Initial reports indicated that the C-terminal SPRY domain of TRIM5α was sufficient to cause HIV-1 restriction, however recent data suggest partial involvement of the proteasome system (Yap *et al*., 2005, Campbell *et al*., 2008). In addition, the ability of TRIM5α to obstruct HIV-1 infection in the cytoplasm at the postentry/preintegration phase by disrupting the uncoating of the viral capsid, has made it an attractive target in the development of gene therapy vectors for the treatment of AIDS (Anderson *et al*., 2009).

The transcriptional repressor properties of TIF1β have also been implicated in the antiviral response of cells infected with MLV and KSHV. During MLV infection, TIF1β is required for the primer binding site-dependent restriction of virus replication in embryonic stem and embryonic carcinoma cells (Wolf and Goff, 2007). While KSHV has been found to silence gene expression, and maintain a state of latency, by exploiting the chromatin remodelling functions of TIF1β. In this model, the switch from viral latency to lytic replication during KSHV infection is mediated via the viral protein kinase (vPK)-dependent phosphorylation of TIF1β which causes a decrease in TIF1β SUMOylation and consequently in its ability to condense chromatin on viral promoters (Chang *et al*., 2009). In addition, TIF1β has been reported to associate with the E8-E2C HPV gene during the inhibition of transcription and DNA replication, and with the EBV origin, oriLyt, during lytic DNA replication (Liao *et al*., 2005, Ammermann *et al*., 2008).

Of great significance to the current study is research carried out by Yondola and Hearing in 2007 who used a proteomic approach to identify novel cellular proteins that interact with the Ad E4ORF3 protein. Through mass spectrometry the authors identified TIF1α as a putative binding partner and successfully confirmed a direct interaction both *in vivo* and *in vitro*. This finding was particularly noteworthy because, unlike previously identified E4ORF3-binding

proteins, TIF1α was not found to be degraded by the 26S proteasome however it was relocalized into PML-containing nuclear tracks (Yondola and Hearing, 2007). Indeed, it was discovered that TIF1α relocalization was dependent on Ad E4ORF3 targeting the N-terminal TRIM domain of the protein which possesses E3 ubiquitin ligase activity, however the significance of this interaction was undetermined.

The authors did however speculate that Ad-mediated relocalization of TIF1α may cooperate with E4ORF3 in the activation of the glucocorticoid nuclear receptor which, amongst others, regulates genes controlling the immune response (Wienzek and Dobbelstein, 2001, Teyssier *et al*., 2006, Yondola and Hearing, 2007). Furthermore, the transcriptional nature of TIF1α suggests a possible role for the protein in the activation of viral gene expression, or equally suggests that sequestration by E4ORF3 prevents TIF1α from regulating cellular gene expression. While the functional consequence of the interaction between E4ORF3 and TIF1α remains elusive, due to its conserved nature among all serotypes so far investigated it is likely to be of critical importance to Ad infection.

While other members of the TIF1 family are yet to be implicated in Ad infection, it is interesting to note that several cellular process involving TIF1β and TIF1γ are abrogated during Ad infection such as the DDR and TGF-β signalling, respectively (Tarakanova and Wold, 2003, Weitzman *et al*., 2010).

CHAPTER 2

MATERIALS AND METHODS

2.1 TISSUE CULTURE TECHNIQUES

2.1.1 Cell lines

The cell lines used throughout this study are summarised in Table 2.1 below.

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
H1299	epithelial	large cell lung carcinoma	human	DMEM/puro	CRL-5803
H1299 shCUL5	epithelial	large cell lung carcinoma	human	DMEM/puro	(Cheng *et al*., 2007)
HEK293	epithelial	embryonic kidney	human	RPMI	CRL-1573
HeLa	epithelial	cervical carcinoma	human	DMEM	CCL-2
HER10	epithelial	embryonic retinoblast	human	RPMI	(Grabham *et al*., 1988)

2.1.2 Cell culture media

Cell lines were maintained in either DMEM (Dulbecco's modified Eagle's medium) or RPMI (Roswell Park Memorial Institute) medium (both Invitrogen) supplemented with 2 mM L-glutamine and 8% foetal calf serum (FCS; Invitrogen) and stored at 4 °C. H1299 cells were supplemented with 5 μg/ml puromycin (puro) which was removed prior to viral infection.

2.1.3 Maintenance and passage of cell lines

All tissue culture techniques were carried out in specialised flow hoods under sterile conditions. Cell passage involved removing existing medium, washing twice with phosphate buffered saline (PBS), adding 1 ml of trypsin (Invitrogen) per dish and incubating at 37 °C for 5 minutes. Culture medium was then added to completely detach cells, which were then pelleted by centrifugation at 428 x g for 5 minutes. Pellets were resuspended in fresh medium pre-warmed to 37 °C, and re-plated at a calculated dilution before being transferred to humidified incubators set at 37 °C and supplied with 5% CO_2.

2.1.4 Cryopreservation of cell lines

Cells were trypsinised and pelleted, as detailed above, and resuspended in 10% of the cryoprotectant dimethyl sulphoxide made up in DMEM or RPMI supplemented with 8% (v/v) FCS. Aliquoted cells were cooled gradually to -80 °C in isopropanol overnight and subsequently transferred for long term storage in liquid nitrogen tanks at -180 °C. When required, cells were rapidly thawed to 37 °C, pelleted, washed once with the appropriate culture medium and resuspended in fresh culture medium.

2.2 CELL BIOLOGY TECHNIQUES

2.2.1 Viruses

Ad3, Ad4, Ad5, Ad7, Ad11 and Ad12 *wt* viruses were either obtained from the American Type Culture Collection (ATCC) or were a gift from Prof. Joe Mymryk (University of Western Ontario, Canada). Ad9 was a kind gift from Prof. Ronald Javier (Baylor College of

Medicine, Texas). Several Ad5 and Ad12 mutant viruses were also used in this study. Ad5 *dl*1520 is an E1B55K-null virus generated by deletional mutagenesis, with two stop codons at nucleotides 3 and 3336 and a deletion from nucleotides 2496 to 3323 (Barker and Berk, 1987). Ad5 *dl*355 is an E4ORF6-null virus which lacks 14 base pairs (bp) between nucleotides 2331 and 2346 (Halbert *et al*., 1985). Ad5 *dl*H5*pm*4155 was a kind gift from Prof. Thomas Dobner (HPI, University of Hamburg, Germany) and contains two separate 1 bp deletions which result in the knockout of E4ORF3 and E4ORF6 (Forrester *et al*., 2011). The two Ad12E1B54K-null viruses used in this study were *dl*620 and *hr*703 which contain an in-frame deletion in the large E1B open reading frame from nucleotides 2129 to 2825 and a point mutant-derived stop codon, respectively (Byrd *et al.*, 1988).

2.2.2 Viral infections

Prior to infection cells were grown to 80% confluency and washed twice in DMEM without FCS. Viruses were applied at a multiplicity of infection (MOI) of 20 and diluted in DMEM without FCS before being added to 6 cm or 10 cm tissue culture dishes, or directly applied onto individual wells of glass multispot microscope slides (Hendley-Essex). Infected cells were incubated at 37 °C with agitation every 15 minutes. After 2 hours, virus-containing medium was removed and replaced with fresh culture medium supplemented with 8% (v/v) FCS.

2.2.3 RNA interference (RNAi)

Individual control small-interfering RNAs (siRNAs) and individual specific siRNAs targeting *CUL1*, *CUL3*, *CUL4A*, *CUL4B*, *CUL7*, *TIF1α* and *TIF1γ* gene expression are shown in Table 2.2. *CUL2* and *CUL5* were silenced using SMARTpool siRNAs where four separate pools of siRNA are combined to increase silencing and reduce off-target activities.

Table 2.2. siRNAs used in this study.

Target	siRNA	Sense sequence	Supplier
control/non-silencing	AllStars	Proprietary	Qiagen
CUL1	s16054	5’ CCGUCAGAGUUGGAACGUAtt 3’	Ambion
CUL2	SMARTpool	5’ GGAAGUGCAUGGUAAAUUU 3’ 5’ CAUCCAAGUUCAUAUACUA 3’ 5’ GCAGAAAGACACACCACAA 3’ 5’ UGGUUUACCUCAUAUGAUU 3’	Dharmacon
CUL3	s16050	5’ CCAGCGUAAGAAUAACAGUtt 3’	Ambion
CUL4A	s16045	5’ GGUUUAUCCACGGUAAAGAtt 3’	Ambion
CUL4B	s16044	5’ GAAGCUAUUCAGAAUAGUAtt 3’	Ambion
CUL5	SMARTpool	5’ GACACGACGUCUUAUAUUA 3’ 5’ GCAAAUAGAGUGGCUAAUA 3’ 5’ UAAACAAGCUUGCUAGAAU 3’ 5’ CGUCUAAUCUGUUAAAGAA 3’	Dharmacon
CUL7	s18991	5’ CCACUUUUGAGCAUUAUUAtt 3’	Ambion
TIF1α	s16786	5’ CGACUGAUUACAUACCGGUtt 3’	Ambion
TIF1γ	Custom	5’ CCUGCAUCUAGAAAGUGAAtt 3’	Ambion

siRNA transfection was carried out in both HeLa and H1299 cell lines. 40 x 10^4 cells were plated into 6 cm tissue culture dishes 24 hours prior to transfection in DMEM supplemented with 8% (v/v) FCS. The following day the transfection reagent Oligofectamine (OF; Invitrogen) was used to deliver siRNA duplexes into cells at 40% confluency as follows. Per dish, 20 µl of OF was added to 80 µl of Opti-MEM (Invitrogen) and left in a tube for 5 minutes, after which time 8.5 µl of the appropriate siRNA (40 µM) and 350 µl of Opti-MEM was added to the OF mixture. OF-siRNA complexes were left to form at room temperature for 20 minutes; during which time the cells to be transfected were washed twice with Opti-MEM pre-warmed to 37 °C. Finally OF-siRNA complexes were added to plated cells with a further 1 ml of Opti-MEM and incubated for 4-6 hours at 37 °C. After this time transfection medium was removed and replaced with fresh DMEM supplemented with 8% (v/v) FCS and returned to a humidified incubator at 37 °C for 72 hours. Where appropriate cotransfections were carried out with two siRNA molecules. As, and when required, cells were infected with virus 24 hours post-RNAi treatment.

2.2.4 Transient DNA transfections

The following plasmids were used in this study: pCS2 TIF1γ, *wt* and mutant, which were FLAG-tagged and a kind gift from Prof. Stefano Piccolo (Dupont *et al.*, 2005); pCMV-Ad5E4ORF3; pCMV-Ad5E4ORF6; pXC15-Ad5E1B55K; pcDNA3Ad12E4ORF3; pcDNA3Ad12E4ORF6; pcDNA3Ad12E1B54K. All adenovirus constructs were HA-tagged. Plasmid DNA was introduced into HeLa cells using a calcium phosphate transfection protocol. 80 x 10^4 cells were plated into 6 cm tissue culture dishes 24 hours prior to transfection in DMEM supplemented with 8% (v/v) FCS. The following day a solution

containing 22 µl $CaCl_2$, 5 µg DNA and 157 µl H_2O was added drop wise into a tube containing 180 µl of 2x HEPES buffered saline (HBS) pH 7.0 (50 mM HEPES [4-(2-hydoxyethyl)piperazine-1-ethanesulfonic acid], 280 mM NaCl, 1.5 mM $Na_2HPO_4[7H_2O]$). This transfection medium was left at room temperature for 30 minutes to allow the formation of calcium-DNA precipitate, which was subsequently directly added drop wise and evenly onto plated HeLa cells at 90% confluency. Transfected cells were then incubated at 37 °C and harvested at the appropriate times. TIF1γ transfections were carried out 48 hours prior to Ad infection, and where indicated cotransfections were carried out with two DNA plasmids.

2.2.5 Drug treatments

Following Ad infection, and where indicated, cells were treated with 10 µM of the proteasome inhibitor MG132 (N-carbobenzoxy-L-leucinyl-L-leucinyl-L-leucinal), 30 µM of the DNA damaging agent HU, 5 mM of the ATR kinase inhibitor caffeine or 20 µM of the CDK kinase inhibitor roscovitine (all Sigma-Aldrich). All drugs were solubilized in DMSO, except for HU which was solubilized in water. Mock treated cells were treated with solubilizing liquid alone.

2.2.6 IR and UV irradiation

HeLa cells were either mock-irradiated or irradiated from a 137Cs ionizing γ-ray source at a dose rate of 2.5 Gy/minute and left to recover for 1 hour. In the case of UV treatment, prior to UV irradiation cells were washed with PBS and mock-irradiated or irradiated with a 25 J/m2

dose of UV light from a 254 nm UV light source, after which the culture medium was replaced.

2.3 PROTEIN BIOCHEMISTRY TECHNIQUES

2.3.1 Preparation of whole cell lysates

Cell lysates were prepared by initially removing culture medium and washing twice in ice-cold saline. Samples were lysed and harvested by scraping in a denaturing buffer (9 M urea, 50 mM Tris-HCl [pH 7.3], 150 mM β-mercaptoethanol). Lysed cells were collected and sonicated twice for 15 seconds on ice and subsequently centrifuged for 20 minutes at 16 000 x g to remove cell debris. The supernatant was then removed with a syringe into new sample tube ready for use or storage at -80 °C.

2.3.2 Quantification of protein concentration

Protein concentrations were determined by Bradford assay (Bio-Rad) using six bovine serum albumin (BSA) standards at known dilutions ranging from 0 - 50 µg/ml made up in deionised H_2O; 10 µl of each standard was added to 4 wells of a flat bottomed 96 well microplate. Typically, 5 µl of each prepared protein lysate was then added to 45 µl of deionised H_2O and 10 µl of this mixture was also added to 4 wells of a flat bottomed 96 well microplate. 200 µl of Bradford reagent (Bio-Rad) diluted 1:4 with deionised H_2O was then added to each standard and sample well and gently mixed by pipetting. Finally absorbance readings were determined using a microplate reader (Bio-Rad) at 595 nm and sample concentrations were calculated by generation of a standard curve.

2.3.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Clarified cell lysates were separated based on their molecular weight by SDS (sodium dodecyl sulphate)-PAGE. Polyacrylamide gels were made using a stock 30% (w/v) Acrylamide/Bis solution (Severn Biotech Ltd) diluted with deionised H_2O to the appropriate percentage (8% or 12% depending on molecular weight of the protein) in the presence of 100 mM Tris, 100 mM Bicine, 0.1% (w/v) SDS and 0.3% (v/v) N,N,N',N' - tetramethylethylenediamine-1,2-diamine (TEMED). Acrylamide polymerisation was initiated by the addition of ammonium persulphate (APS) to a final concentration of 0.06% (v/v). Gel assembly was carried out according to the manufactures' instructions (Hoeffer Scientific) and run in a buffer containing 100 mM Tris, 100 mM Bicine and 0.1% (v/v) SDS. Protein cell lysate (50 μg) or Protein G-Sepharose beads were denatured by the addition of Laemmli Sample Buffer (Bio-Rad) and incubation at 90 °C for 5 minutes. Denatured samples were then loaded into gels along with pre-stained protein molecular weight markers (Fermentas) using a long syringe (Hamilton). Gels were then run overnight at a constant current depending on the molecular weight of the protein of interest.

2.3.4 Staining of polyacrylamide gels

Following electrophoresis, gels were placed in a staining solution containing 0.1% (v/v) Coommassie Brilliant Blue G-250 (Sigma-Aldrich), 1.6% (v/v) orthophosphoric acid, 8% (w/v) ammonium sulphate and 20% (v/v) methanol in deionised H_2O and agitated at room temperature for 10 minutes. Destaining was carried out in 10% (v/v) glacial acetic acid and 20% (v/v) methanol made up in deionised H_2O until protein banding was visible.

2.3.5 Preparation of proteins for analysis by mass spectrometry

Protein bands detected by Coomassie Brillaint Blue G-250 staining were excised under sterile conditions using a scalpel blade. Excised bands were then washed twice in 400 µl of 50% (v/v) acetonitrile and 50 mM ammonium bicarbonate by agitation for 45 minutes at 37 °C. The wash buffer was then removed and the proteins were reduced for 1 hour at 56 °C in 250 µl of 50 mM dithiothrietol (DTT) made up in 10% (v/v) acetonitrile and 50 mM ammonium bicarbonate. The supernatant was then removed and replaced with 50 µl of an alkylating solution containing 200 mM iodoacetamide, 10% (v/v) acetonitrile and 50 mM ammonium bicarbonate in which samples were incubated at room temperature for 30 minutes in the dark. Samples were then washed 3 times in 10% (v/v) acetonitrile and 40 mM ammonium bicarbonate by agitation for 15 minutes at room temperature, and dried in a DNA mini centrifugal evaporator (Heto Holten) for 1 hour.

Proteins were digested by rehydration in 20 µl of 12.5 µg/ml modified trypsin (Sigma-Aldrich) which cleaves C-terminal to arginine and lysine residues. Following a 1 hour incubation at room temperature, 20 µl of 10% (v/v) acetonitrile and 40 mM ammonium bicarbonate was added to the trypsinized sample and left to incubate with agitation overnight at 37 °C. The next day the supernatant was removed and stored in a specialised mass spectrometry tube. The digested proteins in the remaining gel slice were then twice eluted with 40 µl of 3% (v/v) formic acid, 10% (v/v) acetonitrile and 40 mM ammonium bicarbonate for 30 minutes at room temperature with agitation before the supernatant was added to the mass spectrometry tube. Finally, the resulting peptides were then separated using an AmaZon ion trap electron-transfer dissociation (ETD)-enabled mass spectrometer (Bruker), and processed and analysed by the ProteinScape central bioinformatic platform

(Bruker). Mass spectrometric separation and analysis of peptides was provided as an internal service by Ashley Martin and Neil Shimwell.

2.4 IMMUNOCHEMISTRY TECHNIQUES

2.4.1 Antibodies

All antibodies used in this study are described in Table 2.3.

Table 2.3. Antibodies used in this study.

Antigen	Antibody	Origin	Use	Supplier	Reference
Ad12E1A	M13	Mouse	WB, IF	Ed Harlow	Harlow *et al*., 1985
Ad12E1B54K	XPH9	Mouse	WB, IF, IP	In-house	Merrick *et al*., 1991
Ad5E1A	M73	Mouse	WB	Ed Harlow	Harlow *et al*., 1985
Ad5E1B55K	2A6	Mouse	WB, IF, IP	Arnold Levine	Sarnow *et al*., 1982b
Ad5E4ORF3	6A11	Mouse	WB, IF	Thomas Dobner	Nevels *et al*., 1999
Ad5E4ORF6	RSA1	Mouse	WB, IF	Thomas Dobner	Marton *et al*., 1990
ATR	N-19	Goat	WB	Santa Cruz	-
Chk1	G-4	Mouse	WB	Santa Cruz	-
CUL1	105781	Rabbit	WB	Gene Tex	-
CUL2	Ab1870	Rabbit	WB	Abcam	-
CUL3	111274	Rabbit	WB	Gene Tex	-

CUL4A	113876	Rabbit	WB	Gene Tex	-
CUL4B	113875	Rabbit	WB	Gene Tex	-
CUL5	a302-173a	rabbit	WB	Bethyl	-
CUL7	109785	Rabbit	WB	Gene Tex	-
Cyclin E	M-20	Rabbit	WB	Santa Cruz	-
Daxx	M1/2	Mouse	WB	Santa Cruz	-
DNA ligase IV	LIGIV	Rabbit	WB	Stephen Jackson	Critchlow *et al*., 1997
Flag	Anti-Flag M2	Mouse	WB, IF	Stratagene	-
H2AX	07-627	Rabbit	WB	Millipore	-
HA	Anti-HA 12CAS	Mouse	WB, IF	Sigma-Aldrich	-
MDM2	2A10	Mouse	WB	Arnold Levine	Chen *et al*., 1993
Mre11	12D7	Mouse	WB, IF	GeneTex	-
NBS1	1C3	Mouse	WB	GeneTex	-
p21	DCS60	Rabbit	WB	Cell Signalling	-
p53	DO-1	Mouse	WB, IF	David Lane	Vojtěsek *et al*., 1992
p-Chk1 S345	133D3	Rabbit	WB	Cell Signalling	-
PML	PG-M3	Mouse	IF	Santa Cruz	-
PML	H-238	Rabbit	IF	Santa Cruz	-
p-RPA S468	a300-245	Rabbit	WB	Bethyl	-
p-TIF1β S824	5824	Rabbit	WB	Bethyl	-
pRB	RB1	Mouse	WB	Fitzgerald Industries	-

RPA32	Ab-2	Mouse	IF	Calbiochem	-
RPA32	ab10359	Rabbit	IF	Abcam	-
TIF1α	A300-815A	Rabbit	WB, IF	Bethyl	-
TIF1β	A300-274A	Rabbit	WB, IF	Bethyl	-
TIF1γ	197	Rabbit	WB, IF, IP	In-house	Sedgwick and Turnell, unpublished
TOPBP1	(5H)52	Rabbit	WB, IF	Ian Morgan	Boner *et al*., 2002
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® 555 anti-rabbit	Goat	IF	Invitrogen	-
Mouse IgG	Alexa Fluor® 488 anti-mouse	Goat	IF	Invitrogen	-

2.4.2 Immunoprecipitation

Cells cultured in a 10 cm diameter tissue culture dish were harvested by washing twice in ice cold saline and subsequently solubilized in 1 ml of immunoprecipitation (IP) buffer containing 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% (v/v) Nonidet P-40, 25 mM NaF and 25 mM β-glycerophosphate. For immunoprecipitations carried out for mass spectrometric analysis, the IP buffer used additionally contained 1 mM Na_3VO_4 to act as a phosphatase inhibitor. Cell lysates were then homogenized twice with 10 strokes while being kept on ice and centrifuged at 16 000 x g for

30 minutes at 4 °C. Supernatant was then removed from cell debris using a syringe and the appropriate antibodies were added to samples to form antigen-antibody complexes at 4 °C overnight with rotation. After this time 40 µl of Protein G-Sepharose beads (Sigma-Aldrich) were added to all samples to capture and isolate immune complexes for 2 hours at 4 °C with rotation. The beads were then washed five times by centrifugation at 800 x g in ice cold IP buffer, eluted in 30 µl of Laemmli sample buffer (Bio-Rad) and ran on SDS-PAGE gels for Western blotting.

2.4.3 Western blotting

Following separation by SDS-PAGE, proteins where transferred onto a nitrocellulose membrane (PALL) using a Hoeffer Scientific transfer system. Briefly, gels were placed onto nitrocellulose membranes pre-soaked in blotting buffer (50 mM Tris, 190 mM glycine, 20% (v/v) methanol) and sandwiched between two pieces of 3MM blotting filter paper (Whatman) and two blotting sponges. These were then placed in plastic cassettes and slotted into the blotting tank (Hoeffer Scientific). The electro-transfer was set at 280 mA and continued for approximately 6 hours, after which time the membrane was placed in a staining solution containing 0.1% (w/v) Ponceau S (Sigma-Aldrich) and 3% (w/v) trichloroacetic acid for 2 minutes and rinsed with deionised water several times to detect protein bands. The stain was then removed by washing with 0.1% (v/v) Tween 20 in Tris-buffered saline (TBS) containing 150 mM NaCl and 20 mM Tris-HCl (TBST) pH 7.3.

Nitrocellulose membranes were blocked in 5% (w/v) dried milk powder in TBST for 45 minutes at room temperature with agitation, and subsequently washed in TBST. Membranes were often trimmed down to the approximate size of the protein of interest and blot sections

were incubated overnight with antibodies made up in TBST containing 5% (v/v) milk at 4 °C with agitation. The following day, membranes were washed four times in TBST and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Dako) made up in TBST containing 5% (v/v) milk at room temperature for 2 hours with agitation. Finally, membranes were washed four times in TBST and antigens were detected using enhanced chemiluminescence (ECL) reagents (GE Healthcare) and autoradiography film (Kodak).

2.4.4 Immunofluorescence

Cells were grown on glass 12-well multispot microscope glass slides (Hendley-Essex) at densities of 2 x 10^4 cells/well. Slides were gently washed twice in PBS, and prepared by treatment in ice cold 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 fixing in 4% (w/v) paraformaldehyde in PBS for 10 minutes. Following three PBS washes, cells were blocked in 10% (v/v) FCS in PBS for 1 hour at room temperature. Primary antibodies were diluted with 8% (v/v) FCS in PBS and incubated on cells for 1 hour at room temperature, after which time slides were washed three times in PBS and incubated for a further hour at room temperature in the dark with fluorescent Alexa Fluor® secondary antibodies (Invitrogen) diluted with 8% (v/v) FCS in PBS. Finally slides were washed 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 using a LSM 510 META confocal laser scanning microscope (Carl Zeiss) equipped with a 364 nm UVA laser and two visible laser lines (488 and 543 mn). Typically a 40x oiled objective was utilised with each channel (laser) set to an equal optical slice and a pinhole diameter of approximately 1 Airy unit. Final 8 bit images at 1024 pixels of resolution were acquired using a scan speed of 6 and a scan average of 4 to achieve a high resolution image with a good signal-to-noise ratio. These images were then imported into a Zeiss LSM image browser for formatting.

2.4.5 Irradiation-induced DNA damage

DNA damage was induced in cells following a protocol published by Kruhlak and colleagues in 2009. Cells were grown to 70% confluency in chambered coverglass dishes (Thermo Scientific) and pre-sensitized at 37 °C with 7.5 μg/ml of the DNA binding dye Hoechst 33342 (Sigma-Aldrich) in phenol red-free DMEM microscopy medium containing 2 mM L-glutamine and 25 mM HEPES (Gibco) for 30 minutes. Pre-sensitized cells were then rinsed once in, and supplemented with, 400 μl of microscopy medium not containing the Hoechst dye. Irradiation was carried out using a LSM 510 Meta confocal laser scanning microscope (Carl Zeiss) equipped with a 364 nm UVA laser and temperature controlled chamber pre-heated to 37 °C. Briefly, the configurations used included a 50% UVA laser output, a scan speed of 7 and 10 iterations of the region of interest (ROI). Following UVA treatment, cells were harvested and immunofluorescence was carried out as described in section 2.4.4. A comprehensive protocol for this technique was reported previously (Kruhlak *et al*., 2009).

2.5 MOLECULAR BIOLOGY TECHNIQUES

2.5.1 Preparation of DNA for pulsed field gel electrophoresis (PFGE)

DNA for PFGE was prepared from Ad infected HeLa cells according to the manufacturer's instructions (Bio-Rad) and as described previously (Formstone *et al*., 1993). Forty-eight hours post-infection cells were trypsinized, pelleted and washed twice in PBS before being resuspended in PBS at a concentration of 2 x 10^7 cells/ml. A 1% (w/v) solution of InCert agarose (Bio-Rad) in PBS was prepared and cooled to 45 °C. Equal volumes of the cell suspension and InCert agarose were mixed to give a final concentration of 1 x 10^7 cells/ml, and 200 µl of this mixture was immediately decanted into plug moulds (Bio-Rad) which were left to set at 4 °C for 30 minutes. After the plugs had set they were carefully removed and immersed in filtered NDS lysis buffer (1% [w/v] N-lauroyl sarcosine, 0.5 M EDTA, 10 mM Tris) at pH 9.5, containing proteinase K (1 mg/ml), and left at room temperature for 30 minutes. They were then incubated at 50 °C overnight. The following day plugs were removed into fresh NDS buffer containing proteinase K and incubated for a further 20 hours at 50 °C. They were then rinsed twice for 1 hour in NDS buffer minus proteinase K, and stored in fresh NDS buffer at 4 °C until required.

2.5.2 PFGE

DNA was separated using a contour-clamped homogeneous electric field (CHEF-DR II) electrophoresis system (Bio-Rad) which is a hexagonal array of 24 electrodes. Electrophoresis was carried out in 2 litres of 0.5 x TBE running buffer (45 mM Tris, 45 mM Boric acid, 1 mM EDTA) which was pre-cooled to 4 °C for 1 hour and circulated at a rate of 1 litre/minute using a circulating pump (Bio-Rad). 1% (w/v) agarose (Bio-Rad) gels made in

0.5 x TBE were cast using specialised combs. After the gel had set, sample plugs and lambda (λ) ladder standard plugs (Bio-Rad) were loaded into the front of the gel wells and sealed using 1% (w/v) InCert agarose. Gels were submerged into the electrophoresis chamber and DNA was subjected to PFGE for 22 hours before being stained in 0.5 μg/ml ethidium bromide made in 0.5 x TBE for 30 minutes, and then destained in 0.5 x TBE for 1 hour. DNA was visualized and digital images were captured using a UV transilluminator with an integrated camera (Bio-Rad).

2.5.3 p53 reporter assay

The effect of infection on p53 transcriptional activity was determined using a dual-luciferase reporter assay system (Promega). H1299 cells, which carry a homologous deletion of the p53 gene, were grown in 6 cm dishes and transfected with the pBK-CMV vector with a p53 cDNA insert and the PG13-Luc reporter construct which contains p53-binding sites upstream of the luciferase gene (both kind gifts from Dr Ester Hammond), together with the control Renilla luciferase reporter plasmid pRL-TK (a kind gift from Dr John O'Neil). Transfection was performed using Lipofectamine LTX according to the manufacturer's instructions (Invitrogen). After 24 hours cells were Ad infected, harvested after a further 24 hours, and lysed using a passive lysis buffer (Promega). The luciferase activity was measured using a luminometer according to the manufacturer's instructions (Promega).

2.5.4 Semiquantitative reverse transcriptase PCR (RT-PCR)

Semiquantitative RT-PCR was carried out in HeLa and A549 cells. Total RNA was extracted from cell lysates using the RNeasy mini kit (Qiagen). Total RNA (1 μg) was converted into single-strand cDNA using the Reverse Transcriptase System (Promega) and semiquantitative-PCR was performed using the following primers: *p21* forward, 5'-CGACTGTGATGCGCTAATGG-3'; *p21* reverse, 5'-CCGTTTTCGACCCTGAGA-3'; *gapdh* forward, 5'-ACCCCTTCATTGACCTCA-3'; *gapdh* reverse, 5'-CAGCGCCAGTAGAGGCAG-3'. Semiquantitative RT-PCR thermal cycler conditions were as follows: *p21*: 1 cycle of 94 ^{o}C for 3 minutes; 30 cycles of 94 ^{o}C for 30 seconds, 55 ^{o}C for 30 seconds, 72 ^{o}C for 2.5 minutes followed by a 10 minute extension at 72 ^{o}C; *gapdh:* 1 cycle of 94 ^{o}C for 3 minutes; 30 cycles of 94 ^{o}C for 30 seconds, 58 ^{o}C for 30 seconds, 72 ^{o}C for 2.5 minutes followed by a 10 minute extension at 72 ^{o}C.

2.5.5 Agarose gel electrophoresis

PCR products were analysed by agarose gel electrophoresis. Agarose powder (0.8 g; Sigma-Aldrich) was dissolved in 100 ml of 1 x TBE buffer (89 mM Tris, 89 mM Boric acid, 2 mM EDTA) and heated to boiling point. The agarose was allowed to cool before ethidium bromide was added to a final concentration of 0.5 μg/ml. Samples were diluted with 6 x loading buffer containing 0.25% (w/v) bromophenol blue and 30% (v/v) glycerol made in a 10 mM Tris, 1 mM EDTA, (pH 8.0) solution. Samples were then loaded and gel electrophoresis was performed in 1 x TBE at 120 V for 30 minutes. DNA was visualized and digital images were captured using a UV transilluminator with an integrated camera (Bio-Rad).

CHAPTER 3

ADENOVIRAL SEROTYPES DIFFERENTIALLY REGULATE THE DNA DAMAGE RESPONSE

3.1 INTRODUCTION

The introduction of linear double-stranded adenoviral DNA into cells represents a target for cellular DDR pathways which recognise viral DNA as DSBs, and it has been determined that activation of DDR pathways limit Ad replication. The first evidence for this came from observations that infection with Ad5 mutant viruses harbouring deletions in the E4 region resulted in the formation of large covalently-linked concatemers of viral DNA (Weiden and Ginsberg, 1994).

In order to identify the cellular proteins involved in concatemer formation, nearly a decade later a similar mutant Ad5 E4-deletion virus, *dl*1004, virus was used to infect human cell lines carrying mutations in DNA repair genes (Stracker *et al*., 2002). This study reported that *dl*1004 infection of Mre11-deficient ATLD cells rescued the concatemer phenotype seen in normal fibroblasts, suggesting a role for Mre11 in viral genome concatemerization (similar findings were reported for cells lacking DNA-PKcs and DNA ligase IV; Stracker *et al*., 2002). As the MRN complex is involved in the activation of the ATM and ATR kinases during DNA damage signalling, it was hypothesized that cells infected with the E4 mutant virus were unable to inhibit Mre11, such that both ATM and ATR signalling cascades were activated leading to viral genome concatemerization (Carson *et al*., 2003). Further research demonstrated members of the MRN complex being relocalized into PML (also known as TRIM19)-containing foci adjacent to sites of viral replication (VRCs), and suggested that Mre11 degradation by the virus occurs in a proteasome-dependent manner which is mediated by a cullin-containing E3 ubiquitin ligase complex (CRL); this process is now known to correlate with the onset of viral DNA replication (Mathew and Bridge, 2007). The importance of Ad-mediated degradation during Ad infection and the role of E3 ubiquitin

ligases in this process are discussed further in this Chapter and also in the Introduction and Chapter 5.

Other components of the DNA repair machinery are also targeted for proteasomal degradation by adenoviruses to optimise the cellular environment, including p53, DNA ligase IV, TOPBP1, BLM and Daxx (Nevels *et al*., 1997, Steegenga *et al*., 1998, Baker *et al*., 2007, Blackford *et al*., 2010, Schreiner *et al*., 2010, Orazio *et al*., 2011). In addition, the Ad5 coreceptor integrin alpha 3 has also been identified as an Ad5 substrate (Salone *et al*., 2003, Dallaire *et al*., 2009b).

In many cases, the degradation of these substrates is preceded by their relocalization by E4ORF3 into distinctive nuclear tracks, or for TOPBP1 into VRCs (Blackford *et* al., 2010). Interestingly several reports have observed that nuclear tracks eventually surround sites of viral replication during late times of infection (Doucas *et al*., 1996). Although the significance of this is unknown, it may be hypothesized that sequestering proteins into nuclear tracks is a mechanism used to prevent cellular proteins from interfering with viral DNA replication. Significantly, E4ORF3-induced nuclear tracks also contain PML which in unstressed cells is tightly bound to the nuclear matrix in discrete nuclear foci (Carvalho *et al*., 1995). In uninfected cells PML-containing nuclear structures are functionally heterogeneous and are implicated in many cellular processes including the DDR, transcription, senescence, apoptosis and innate immunity (Zimber *et al*., 2004, Bernardi and Pandolfi, 2007). Furthermore they often organize the delivery and storage of proteins essential for these processes (Zimber *et al*., 2004, Bernardi and Pandolfi, 2007).

During Ad infection, interactions with PML by viral proteins have been linked to oncogenic transformation potential (Nevels *et al*., 1999). It was recently reported that Ad5E1B55K

represses p53 through its innate E3 SUMO1 ligase activity, and that this modification is carried out within PML nuclear tracks (Muller and Dobner, 2008, Pennella *et al*., 2010). This suggests that the virus may exploit the dynamic nature of PML to promote the inactivation of the DDR, however the function of these interesting structures remains largely unknown.

With advances in proteomic technology many new Ad targets are being identified amongst members of the DDR pathways. Recently, our lab identified TOPBP1 as a novel Ad substrate which is targeted for ubiquitin-dependent proteasome-mediated degradation during Ad12 infection, but which remains unaffected during Ad5 infection. This leads to the possibility that other serotypes may differentially regulate the DDR, although the majority of research has focused on Ad5 (species C) and Ad12 (species A) serotypes which have been well characterized. There are however over 50 other serotypes, divided into seven species (A to G), that have been relatively overlooked. In the current study previously uncharacterized serotypes (with regard to their relationship to the DDR) Ad3 (species B1), Ad7 (species B1), Ad11 (species B2), Ad9 (species D) and Ad4 (species E) were studied for their effect on the DDR proteins Mre11, p53, DNA ligase IV and TOPBP1 with regard to protein expression and cellular localization. The effect these viruses have on ATM and ATR kinase activity were also studied.

3.2 AIMS

The specific aims of this study were to:

a) Investigate Ad serotype representatives from species A to E for their ability to degrade proteins involved in DDR pathways.

b) Examine the ability of these serotypes to relocalize cellular proteins during Ad infection.

c) Determine the effect Ad infection has on the DDR in relation to ATM and ATR kinase activation.

3.3 RESULTS

3.3.1 Validation of infection with Ad serotypes

All viral serotypes used in this study (Table 1.1) have previously been titred using plaque forming assays. Since the viruses were supplied from various sources, however, they were also authenticated in the HeLa cell line which was predominantly used during this study. Commercial antibodies against viral proteins are generally only available for the more common serotypes (Ad5 and Ad12), therefore in order to test the efficacy of infection by the other viruses two approaches were adopted. Firstly, adenoviral structural proteins expressed in the late stages of infection were visualised by Ponceau S staining. HeLa cells were thus infected with the panel of seven serotypes representative of species A to E (Ad3, Ad4, Ad5, Ad7, Ad9, Ad11 and Ad12) at an MOI of 20 and harvested at intervals over a period of 72 hours. Lysates were subjected to SDS-PAGE and Western blotting, and the nitrocellulose membranes were treated with Ponceau S to stain the proteins (Fig 3.1). Arrows indicate expression of the ~117 k hexon structural protein, which for most serotypes is evident by at least 32 hours post-infection.

Secondly, evidence that all serotypes were entering into a viral lifecycle and replicating their DNA was evident from the establishment of RPA32-positive nuclear viral replication and transcription centres, as visualised by confocal microscopy (Figs 3.6-3.8). These methods demonstrate that all serotypes used in this study were capable of expressing late viral structural proteins and forming VRCs in HeLa cells. Based on the evolutionary divergence between serotypes, one would also expect them to have comparable but different lifecycles and effects on the host cell.

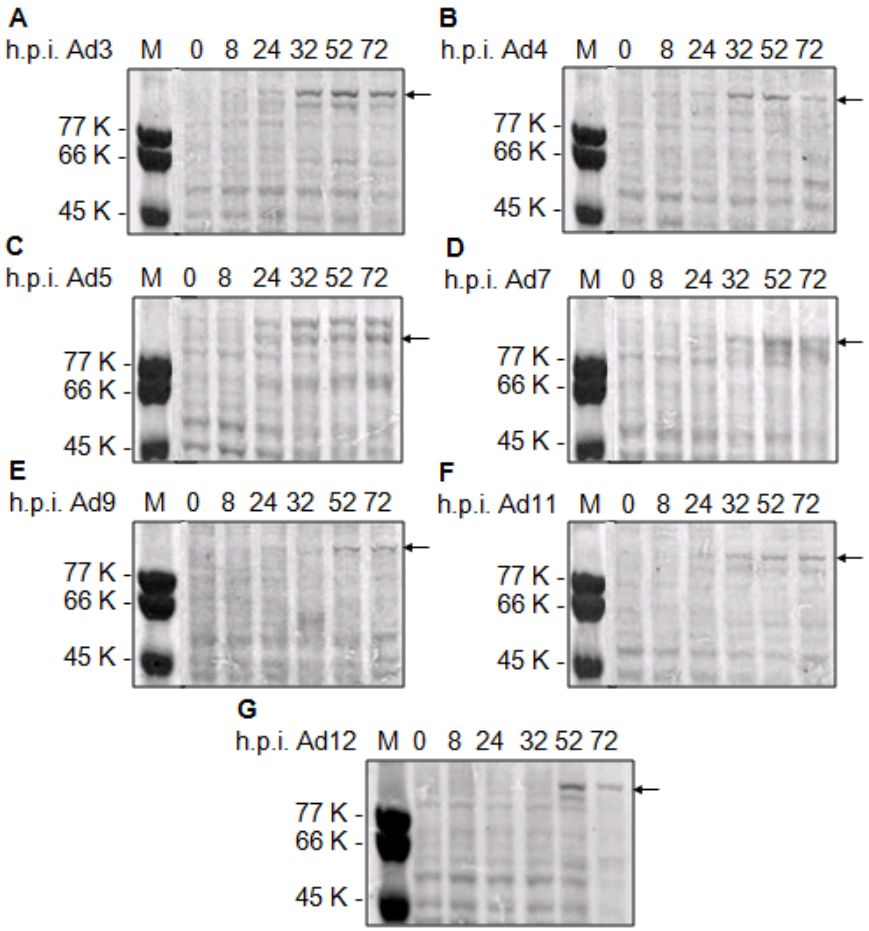


Fig 3.1A-G. Validation of infection with Ad serotypes by visualisation of structural protein production

HeLa cells were infected with Ad3 (A), Ad4 (B), Ad5 (C), Ad7 (D), Ad9 (E), Ad11 (F) and Ad12 (F) and harvested at the indicated time points. Proteins were separated by SDS-PAGE and membranes were stained with Ponceau S. Arrows indicate viral hexon expression.

3.3.2 All serotypes induce reduction of DNA ligase IV protein levels

It is well established that Ad5 and Ad12 inactivate the host cell DDR by facilitating the ubiquitin-dependent proteasome-mediated degradation of Mre11, DNA ligase IV and p53 (Querido *et al*., 2001, Stracker *et al*., 2002, Baker *et al*., 2007). This is achieved through the hijack of various cellular complexes by E1B55K and E4ORF6 viral proteins, to form specific E3 ubiquitin ligase protein complexes (Querido *et al.*, 2001, Harada *et al.*, 2002).

To investigate whether the degradation of Mre11, DNA ligase IV and p53 by Ad5 and Ad12 is representative for all adenoviruses, the panel of serotypes were examined. HeLa cells were infected with viruses at an MOI of 20 and harvested at various time points before being prepared for Western blotting. Several clear differences in protein expression were evident; although p53 was transiently increased (by the action of E1A) before being degraded during Ad4, Ad5 and Ad12 infection, Ad3, Ad7, Ad9 and Ad11 infection all caused the expression of p53 to appreciably increase with no sign of degradation (Fig 3.2C).

More variation was seen with Mre11 protein expression, where Ad4, Ad5 and Ad12 induced rapid and complete depletion starting at 32 hours post-infection, while Ad3, Ad7, Ad9 and Ad11 had no effect even at late time points (Fig 3.2A). Interestingly, all serotypes used in this study were capable of reducing DNA ligase IV protein levels (Fig 3.2B); an observation that was previously only seen with Ad5 and Ad12. In the present study DNA ligase IV depletion by Ad4, Ad5, Ad7, Ad11 and Ad12 was evident 32 hours post-infection, while during Ad3 and Ad9 infection the depletion occurred more gradually and at later time points. This depletion of protein expression by the previously uncharacterized viruses is assumed to be mediated via proteasomal degradation as is reported for Ad5 and Ad12.

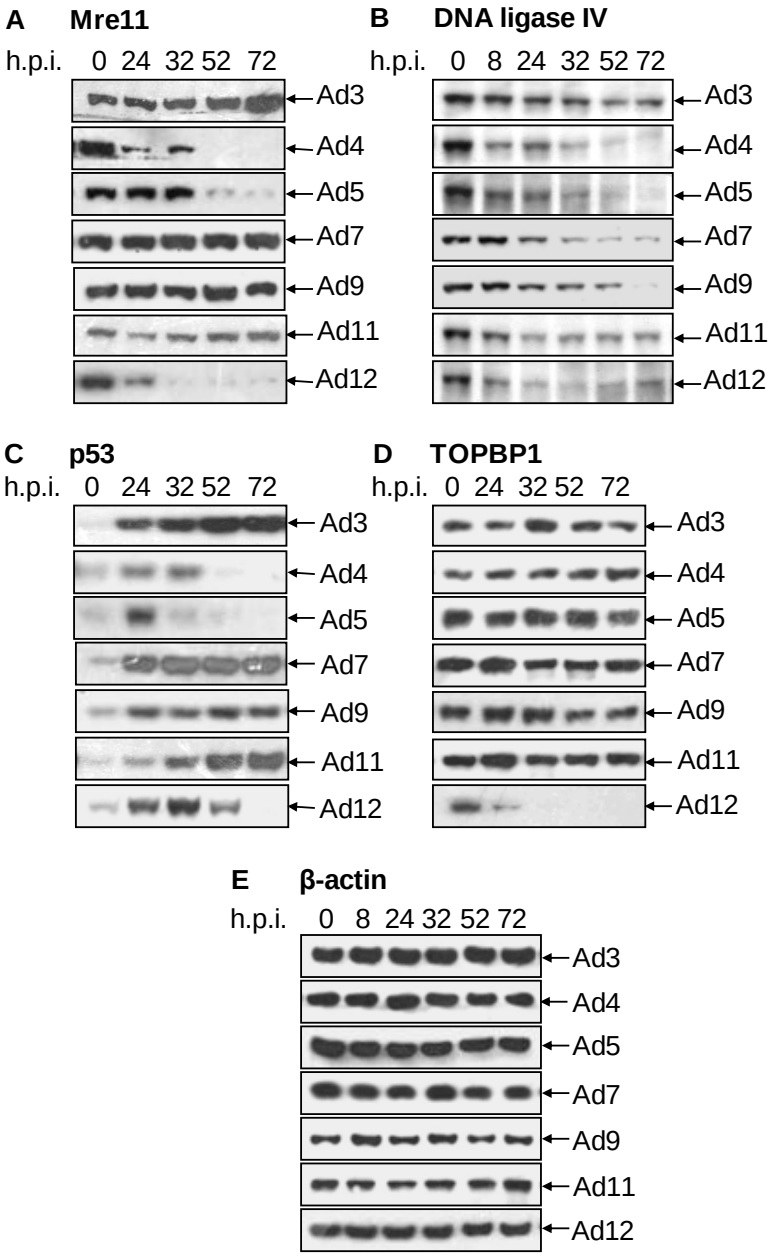


Fig 3.2A-E. Expression of Mre11, DNA ligase IV, p53 and TOPBP1 following Ad infection

HeLa cells were infected with Ad3, Ad4, Ad5, Ad7, Ad9, Ad11 and Ad12 serotypes. Cells were harvested at the indicated times post-infection and examined by Western blotting for Mre11 (A), DNA ligase IV (B), p53 (C), TOPBP1 (D) and β-actin as a loading control (E).

Levels of TOPBP1 protein expression were also investigated during infection with the panel of serotypes. While TOPBP1 was found to be rapidly degraded during Ad12 infection, levels remained stable during infection with all other serotypes (Fig 3.2D). Western blotting with an antibody against β-actin has been included as a loading control (Fig 3.2E), and all data presented here are representatives of at least 3 separate experiments.

It may be hypothesized that variations in the rate of protein depletion may be due to differences in the rate of expression of viral early region proteins. Despite this, all viruses examined had the potential to abrogate the DDR by depletion of one or more key components of the pathway.

3.3.3 Overexpressed p53 during Ad infection is transcriptionally inactive

p53 regulation during Ad infection has been studied in detail and it has been established, at least for Ad5 and Ad12, that during early times of infection E1A acts to stabilize p53 protein expression (Debbas and White, 1993, Lowe and Ruley, 1993). Subsequently, in order to counteract these apoptotic events large E1B proteins act to facilitate p53 inactivation by relocalization and degradation (Berk *et al.*, 2005). Data presented here show Ad4, Ad5 and Ad12 infections that are consistent with this model of Ad-mediated regulation of p53 (Fig 3.2C). Interestingly however, during Ad3, Ad7, Ad9 and Ad11 infection, p53 protein expression was rapidly induced and stabilized up to 72 hours post-infection (Fig 3.2C).

In order to ascertain whether this overexpressed p53 is transcriptionally active, three approaches were adopted. Firstly, Western blotting was performed with the same lysates used in Fig 3.2C to probe for the p53-regulated, and p53-directed, E3 ubiquitin ligase MDM2 (Wu *et al.*, 1993, Fang *et al.*, 2000, Rodriguez *et* al., 2000). Interestingly, infection with all

serotypes resulted in the rapid loss of MDM2 protein expression (Fig 3.3), suggesting that the high levels of p53 protein expressed during Ad3, Ad7, Ad9 and Ad11 infection are transcriptionally inactive.

Secondly, a direct measurement of p53 transcriptional activity was undertaken using a luciferase reporter assay. H1299 cells were cotransfected with a luciferase reporter downstream of multiple copies of a p53 consensus DNA-binding site, and a plasmid expressing p53. After 24 hours cells were mock-treated or infected with Ad3 or Ad7 and harvested after a further 24 hours. Levels of protein expression were detected by Western blotting (Fig 3.4A), and relative luciferase activity was measured using a luminometer (Fig 3.4B). Data indicated that while p53 protein levels were elevated significantly during Ad3 and Ad7 infection, its relative luciferase activity only marginally increased compared to the mock sample, suggesting that the p53 is transcriptionally inactive.

The transcriptional activity of p53 was thirdly assessed by measuring the levels of the p53-responsive gene p21, at the mRNA and protein level following infection. HeLa and A549 cells were infected with Ad3 and Ad7, respectively, and samples were either subjected to Western blotting or semiquantitative RT-PCR. Once again p53 protein levels increased during infection, but despite this p21 protein levels decreased (Fig 3.5A). As a positive control A549 cells were treated with 3 Gy of IR and harvested after 2 hours, and as expected this sample showed a marked increase in both p53 and p21 protein levels. To measure p21 mRNA levels, RNA was extracted from infected cell lysates and converted into cDNA before semiquantitative RT-PCR was performed. These data confirmed that reduced levels of p21 protein expression was due to reduced p21 mRNA; and likewise for the IR-treated A549 cells an increase in p21 mRNA was observed (Fig 3.5B). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as a loading control for this experiment.

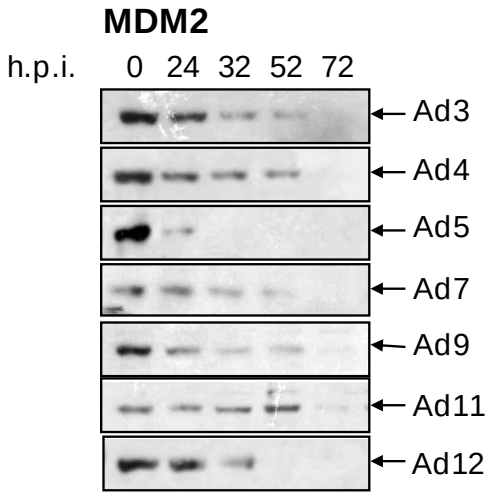


Fig 3.3. Expression of the p53 regulated protein MDM2 following adenoviral infection

HeLa cells were infected with Ad3, Ad4, Ad5, Ad7, Ad9, Ad11 and Ad12 serotypes. Cells were harvested at the indicated times post-infection and examined by Western blotting for MDM2.

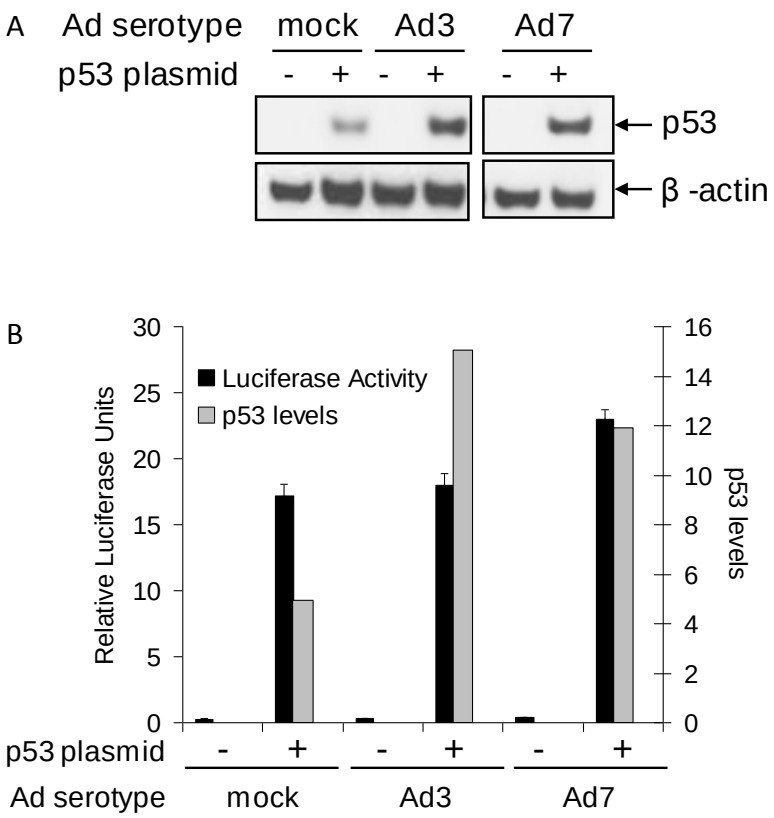


Fig 3.4A and B. Transcriptional activity and protein expression levels of p53 following Ad3 and Ad7 infection

H1299 cells were co-transfected with a luciferase reporter construct and cDNA expressing p53 before being mock-treated or infected with Ad3 or Ad7. Cells were then harvested 24 hours post-infection and Western blotted for p53 expression along with β-actin as a loading control (A). Relative p53 protein levels are shown in grey columns and relative luciferase activity, as measured by luminescence, is shown in black columns (B).

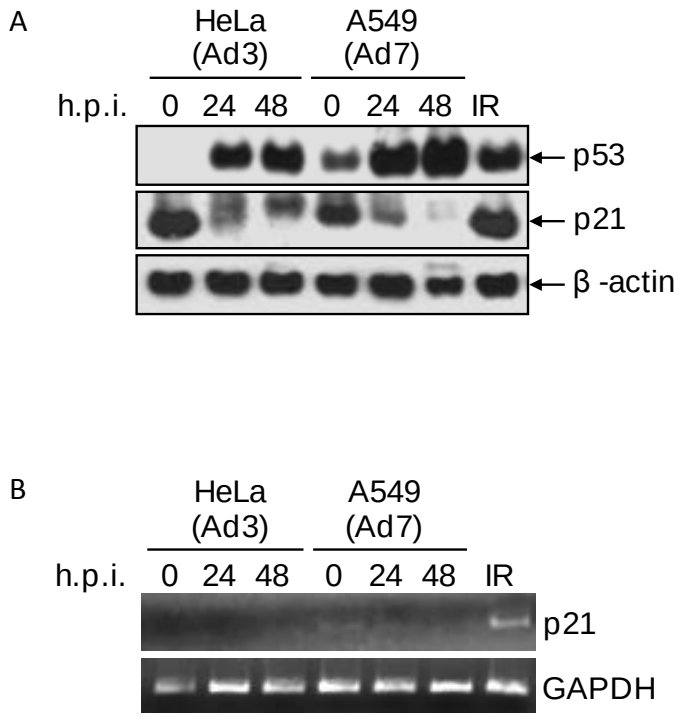


Fig 3.5A and B. Transcriptional activity of p53 directed towards p21 following Ad3 and Ad7 infection

HeLa and A549 cells were infected with Ad3 and Ad7 as indicated and harvested at the indicated times. Cells were examined by Western blotting for p53, p21 and β-actin as a loading control (A). Samples were also subjected to semiquantitative RT-PCR for p21 along with GAPDH as a loading control (B). Right-hand lanes labelled "IR" indicate A549 cells that have been treated with ionizing radiation (3 Gy) and harvested after 2 hours to act as a positive control.

As for those serotypes that caused a reduction in p53 protein levels after infection (Ad4, Ad5 and Ad12), one can reasonably assume that this would automatically prevent it from activating the transcription of target genes. Taken together these experiments suggest that while p53 protein levels largely increase following infection with some serotypes, this overexpressed product is not transcriptionally active (at least towards MDM2 and p21).

3.3.4 Ad serotypes differentially relocalize p53, Mre11 and TOPBP1

Following Ad infection, a number of cellular proteins are sequestered and relocalized in order to negate the host cell's DDR. This deactivation is advantageous for the virus and necessary for viral replication, and perhaps evasion of the cellular immune response.

Significantly, Ad5E1B55K exhibits several distinct staining patterns during infection by localizing to sites of viral replication and transcription, cytoplasmic aggresomes and nuclear track-like structures (Zantema *et al*., 1985, Dosch *et al*., 2001). Since these early reports other proteins have also been detected with similar staining patterns; for example the ssDNA-binding protein replication protein 32 (RPA32) is now commonly used as marker for VRCs when viral antibodies are unavailable, and for the same purpose PML is often used to stain for nuclear tracks.

Immunofluorescence and confocal microscopy was used to investigate the capabilities of the less studied serotypes to relocalize cellular proteins. Briefly, HeLa cells were grown on glass slides before being infected and harvested after 24 and 48 hours by treatment with a pre-extraction buffer and fixation in 4% PFA (see Chapter 2 for more details). Fixed cells were then stained with antibodies against Mre11, p53 and TOPBP1, as well as antibodies against RPA32 and PML to act as markers for the virally-induced structures. Cells were then finally

mounted in a DAPI-containing medium to stain the DNA and visualised by confocal microscopy.

Consistent with previous studies, Mre11 was relocalized into PML-containing nuclear tracks during Ad5 infection, and RPA32-positive VRCs during Ad12 infection (Fig 3.6A and B). However, contrary to a previous study, Ad4 appeared to promote Mre11 accumulation at nuclear tracks and not at VRCs (Fig 3.6C). Infection with Ad3, Ad7 and Ad11, all of which were unable to induce Mre11 degradation, were also unable to relocalize the protein away from VRCs, such that during infection Mre11 colocalized with RPA32 (Fig 3.6B). Interestingly, Ad9, which was also unable to degrade Mre11, still had the capacity to relocalize the protein into nuclear tracks (Fig 3.6A).

Consistent with previous findings, Ad5 and Ad12 infection promoted the accumulation of p53 into nuclear tracks (Fig 3.7B). In this regard, Ad4 infection also promoted p53 relocalization to nuclear tracks prior to degradation (Fig 3.7B). While Western blot analysis revealed that Ad3, Ad7 and Ad11 infection enhanced p53 protein expression to above basal levels (Fig 3.2C), confocal microscopy indicated that p53 is relocalized to VRCs during these infections (Fig 3.7A). Ad9 infection also showed moderate stabilization of p53 protein expression by Western blotting (Fig 3.2C), however, in contrast to Ad3, Ad7 and Ad11, this serotype was capable of relocalizing p53 into nuclear tracks (Fig 3.7B).

Since protein levels of TOPBP1 were solely affected by Ad12 infection (Fig 3.2D), its localization during infection with the other serotypes was also studied. During Ad3, Ad4, Ad5, Ad7, Ad9 and Ad11 infection TOPBP1 was found relocalized from a pan-nuclear state into RPA32-positive VRCs (Fig 3.8). This is the same pattern seen during early times of Ad12 infection before the protein is targeted for ubiquitin-mediated proteolysis.

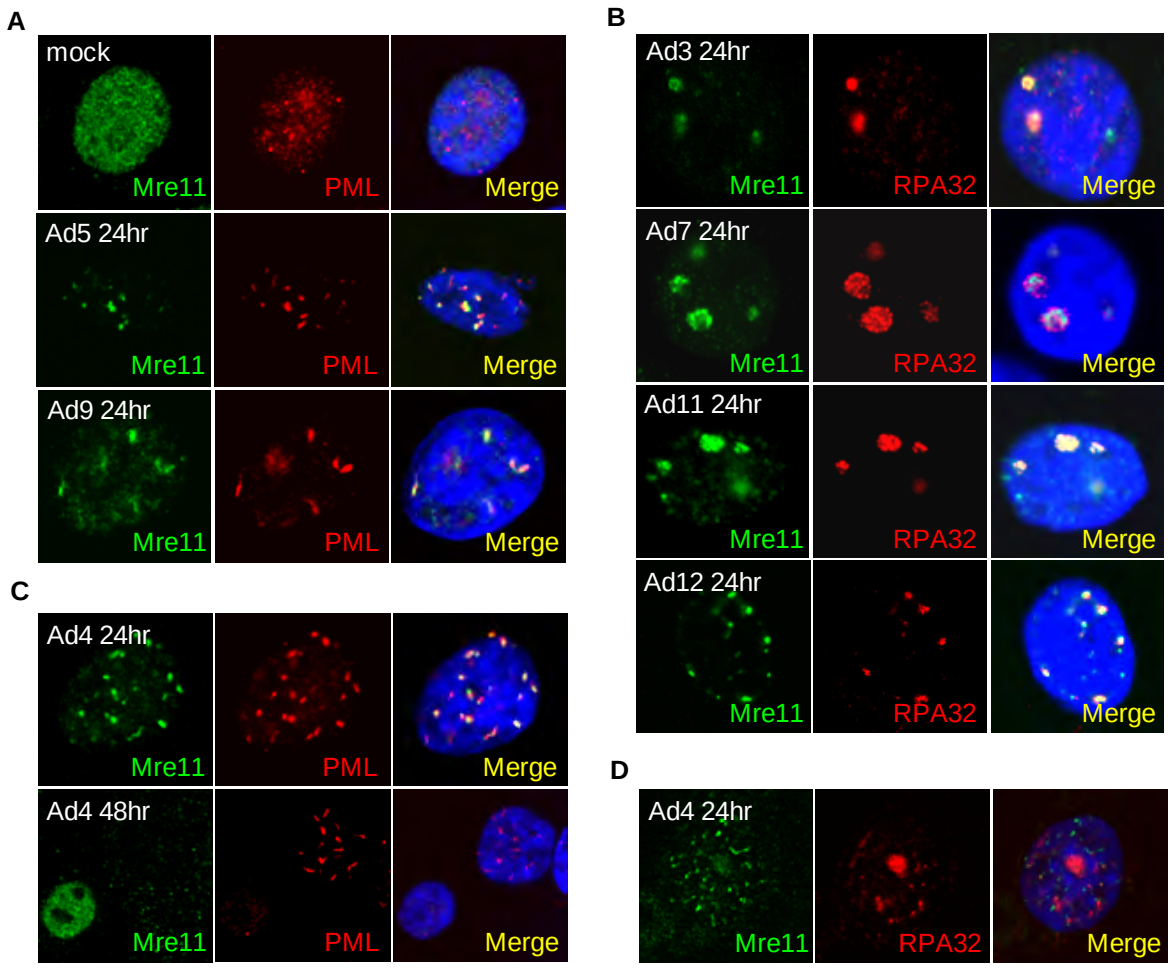


Fig 3.6A-D. Localization of Mre11 following Ad infection

HeLa cells were mock-treated or infected with Ad5 and Ad9 (A), Ad3, Ad7, Ad11 and Ad12 (B), and Ad4 (C and D) for the times shown. Cells were then treated with a pre-extraction buffer and fixed in 4% paraformaldehyde before being stained for Mre11 (green), PML (red), RPA32 (red), and DAPI (blue) and visualised by confocal microscopy. Colocalization of proteins is evident in the right-hand merge column (yellow).

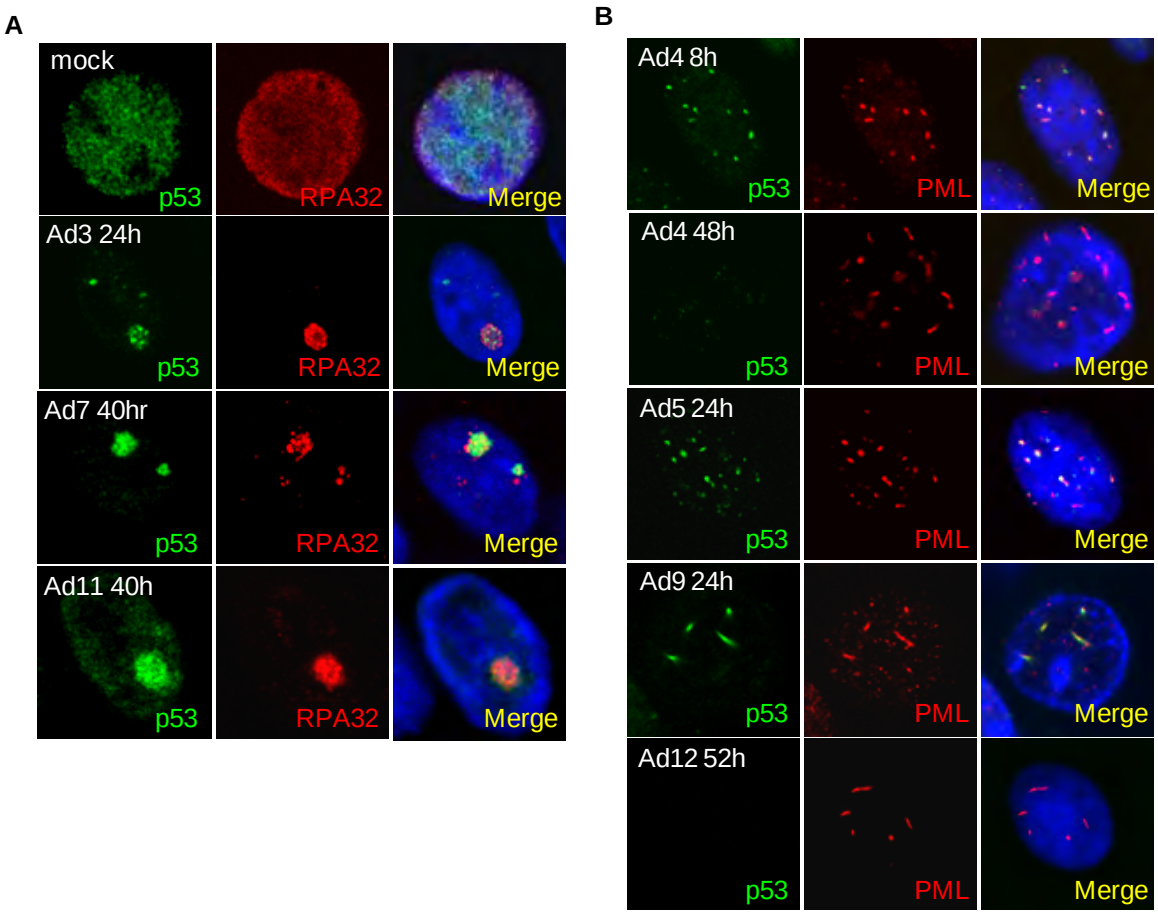


Fig 3.7A and B. Localization of p53 following Ad infection

HeLa cells were mock-treated or infected with Ad3, Ad7 and Ad11 (A), and Ad4, Ad5, Ad9 and Ad12 (B) for the times shown. Cells were then treated with a pre-extraction buffer and fixed in 4% paraformaldehyde before being stained for p53 (green), PML (red), RPA32 (red), and DAPI (blue) and visualised by confocal microscopy. Colocalization of proteins is evident in the right-hand merge column (yellow).

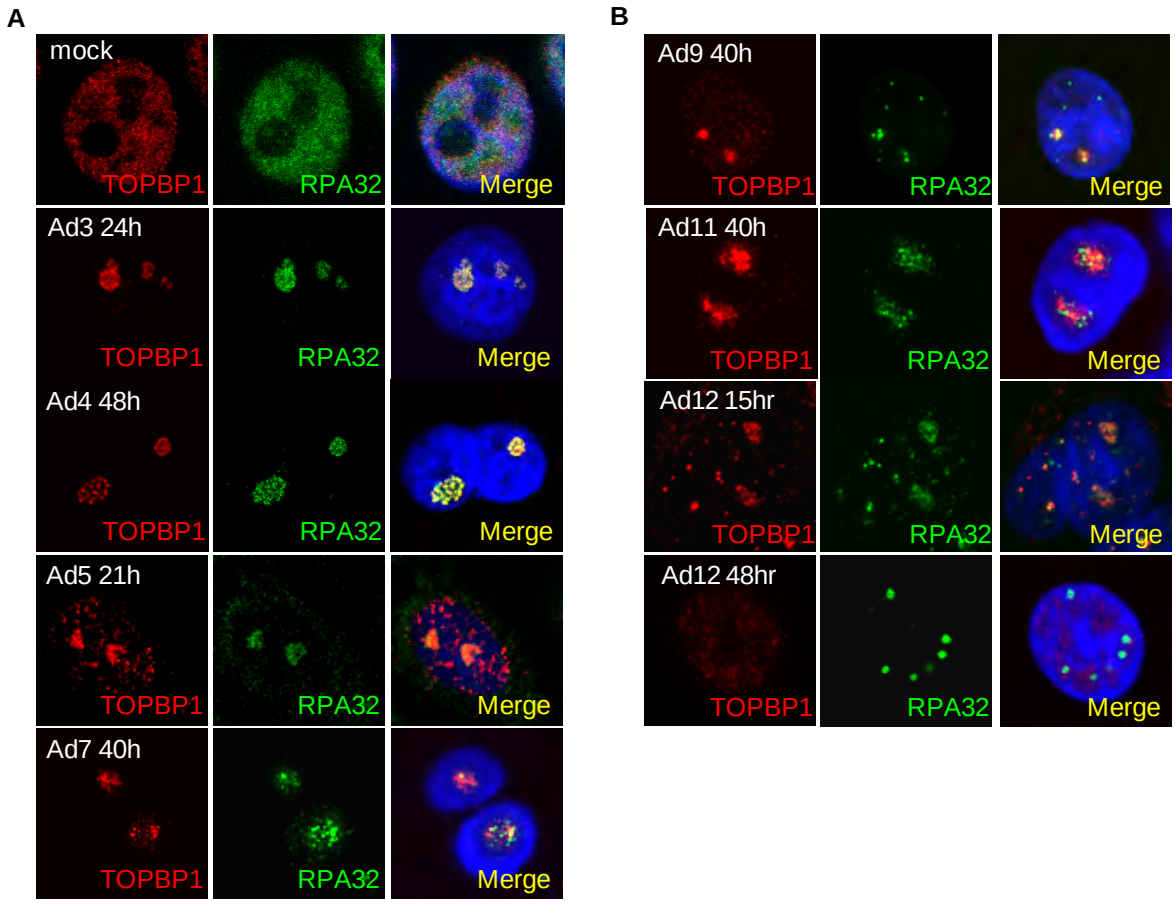


Fig 3.8A and B. Localization of TOPBP1 following Ad infection

HeLa cells were mock-treated or infected with Ad3, Ad4, Ad5 and Ad7 (A), and Ad9, Ad11 and Ad12 (B) for the times shown. Cells were then treated with a pre-extraction buffer and fixed in 4% paraformaldehyde before being stained for TOPBP1 (red), PML (green), RPA32 (green), and DAPI (blue) and visualised by confocal microscopy. Colocalization of proteins is evident in the right-hand merge column (yellow).

Table 3.1. Summary of protein degradation and localization following infection with Ad3, Ad4, Ad5, Ad7, Ad9, Ad11 and Ad12.

Species	Ad serotype	Protein	Upreg.	Transient upreg.	Stable	Degraded	Tracks	VRCs
A	12	p53		✓		✓		
		Mre11				✓		✓
		TOPBP1				✓		✓
		DNA lig IV				✓		
B1	3	p53	✓					✓
		Mre11			✓			✓
		TOPBP1			✓			✓
		DNA lig IV				✓		
B1	7	p53	✓					✓
		Mre11			✓			✓
		TOPBP1			✓			✓
		DNA lig IV				✓		
B2	11	p53	✓					✓
		Mre11			✓			✓
		TOPBP1			✓			✓
		DNA lig IV				✓		
C	5	p53		✓		✓	✓	
		Mre11				✓	✓	
		TOPBP1			✓			✓
		DNA lig IV				✓		
D	9	p53	✓				✓	
		Mre11			✓		✓	
		TOPBP1			✓			✓
		DNA lig IV				✓		
E	4	p53		✓		✓	✓	
		Mre11				✓	✓	
		TOPBP1			✓			✓
		DNA lig IV				✓		

[#]Upreg, upregulated expression; VRCs, VRCs

The localization of endogenous DNA ligase IV was not determined in this study due to the lack of suitable antibodies to detect the protein by immunofluorescence. The data collected from Figures 3.2 and 3.6-3.8 are summarized in Table 3.1.

3.3.5 DDR proteins are differentially phosphorylated during Ad infection

Ad5 and Ad12 infections neutralize host cell DDR pathways to prevent viral DNA concatemer formation, although they have evolved different mechanisms for this evasion (Carson *et al*., 2003). During Ad5 infection, the relocalization and subsequent degradation of Mre11 can negate the DDR, whilst it has been shown that during Ad12 infection TOPBP1 is degraded to inhibit ATR activation (Blackford *et al*., 2010). To date, no evidence of ATM or ATR activation has been observed following *wt* Ad infection, but since Ad5 and Ad12 adopt different mechanisms the ability of the other serotypes to regulate DDR pathways was explored. Thus, Western blots were performed with the same HeLa lysates used in Fig 3.2 to probe for ATR activation via Chk1 serine 345 phosphorylation, and ATM activation via TIF1β serine 824 phosphorylation.

No Chk1 phosphorylation was evident during Ad5, Ad9 and Ad12 infection, which is likely to reflect their ability to degrade Mre11, relocalize Mre11 or degrade TOPBP1, respectively (Fig 3.9). Interestingly, although Ad4 infection promotes Mre11 degradation (Fig 3.2A), it is unable to inhibit Chk1 phosphorylation, and Ad3, Ad7 and Ad11 infections similarly induced the phosphorylation of Chk1 (Fig 3.9). HeLa cells treated for 2 hours with 30 μM of HU were used as a positive control for Chk1 phosphorylation.

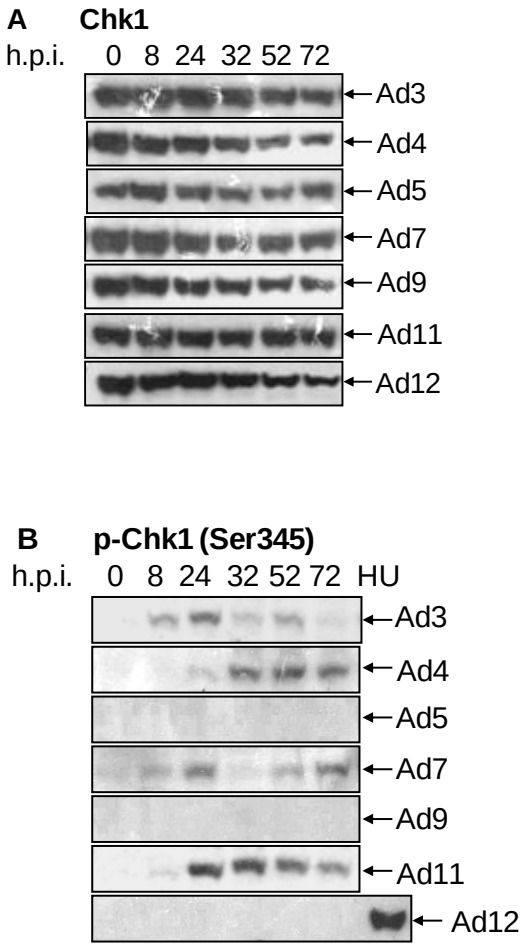


Fig 3.9A and B. Phosphorylation of Chk1 following Ad infection

HeLa cells were infected with Ad3, Ad4, Ad5, Ad7, Ad9, Ad11 and Ad12 serotypes. Cells were harvested at the indicated times points post-infection and examined by Western blotting for Chk1 (A) and phospho-Chk1 (Ser345; B). The final track labelled as HU in the Ad12-infected cells Western blotted for phospho-Chk1 blots shows HeLa cells treated with HU as a positive control.

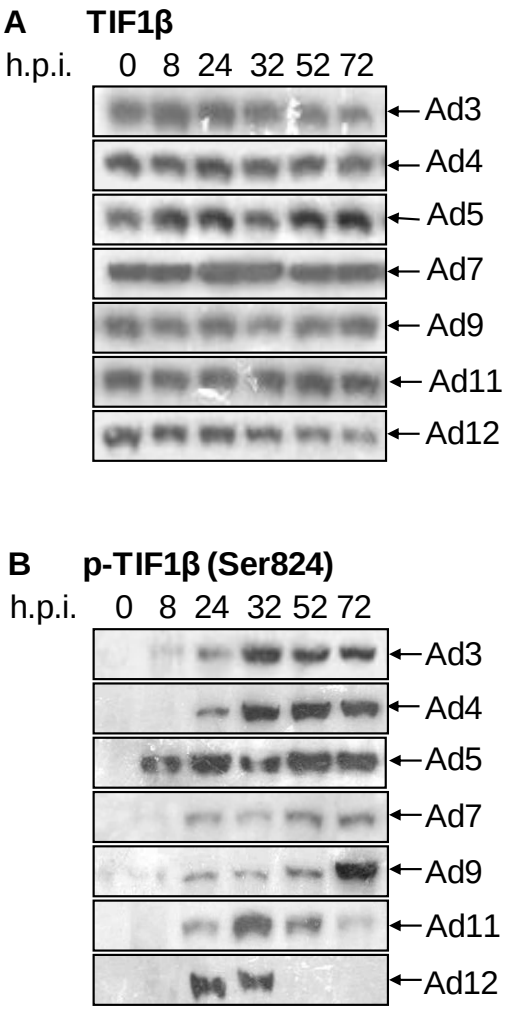


Fig 3.10A and B. Phosphorylation of TIF1β following Ad infection

HeLa cells were infected with Ad3, Ad4, Ad5, Ad7, Ad9, Ad11 and Ad12 serotypes. Cells were harvested at the indicated time point post-infection and examined by Western blotting for TIF1β (A) and phospho-TIF1β (Ser824; B).

TIF1β is phosphorylated at serine 824 by ATM in response to genotoxic stress (White *et al*., 2006, Li *et al*., 2007). Figure 3.10 shows that 24 hours post-infection, all serotypes examined here also induce TIF1β phosphorylation and hence activation of ATM; although the kinetics varied appreciably (Fig 3.10). For Ad3, Ad4, Ad7 and Ad9 serotypes, TIF1β phosphorylation was evident 24 hours post-infection and increased up to 72 hours. Ad5-induced TIF1β phosphorylation was more intense and detected from early to late infection time points. The kinetics of TIF1β phosphorylation during Ad11 and Ad12 infection was transient, with phosphorylation visible at 24 hours but not 72 and 52 hours post-infection, respectively.

In summary, these data indicate that despite targeting different cellular proteins for degradation all serotypes studied here were capable of activating the cellular DDR to some extent, although the presence of VRCs and production of late structural proteins suggest that this aspect of activation is not deleterious to the viral life cycle.

3.3.6 Activation of ATM and ATR signalling pathways during Ad infection does not lead to viral concatemer formation

Early studies showed that a mutant Ad5 virus lacking the E4 region (ΔE4) was defective for DNA replication and mRNA translation (Halbert *et al*., 1985), a failing which was later attributed to the formation of large DNA concatemers by the covalent linkage of viral genomes (Weiden and Ginsberg, 1994). This E4 region is partly responsible for targeting the early DSB sensor protein Mre11 for proteasomal degradation. Therefore during Ad5ΔE4 infection, concatemers are formed due to the host cell's ability to recognise and process linear viral genome ends as DSBs. Since many of the serotypes studied here were unable to deplete or relocalize components of the DDR (specifically Mre11), or inhibit ATM and ATR kinase

activity, the possibility that these infections could lead to viral DNA concatemerization was investigated. HeLa cells were thus infected with the *wt* viruses Ad3, Ad7, Ad9, Ad11 and Ad12, and the Ad5 mutant viruses *dl*355 and H5*pm*4155 which lack the E4ORF6 and E4ORF3/E4ORF6 regions, respectively. Forty-eight hours post-infection, cells were harvested and the DNA was analysed by PFGE. As expected infection with the H5*pm*4155 mutant virus resulted in the formation of multimeric viral DNA of varying sizes (Fig 3.11). Infection with the *dl*355 mutant also resulted in viral genome concatemerization, but to a lesser extent than H5*pm*4155 (Fig 3.11). Infection with Ad3, Ad7 and Ad11 revealed only ~35 Kb monomeric viral DNA, suggesting that although certain DDR pathways were activated by these viruses, concatemerization was avoided.

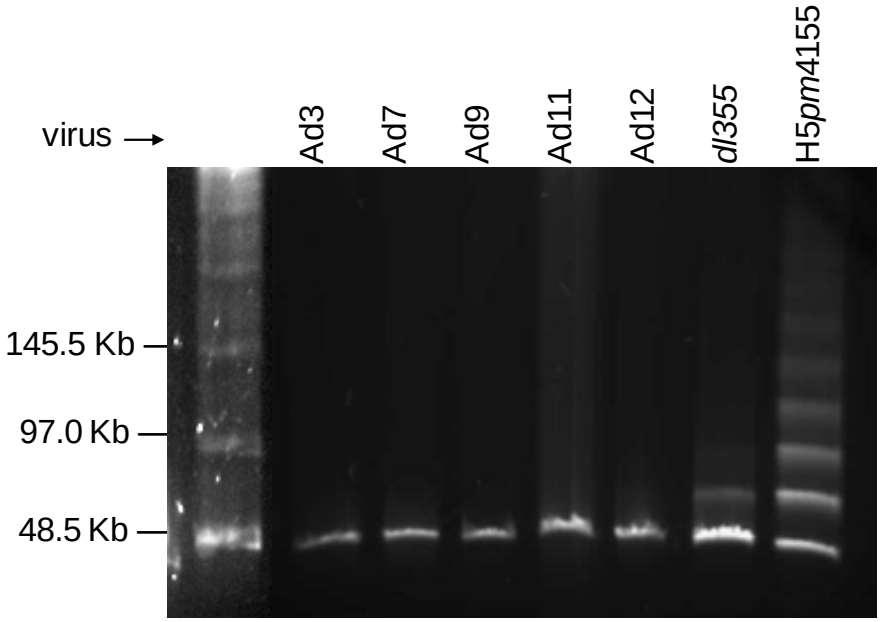


Fig 3.11. Absence of DNA concatemer formation in *wt* adenoviral infections

HeLa cells were infected with *wt* Ad3, Ad7, Ad9, Ad11 and Ad12, as well as mutant viruses *dl*355 (Ad5ΔE1B55K) and H5*pm*4155 (Ad5ΔE4ORF3/E4ORF6). Cells were harvested 24 hours post-infection, set into agarose plugs, digested with proteinase K and subsequently subjected to PFGE. DNA was visualised through ethidium bromide staining.

3.4 DISCUSSION

Previous reports have determined that adenoviruses neutralize the host cell DDR by targeting various proteins for 26S proteasome-dependent degradation, and that viruses can differentially regulate these pathways (Stracker *et al*., 2005, Blackford *et al*., 2010). Results shown here have expanded these findings and examined the effect previously uncharacterized serotypes have on proteins involved in the DDR. All serotypes studied had some effect on the DDR, and although Ad5 and Ad12, as previously published, share broadly similar tactics more diversity has been revealed amongst other serotypes.

Of notable difference was the effect serotypes had on Mre11 and p53. Ad4, Ad5 and Ad12 infection promoted the degradation of both of these proteins as has been previously described in a proteasome-dependent manner (Querido *et* al., 2001; Stracker *et al*., 2005), while during Ad3, Ad7, Ad9 and Ad11 infection p53 protein levels were upregulated and Mre11 protein levels were unaffected (Fig 3.2). The localization of Mre11 also varied; during Ad3, Ad7, Ad11 and Ad12 infection it was found at sites of viral replication, while during Ad4, Ad5 and Ad9 infection it was found colocalizing with PML in nuclear tracks. The Ad4-mediated relocalization of Mre11 reported here (Fig 3.6C) is in contrast to a previous study which reported that another member of the MRN complex, Nbs1, was observed at RPA32-positive VRCs (Stracker *et al*., 2005). This discrepancy may reflect the use of different cells lines or multiplicities of infection, or be because the previous study used Nbs1 as a marker for the MRN complex rather than Mre11. Since the dynamics of this complex during Ad infection is unknown, MRN components may dissociate into discrete cellular compartments. It should also be noted that microscopy images from the previous publication portray only a partial colocalization with RPA32, and the extra diffuse staining rather resembles nuclear tracks. Ad5 infection has been shown to disrupt ATR signalling via relocalization of the MRN

complex into nuclear tracks (Carson *et al*., 2009). The differing result seen here with Ad4 demonstrates that even though Mre11 is relocalized before degradation, the ATR substrate Chk1 is still phosphorylated, suggesting that the MRN complex may exert some effect on ATR activation, or that pathways independent of MRN function promote Chk1 activation following infection.

The study by Carson *et al*. also demonstrated that Mre11 relocalization requires the Ad5E4ORF3 isoleucine residue at position 104, which is specific to species C adenoviruses (Carson *et al*., 2009). The authors found that mutating this residue to arginine (I104R), which is the residue present in all other groups, prevented the ability of Ad5E4ORF3 to relocalize Mre11, but not PML, into nuclear tracks. Interestingly, the current study found that Ad4 (species E) and Ad9 (species D) which both have an arginine at position 104 in E4ORF3, still relocalized Mre11 into nuclear tracks during infection; although Ad9 was unable to induce its degradation. This suggests that E4ORF3 proteins may have evolved alternative methods, and have different requirements, for relocalizing cellular proteins during infection. It is also reasonable to suggest that the Ad9-mediated relocalization of Mre11 into nuclear tracks may inactivate the protein without the necessity of degradation, which would account for the lack of Chk1 phosphorylation observed during Ad9 infection.

Ad3, Ad7, Ad9 and Ad11 infections induced a marked accumulation of the p53 gene product, which was subsequently deemed to be transcriptionally inactive by luciferase assay and semiquantitative RT-PCR analysis of RNA (Fig 3.2-3.5). This is a novel observation for a protein involved in the DDR, and may be attributed to the actions of E1A which is known to induce p53 protein expression during early times of infection via $p19^{ARF}$ activity. The results presented here correlate well with another study where a single amino acid Ad5E1B55K mutant (R250A) bound poorly to p53 in comparison to *wt* Ad5, and was unable to induce p53

degradation (Shen *et al*., 2001). Indeed, this poorly conserved arginine residue is not present in Ad3, Ad7, Ad9 and Ad11 E1B55K proteins which might explain their inability to degrade p53 (Blackford *et al*., 2010). It has also been suggested that enhanced p53 expression does not provoke the assumed pro-apoptotic effect viruses aim to avoid, but instead can be advantageous for the adenoviral lytic cycle by cooperating with Ad5E1A to enhance transcription from the MLP (Royds *et al*., 2006). Moreover, the localization of p53 to VRCs during infection with Ad3, Ad7 and Ad11 infection is likely to reflect lack of the key isoleucine residue in their E4ORF3 proteins as mentioned above.

TOPBP1 has recently been identified as a novel Ad12 substrate, resulting in the inactivation of the ATR pathway. It is targeted for proteasomal degradation through the action of Ad12E4ORF6 alone, without the necessity for Ad12E1B54K (Blackford *et al*., 2010). In the current study only Ad12 infection was able to induce TOPBP1 degradation, while during Ad3, Ad4, Ad5, Ad7, Ad9 and Ad11 infection TOPBP1 levels remained stable. Infection with all serotypes studied led to the accumulation of TOPBP1 at RPA32-positive VRCs. The function of TOPBP1 at these structures remains unknown, however in the case of Ad12 its initial localization to these sites is only evident 24 to 48 hours post-infection before it is targeted for degradation. Presumably, accumulation of TOPBP1 at VRCs by viruses other than Ad12 is sufficient to negate its function.

DNA ligase IV protein levels were reduced during infection with all serotypes studied. NHEJ is thought to promote the illegitimate repair of viral genomes, and since DNA ligase IV, when complexed with XRCC4, catalyzes the final step of this ligation it could inhibit viral replication if not inactivated. Indeed, when human fibroblasts expressing an inactive DNA ligase IV mutant (180BR) were infected with the Ad5 mutant virus *dl*1004, the concatemerization phenotype was rescued (Stracker *et al*., 2002). Despite being targeted by

all viruses, it has previously been shown that DNA ligase IV degradation is not absolutely necessary to inhibit NHEJ as Ad5E1B55K-independent Ad5E4ORF6-dependent dissociation of DNA ligase IV from XRCC4 (which is not degraded) inhibits the function of this complex (Jayaram *et al*., 2008).

MDM2 protein levels were used in this study as an indicator of p53 activity, and helped demonstrate that p53 accumulated during Ad3, Ad7, Ad9 and Ad11 infection was not transcriptionally active. As mentioned, MDM2 and p53 form a negative feedback loop to regulate their expression, however, MDM2 is also able to autoubiquitylate itself leading to its own proteolysis; a scenario induced by DNA damage and which causes p53 stabilization (Fang *et al*., 2000). The balance between MDM2 autoubiquitylation and substrate ubiquitylation is dependent on its association with Daxx and the deubiquitylating enzyme HAUSP, such that MDM2 association with the adaptor protein Daxx induces HAUSP to direct MDM2 ligase activity away from autoubiquitylation and towards p53 ubiquitylation (Li *et al*., 2004, Ronai, 2006). Ad5 infection has recently been shown to induce Ad5E1B55K-dependent Daxx degradation, making it possible that the depletion of MDM2 protein levels seen here is facilitated by dissociation from the Daxx/HAUSP complex (Schreiner *et al*., 2010). Although no changes in HAUSP protein levels were detected during Ad5 and Ad12 infection, Daxx was depleted during Ad4, Ad5 and Ad12 infection (data not shown). Interestingly these were the only serotypes capable of degrading p53, which suggests that during Ad3, Ad7, Ad9 and Ad11 infection a different series of events is likely to account for p53 inactivation, such as inappropriate post-translation modifications. Furthermore, recent evident suggests that DNA damage induces the casein kinase I-dependent (CKI) phosphorylation of MDM2, which targets the protein for destruction by the $SCF^{\beta\text{-}TRCP}$ E3

ligase complex (Inuzuka *et al*., 2010). Thus, more research is needed to determine how MDM2 is regulated during Ad infection, and what relationship this has to p53 regulation.

Data presented here suggest that infection with all serotypes studied lead to some form of DDR activation. Indeed, all serotypes activated ATM kinase activity as demonstrated by TIF1β phosphorylation (Fig 3.10), regardless of Mre11 regulation, which suggests that viral replication is not compromised by some level of DDR pathway activation. This is in contrast to a previous study suggesting that Mre11 degradation during Ad infection inhibits ATM kinase activity towards 53BP1 and Chk2, although low levels of phosphorylation were observed (Carson *et al*., 2003). Furthermore, recent studies have suggested that widespread phosphorylation of H2AX during Ad5 and Ad12 infection, which occurs after Mre11 degradation, is dependent on ATM kinase activity (Blackford *et al*., 2008; Nichols *et al*., 2009). Although the promiscuity of TIF1β as a substrate for phosphorylation has been scrutinized, the latest publications suggest that this modification is ATM-specific (Goodarzi *et al*., 2009); however this awaits confirmation by other laboratories. To determine if ATM is responsible for the TIF1β phosphorylation observed here future experiments could be carried out in ATM shRNA cells.

ATR activation was somewhat varied between serotypes. Infection with Ad3, Ad4, Ad7 and Ad11 initiated ATR kinase activity towards Chk1 (Fig 3.9), which mostly correlates with their inability to degrade Mre11 (Fig 3.2A) or mislocalize it into nuclear tracks (Fig 3.6). Ad4 infection however promoted Mre11 degradation (Fig3.2A) and recruitment to nuclear tracks (Fig 3.6), suggesting that Mre11 relocalization and degradation is not sufficient to ablate Chk1 phosphorylation, and indicates that another protein may activate ATR. In contrast, Ad9 which can relocalize but not degrade Mre11, does inhibit Chk1 phosphorylation suggesting that different serotypes have evolved different mechanisms to activate or inhibit DDR

pathways. Despite these wide variations in kinase activation, the genomes of all the serotypes studied here were present as momomers with no trace of concatemer formation suggesting that the presence of Mre11 and p53 during infection may not be detrimental to the viral lifecycle. It is assumed however, that Mre11 and p53 are functionally inactivated in these cells; indeed this was determined for p53 at least (Fig 3.3-3.5). Given these data, it is possible that the degradation of DNA ligase IV may be sufficient to obstruct the DDR during Ad3, Ad7 and Ad11 infection, or that these viruses have evolved novel mechanisms to prevent viral genome concatemerization.

It was also observed through PFGE that the *dl*355 mutant lacking the E4ORF6 region formed DNA concatemers, although not to the extent of H5*pm*4155 (ΔE4ORF3/ORF6). This may be attributable to the action of E4ORF3, which is sufficient to relocalize Mre11 into nuclear tracks and subsequently E1B55K-postive cytoplasmic aggresomes (Liu *et al*., 2005). Also as E4ORF3 and E4ORF6 encode redundant gene products, either protein is sufficient for DNA replication and lytic viral infection (Weiden and Ginsberg, 1994).

Data presented here are consistent, to some extent, with another recent study examining the substrate specificity of a separate panel of adenoviruses (Cheng *et al*., 2011). This independent study cloned and expressed the coding sequences of E1B55K and E4ORF6 from Ad12 (species A), Ad16 (species B1), Ad34 (species B2), Ad5 (species C), Ad9 (species D), Ad4 (species E) and Ad40 (species F). Consistencies between the two studies include the observations that infection with all serotypes initiated the degradation of DNA ligase IV but had varying effects on p53 and Mre11 degradation; traits that were attributed to heterogeneity in ubiquitin ligase complex composition (Cheng *et al*., 2011). There are a few discrepancies between the current study and the study by Cheng *et al*. For instance, Cheng *et al*. indicated that Ad4E1B55K/E4ORF6 expression did not promote the degradation of p53 or Mre11,

whereas data presented here, in agreement with previous reports, revealed that Ad4 infection does promote p53 and Mre11 degradation (Fig 3.2C; Stracker *et al*., 2005). Cheng *et al*. also showed some Mre11 degradation with Ad9E1B55K/E4ORF6, whereas the current study did not observe this (Fig 3.2A). Similarly, their E1B55K/E4ORF6 constructs from species B1 (Ad16) and species B2 (Ad34) viruses also promoted some Mre11 degradation, in contrast to the species B1 (Ad3 and Ad7) and species B2 (Ad11) viruses used here which did not (Fig 3.2A).

Since both studies have utilised only a representative serotype from each Ad group, directly comparing different serotypes from the same species might be inappropriate. Differences may also have arisen from the different techniques utilised; Cheng *et al*. transfected E1B55K and E4ORF6 plasmid DNA into H1299 cells, whereas the current study used HeLa cells in the context of a full infection, and it is possible that other viral gene products may regulate their capacity to target cellular proteins and pathways.

Cheng *et al*. also examined Ad40, one of two serotypes from species F. They found that E1B55K and E4ORF6 proteins from this virus were capable of degrading p53, Mre11 and DNA ligase IV in a manner highly comparable to Ad12. This similarity was also extended by the ability of Ad12 and Ad40 E4ORF6 proteins to form CUL2-based E3 ubiquitin ligase complexes, while all other viruses conveyed CUL5 specificity (Cheng *et al*., 2011). This result might have been anticipated, since sequence homology data suggests that serotypes from species A and F are highly related and the most divergent from those of other species (Bailey and Mautner, 1994). Given this evidence it is possible that species F serotypes, akin to Ad12, have the ability to degrade TOPBP1 in a CUL2-dependent manner, and broader studies will reveal if all members from each species evade the DDR by the same mechanism.

It will be interesting to analyse the ability of the serotypes used in this study to degrade integrin alpha 3 and BLM, two recently identified Ad5E1B55K/E4ORF6 substrates (Dallaire *et al*. 2009b, Orazio *et al*., 2011). During the early stages of Ad5 infection BLM is relocalized into VRCs (a novel observation for an Ad5 substrate), and its degradation is an independent event from Mre11 degradation (Orazio *et al*., 2011). As there is no obvious homology among E1B55K/E4ORF6 substrates, it is also possible that different regions of these viral proteins mediate interactions with distinct substrates. In view of the ever emerging variations regarding Ad substrate specificities and their abilities to evade the host cells defence mechanisms, some of which are presented here, there are likely to be many as yet unidentified cellular targets for adenoviruses.

It cannot be discounted that variations seen amongst the serotypes used in this study arise from differing rates of infections and expression of viral proteins. Although visualisation of late hexon proteins has provided some validation, further authentication could be obtained by analysing the cell cycle distribution of infected cells by FACS analysis (as cells arrest in S-phase in response to E1A expression; Grand *et al*., 1998), or by using RQ-PCR to measure viral genome accumulation. It should also be noted that although Ad3, Ad7 and Ad11 infections failed to relocalize any of the DDR proteins studied here into nuclear tracks, these track structures were still established within the cell as observed by PML staining (data not shown).

In conclusion, there was no observed correlation between the degradation and localization of cellular proteins following infection with a broad spectrum of Ad serotypes, raising the likelihood that viruses from specific species have evolved varying strategies to circumvent host cells' defence mechanisms and stimulate viral replication. However, it seems likely that the importance of degrading DNA ligase IV throughout Ad evolution has been maintained.

Please note that the data presented in this Chapter has been published; Forrester *et al*., (2011) J.Virol 85:2201-2211.

CHAPTER 4

IDENTIFICATION OF TIF1γ AS AN ADENOVIRUS E1B55K- AND E4ORF3-INTERACTING PROTEIN

4.1 INTRODUCTION

Ad oncoproteins act rapidly to target and degrade cellular proteins that would otherwise interfere with their own viral lifecycle. For example, proteins involved in DDR pathways such as p53 and Mre11, are bound by Ad E1B55K/E4ORF6 complexes and targeted for proteasomal degradation (Nevels *et al*., 1997, Querido *et al*., 2001, Stracker *et al*., 2002). Previously, novel Ad substrate and co-activator proteins have been identified using human mutated cell lines (ATLD and 180BRM; Stracker *et al*., 2002), by conventional immunoprecipitation and Western blotting techniques (Baker *et al*., 2007, Blackford *et al*., 2010) and through the use of mass spectrometry (Querido *et al*., 2001, Harada *et al*., 2002, Dallaire *et al*., 2009a).

Mass spectrometry is a proteomic technique that can be used to study protein complexes by the analysis of fragmented peptides, following collision-induced dissociation (CID) or electron-transfer dissociation (ETD). CID is useful for the identification of peptides from proteins isolated following immunoprecipitation, but provides limited peptide-sequence information and unstable post-translational modifications such as phosphorylation, glycosylation and acetylation are often cleaved off the backbone during fragmentation (Mikesh *et al*., 2006). In contrast, ETD is capable of providing extensive sequence coverage and unstable modifications such as phosphorylation are preserved on the peptide chain (Sobott *et al*., 2009). Modifications such as phosphorylation play a major role in regulating protein activity and stability, localizing proteins to specific cellular compartments and facilitating interactions with other proteins. Indeed, Ad infection is known to stimulate the phosphorylation of several DDR proteins including RPA32 and H2AX (Blackford *et al*., 2008, Nichols *et al*., 2009). Furthermore, data presented in Chapter 3 show that TIF1β and Chk1 are phosphorylated as a consequence of Ad infection.

4.2 AIMS

The specific aim of this study was to use a proteomic approach to identify novel Ad12E1B54K-binding proteins, and then to:

a) Corroborate any novel findings using immunological techniques.

b) Determine the status of these proteins during infection with regard to their expression and cellular localization.

c) Examine if/how these cellular proteins are regulated by Ad proteins.

d) Investigate possible novel roles for these proteins during Ad infection.

e) Investigate possible novel roles for these proteins in the DDR.

Theoretically, proteins which interact with each other may be involved in the same biological pathways; therefore it was hoped that these experiments would also provide general insight into Ad-mediated regulation of the cellular environment. The results of findings from these studies are presented in this Chapter.

4.3 RESULTS

4.3.1 Mass spectrometric analysis of anti-Ad12E1B54K immunoprecipitates

In order to identify novel Ad12E1B54K-binding proteins, Ad12 E1-transformed HER10 cells were harvested in NETN lysis buffer and an anti-Ad12E1B54K antibody was used to immunoprecipitate Ad12E1B54K, and associated proteins, from the lysate (Merrick *et al*., 1991). These immunoprecipitates were incubated with Protein G-Sepharose beads to form immunocomplexes, and bound proteins were eluted by boiling in SDS sample buffer before being separated by SDS-PAGE. Two negative control samples were also included, one being control mouse IgG with cell lysate and Protein G-Sepharose, and the other being anti-Ad12E1B54K antibody with lysis buffer but without cell lysate. Gels were immersed in a solution of Coomassie Brillaint Blue G-250 protein stain, however, curiously no obvious protein bands were detected, so random gel slices were excised using a clean scalpel blade and digested with modified trypsin which cleaves C-terminal to arginine or lysine residues. The resulting peptides were then separated using an AmaZon ion trap ETD-enabled mass spectrometer, and processed and analysed by the ProteinScape central bioinformatic platform.

A number of peptides were identified in this screen, many of which were specifically detected in test samples and were not found in negative controls (Table 4.1). Table 4.1 is divided into known and unknown Ad12E1B54K-interacting proteins, and the third column refers to the number of peptides identified from each protein, which along with Mascot scores help determine the likelihood that the protein interaction is valid. Thus, if two or more peptides are identified with a Mascot score of 21 or above, it represents a p-value of less than 0.01 per peptide (Perkins *et al*., 1999).

Table 4.1. Summary of Ad12E1B54K-interacting proteins identified by mass spectrometric analysis.

Protein	MW (kDa)	Number of peptides	Sequence coverage
Known adenoviral binding proteins			
HNRNPU1	90	10	10%
BLM	159	6	5.2%
Mre11	81	2	3.3%
Other DNA damage response/repair proteins			
DDB1	127	13	11.9%
BRCA2	384	2	1.0%
UBR5	309	2	1.0%
TIF1 family members			
TIF1γ	122	17	16.3%
TIF1β	89	9	11.1%
TIF1α	117	2	2.8%
Ubiquitin pathway proteins			
USP9/FAM	289	7	3.2%
CUL7	191	4	2.8%
SMURF2	86	1	1.5%

Three previously verified Ad12E1B54K-interacting proteins were identified in this screen supporting the validity of the technique, these were Mre11, HNRNPUL1 (also known as E1B55K-associated protein 5; E1B-AP5) and BLM (Stracker *et al*., 2002, Blackford *et al*., 2008, Orazio *et al*., 2011). Of the numerous peptides identified from proteins previously unknown to interact with Ad12E1B54K, six will be discussed in more detail below.

Three of these proteins, DDB1, BRCA2 and UBR5 (E3 ubiquitin-protein ligase 5) are known components of the DDR. Thirteen peptides from DDB1, which is involved in nucleotide excision repair, were detected in HER10 immunoprecipitates and indeed this protein was subsequently found to bind Ad5E1B55K (data not shown). BRCA2 and UBR5 have roles in HR and DNA damage-induced Chk2 activation, respectively; the ability of these proteins to bind Ad12E1B54K awaits verification.

Interestingly three members from the TIF1 family of transcriptional repressors (TIF1α, TIF1β and TIF1γ) were also detected in immunocomplexes with Ad12E1B54K. Two peptides were identified from TIF1α, 9 peptides were identified from TIF1β and 17 peptides were identified from TIF1γ, all of which had Mascot scores of 142 or above. TIF1 family members are defined by the presence of an N-terminal TRIM domain, a TSS and tandem PHD and BRD domains (Yan *et al*., 2004).

TRIM domain proteins possess E3 ubiquitin ligase activity through their RING finger motif and while TIF1γ serves as an antagonist of TGF-β signalling by ubiquitylation SMAD4, TIF1α has been found to directly ubiquitylate p53 (Dupont *et al*., 2005, Allton *et al*., 2009). Interestingly, TIF1α has also been previously identified as a target for E4ORF3-mediated relocalization during Ad5 infection (Yondola and Hearing, 2007). And TIF1β is an ATM kinase substrate during the DDR which may make it an attractive target for Ad proteins

during infection (White *et al*., 2006, Li *et al*., 2007). More detail on TIF1 function can be found in Chapter 1.

Other potentially novel Ad12E1B54K-interacting proteins that function in ubiquitin pathways were also identified, including USP9 (a DUB also known as FAM) and SMURF2 which both have roles in the TGF-β signalling pathway. CUL7, a cullin family member was also identified, however, the known Ad12E1B54K-binding protein CUL2 was not (Table 4.1)

4.3.2 TIF1γ and TIF1β bind to adenoviral proteins *in vivo*

Proteomic approaches to identify protein-protein interactions are not definitive and further immunological analysis is required to corroborate any novel interactions. To validate TIF1γ association with Ad12E1B54K, and to determine whether it also binds to Ad5E1B55K, co-immunoprecipitation experiments were carried out using lysates from Ad12 E1-transformed HER10 and Ad5 E1-transformed HEK293 cell lines. Briefly, Ad12E1B54K, Ad5E1B55K and TIF1γ were immunoprecipitated from 4 mg of cell lysate, and bound proteins were eluted, resolved by SDS-PAGE and detected by Western blotting using the appropriate antibodies.

In reciprocal co-immunoprecipitation experiments TIF1γ was found to interact with both Ad12E1B54K and Ad5E1B55K (Fig 4.1). To confirm this interaction during viral infection, HeLa cells were mock-treated or infected with Ad12 and harvested after 24 hours. Ad12E1B54K and TIF1γ were then immunoprecipitated from lysates and assayed as described for Fig 4.1. Western blot analysis revealed that TIF1γ interacts with Ad12E1B54K in infected cells (Fig 4.2), and interestingly that TIF1γ protein levels are reduced following Ad infection (Fig 4.2, *cf*. input lanes 11 and 12).

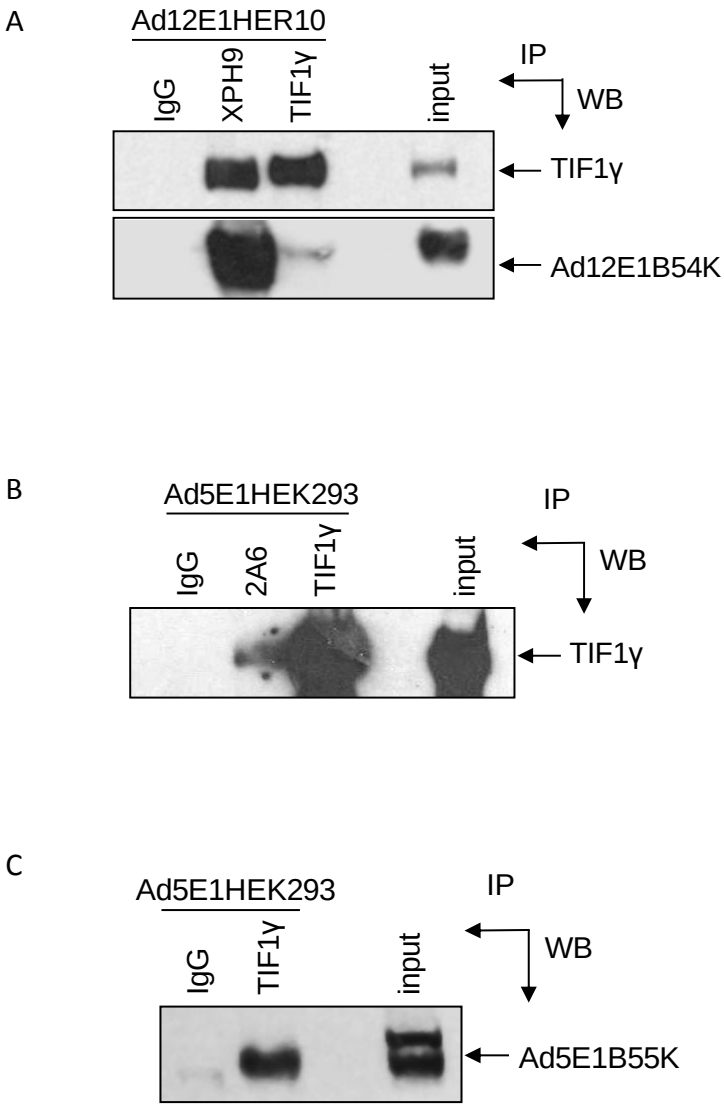


Fig 4.1A C. Ad12- and Ad5E1B55K interacts with TIF1γ in transformed cell lines

Ad12E1B54K, Ad5E1B55K and TIF1γ were immunoprecipitated from 4 mg of HER10 (anti-XPH9 antibody) and HEK293 (anti-2A6 antibody) cell lysate, respectively. After incubation with Protein G-Sepharose beads, samples were washed, eluted and resolved by SDS-PAGE. Bound proteins were detected by Western blotting.

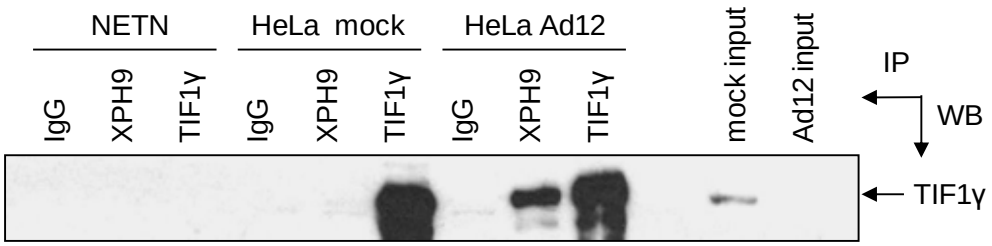


Fig 4.2. Ad12E1B54K interacts with TIF1γ in infected cells

Ad12E1B54K was immunoprecipitated from 4 mg of mock-treated and Ad12-infected HeLa lysate with an anti-XPH9 antibody. After incubation with Protein G-Sepharose beads, samples were washed, eluted and resolved by SDS-PAGE. Bound TIF1γ proteins were detected by Western blotting.

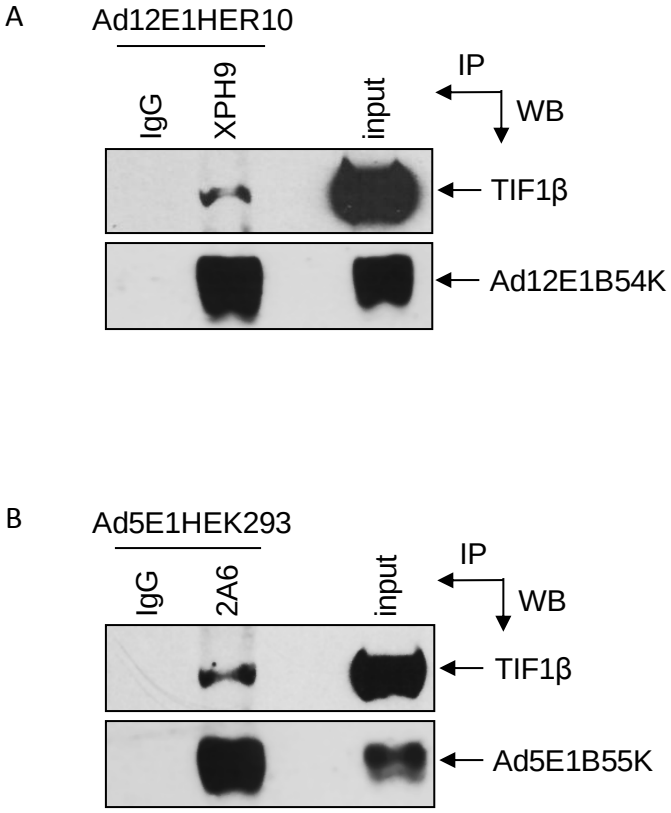


Fig 4.3A and B. Ad12- and Ad5E1B55K interact with TIF1β in transformed cell lines

Ad12E1B54K and Ad5E1B55K were immunoprecipitated from 4 mg of HER10 (anti-XPH9 antibody) and HEK293 (anti-2A6 antibody) cell lysate, respectively. After incubation with Protein G-Sepharose beads, samples were washed, eluted and resolved by SDS-PAGE. Bound TIF1β proteins were detected by Western blotting.

TIF1β was also identified as a possible Ad12E1B54K-binding protein. To investigate this further Ad12E1B54K and Ad5E1B55K were immunoprecipitated from HER10 and HEK293 cell lysates, respectively, and analysed for co-immunoprecipitation with TIF1β as described above. Western blot analysis revealed an association between TIF1β and both Ad5 and Ad12 large E1B viral proteins (Fig 4.3A and B). These data support the mass spectrometry results, and identify TIF1γ and TIF1β as novel Ad5 and Ad12 large E1B-binding proteins.

4.3.3 Ad12E1B54K/E4ORF6-mediated substrate degradation is not affected by TIF1γ depletion

As discussed in Chapter 1 and Chapter 3, E1B55K and E4ORF6 hijack CRL complexes and utilise them to ubiquitylate and degrade key members of the DDR pathway (e.g. Mre11 and p53). These cellular E3 ubiquitin ligase complexes are reported to be CUL2- or CUL5-based and contain an RBX1 domain and Elongins B and C (Querido *et al.*, 2001, Harada *et al.*, 2002, Blackford *et al.*, 2010). Given recent evidence contesting the involvement of CUL2 or CUL5 in the degradation of Mre11, it is possible that another E3 ubiquitin ligase may be responsible for the degradation of cellular proteins during Ad infection (Forrester *et al.*, 2011, and Chapter 5 of this thesis). As TIF1γ functions as an E3 ubiquitin ligase in the TGF-β signalling pathway (Dupont *et al.*, 2005, Morsut *et al.*, 2010), it is possible that it might also participate in the ubiquitin-mediated degradation of Mre11 during Ad infection.

To investigate this possibility HeLa cells were treated with control non-silencing siRNA and siRNA targeting TIF1γ and cultured for 24 hours, after which time they were either mock-treated or infected with Ad12. Samples were harvested over a period of 72 hours and examined by SDS-PAGE and Western blotting. Results indicated that even though TIF1γ

protein levels were reduced significantly by RNAi, this depletion had no effect on Mre11 degradation which was still evident 24 hours post-Ad12 infection (Fig 4.4). Interestingly, this experiment also revealed that Ad infection resulted in a loss of TIF1γ protein expression from cells treated with either non-silencing, or TIF1γ siRNA (Fig 4.4).

4.3.4 TIF1γ protein levels are reduced following Ad infection

As results presented in Figures 4.2 and Fig 4.3 suggest that TIF1γ protein levels might be reduced during Ad12 infection, this observation was examined in more detail. Thus, HeLa cells were mock-treated or infected with Ad12 and harvested over a 72 hour period before being subjected to Western blotting. As expected, Ad12 infection depleted DNA ligase IV protein levels, but interestingly and consistent with earlier findings, TIF1γ protein levels were also reduced (Fig 4.5A).

To expand upon this observation, cells were infected with Ad3, Ad5, Ad7, Ad11 and Ad12, harvested, and analysed by Western blotting to determine the protein levels of all TIF1 family members during infection. Levels of TIF1β displayed no variation during infection with all serotypes, confirming results from Chapter 3 (Fig 3.11). This was also the case for TIF1α protein levels; however there was some variation between samples at late time points following Ad3, Ad5 and Ad7 infection (Fig 4.5B). It was evident however, that infection with all serotypes tested resulted in the loss of TIF1γ expression (Fig 4.5B). Ad12 and Ad5 resulted in a decrease in TIF1γ protein levels by 24 hours post-infection, while TIF1γ depletion was not evident until 48 hours post-Ad3, -Ad7 and -Ad11 infection (Fig 4.5B). During all infections however, TIF1γ depletion was marked with virtually no protein present after 72 hours. A Western blot of Nbs1 was included as a control for this experiment, and its

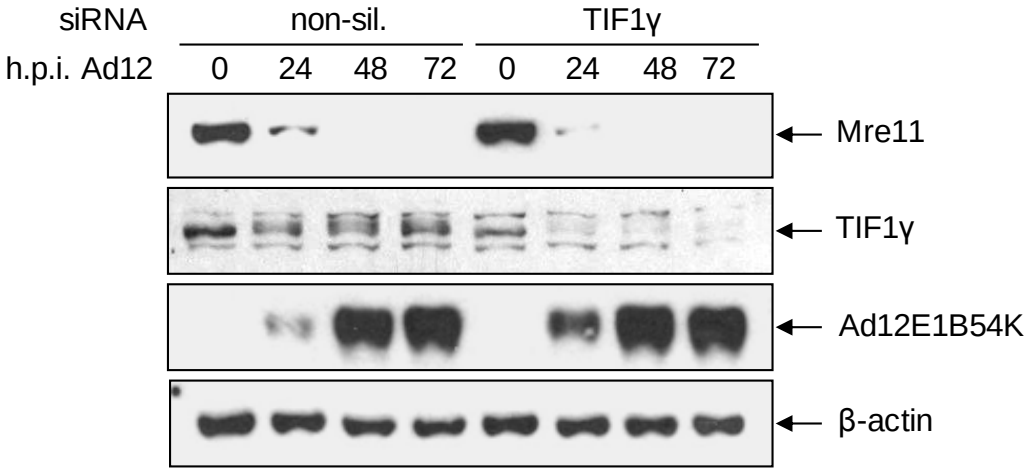


Fig 4.4. Effect of TIF1γ depletion on Mre11 protein degradation during Ad12 infection

HeLa cells were treated with non-silencing siRNA or siRNA targeting TIF1γ before being infected with Ad12. Cells were then harvested at the indicated time points and examined by Western blotting using antibodies against Mre11, TIF1γ, Ad12E1B54K and β-actin as a loading control.

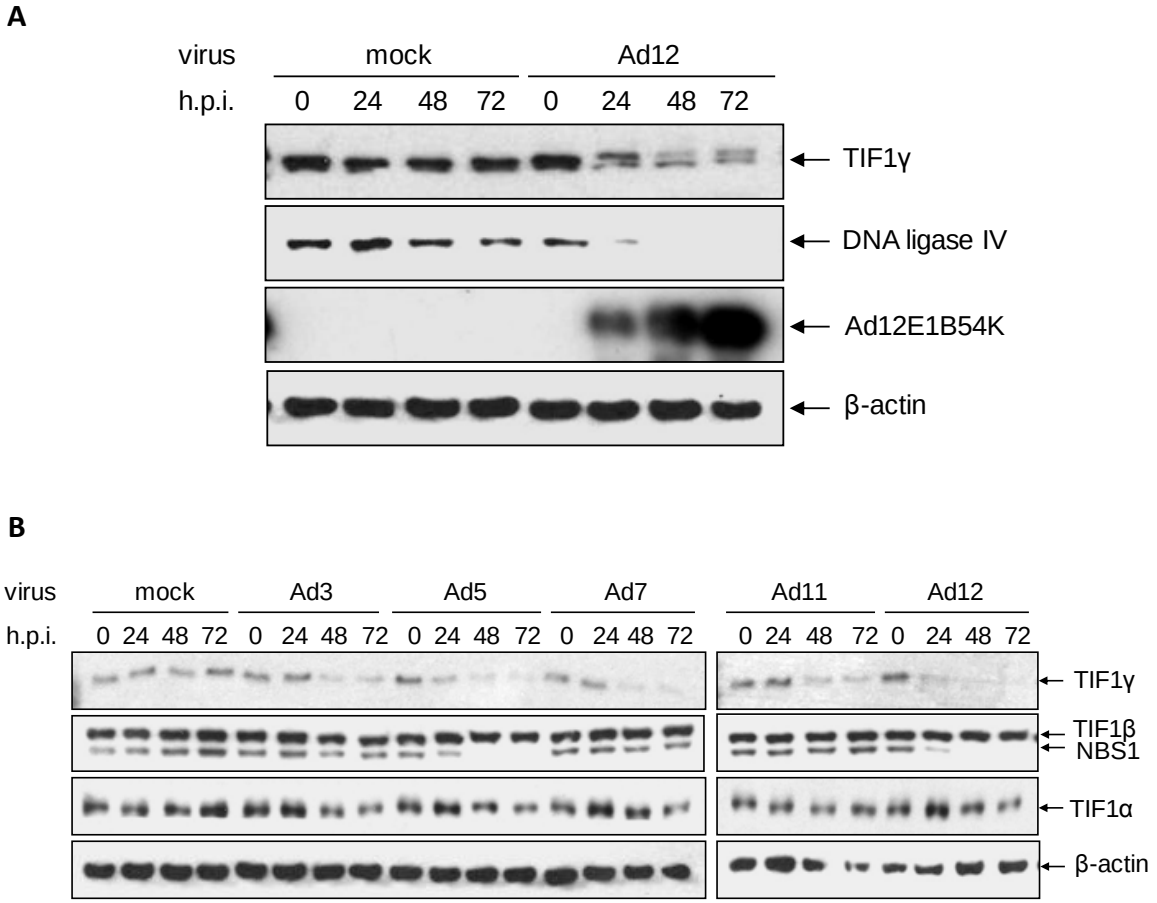


Fig 4.5A and B. Members of the TIF1 family of proteins are degraded during adenovirus infection

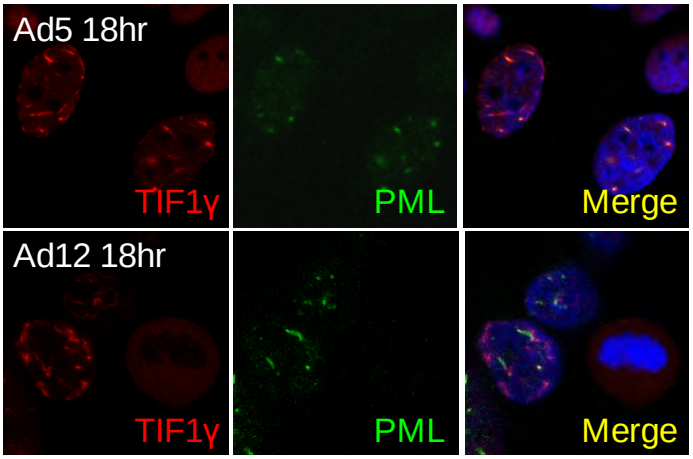
HeLa cells were mock-treated, infected with Ad12 (A) or infected with Ad3, Ad4, Ad5, Ad7, Ad9, Ad11 and Ad12 serotypes (B). Cells were harvested at the indicated time points and examined by Western blotting using antibodies against TIF1γ, TIF1β, TIF1α, DNA ligase IV, Ad12E1B54K and β-actin as a loading control.

level was appropriately reduced during Ad5 and Ad12 infections with no change seen during Ad3, Ad7 and Ad11 infection (Fig. 4.5B). These data indicate that TIF1γ expression is commonly reduced following infection with a number of different Ad serotypes.

4.3.5 Endogenous TIF1γ and TIF1α are relocalized into PML-containing nuclear tracks during Ad5 and Ad12 infection

E4ORF3 acts to reorganise the tumour suppressors PML and p53 into "track-like" structures throughout the nucleus during Ad infection (Evans and Hearing, 2003). Therefore, the localization of the TIF1γ protein was also examined as it is likely that TIF1γ may be regulated in a similar manner. To follow the localization of endogenous TIF1γ during infection immunofluorescence was performed. HeLa cells were seeded onto glass slides before being mock-treated or infected with Ad5 or Ad12. 18 hours post-infection cells were harvested by treatment with a pre-extraction buffer and fixation in 4% PFA. Cells were then stained for TIF1γ, RPA32 and PML and mounted in a DAPI-containing mounting medium to stain the DNA; RPA32 and PML were used as surrogate markers for VRCs and nuclear tracks, respectively. Images were analysed using a LSM 510 META confocal laser scanning microscope (Carl Zeiss). Results clearly illustrate that TIF1γ relocalizes from a pan-nuclear state before infection, into PML-containing nuclear tracks during both Ad5 and Ad12 infection (Fig 4.6A). This was confirmed by the lack of TIF1γ-RPA32 colocalization, suggesting that TIF1γ is not relocalized to sites of viral replication (Fig 4.6B). TIF1α was also relocalized into nuclear tracks 18 hours post-Ad5 and -Ad12 infection (Fig 4.7).

A



B

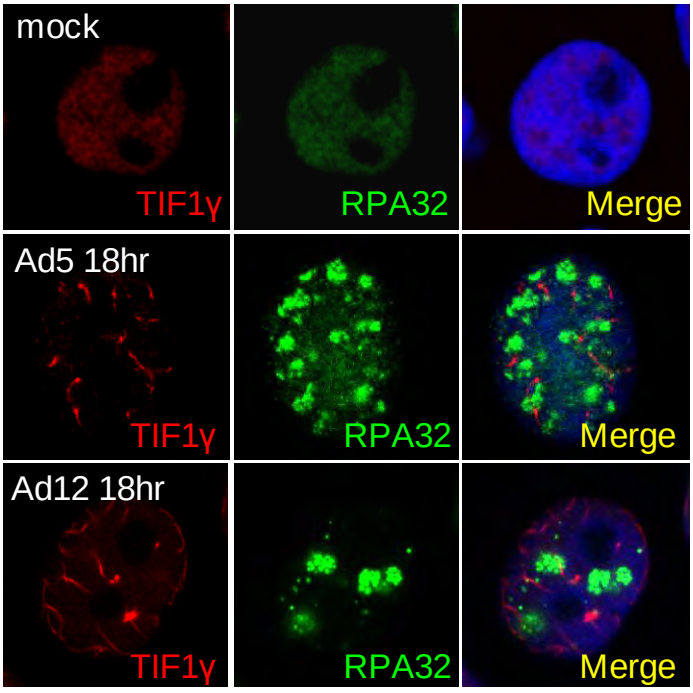


Fig 4.6A and B. Localization of TIF1γ following adenovirus infection

HeLa cells were mock-treated or infected with Ad5 and Ad12 and harvested at the indicated time points. Cells were treated with a pre-extraction buffer and fixed in 4% paraformaldehyde before being stained for TIF1γ (red), PML (green; A), RPA32 (green; B) and DAPI (blue) and visualised by confocal microscopy. Colocalization of proteins is evident in the right-hand merge column (yellow).

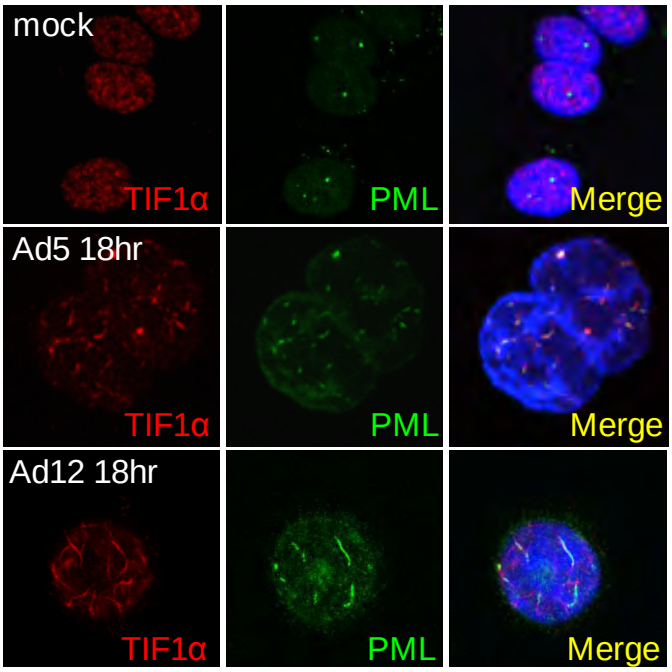


Fig 4.7. Localization of TIF1α following adenovirus infection

HeLa cells were mock-treated and infected with Ad5 and Ad12 and harvested at the times shown. Cells were treated with a pre-extraction buffer and fixed in 4% paraformaldehyde before being stained for TIF1α (red), PML (green) and DAPI (blue) and visualised by confocal microscopy. Colocalization of proteins is evident in the right-hand merge column (yellow).

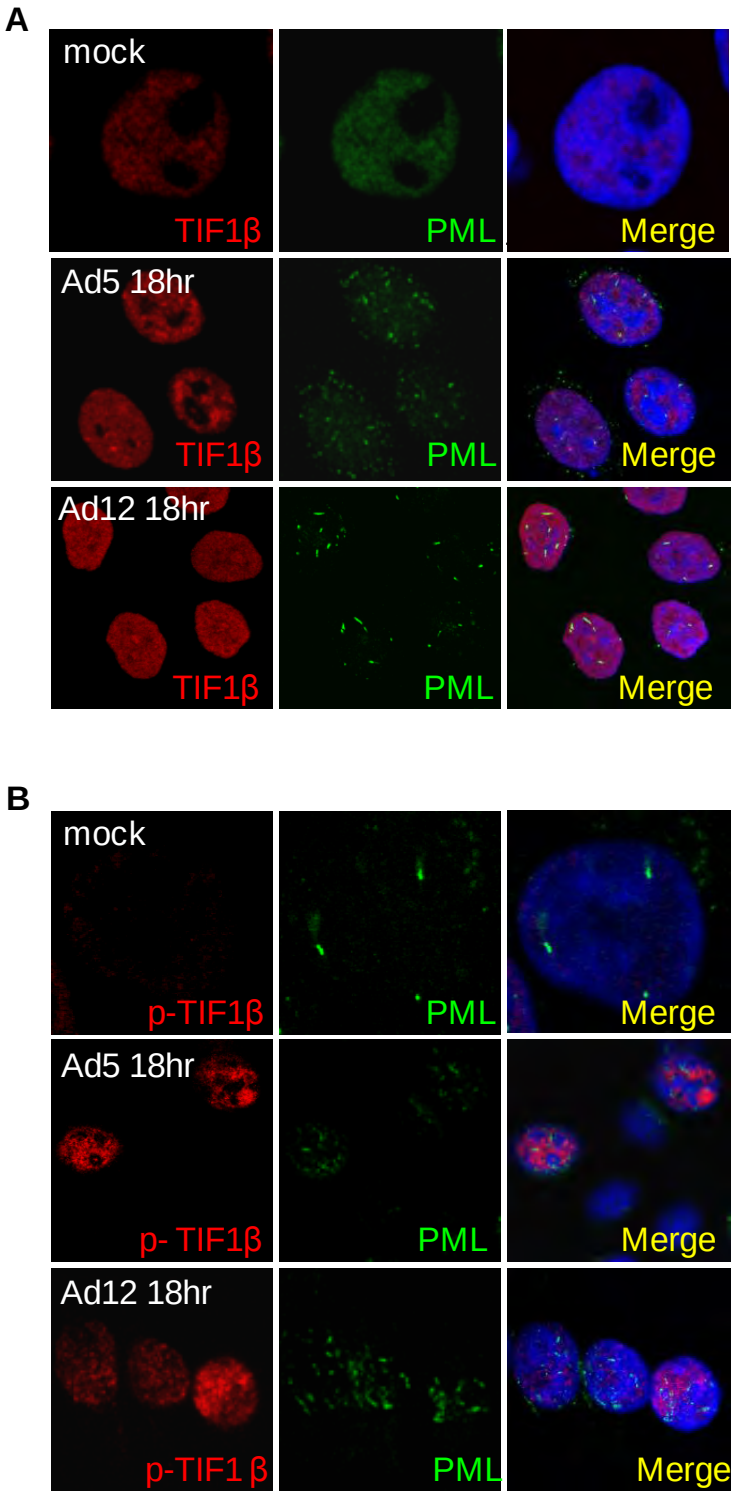


Fig 4.8A and B. Localization of TIF1β and phospho-TIF1β (Ser842) following adenovirus infection

HeLa cells were mock-treated and infected with Ad5 and Ad12 and harvested at the indicated time points. Cells were treated with a pre-extraction buffer and fixed in 4% paraformaldehyde before being stained for TIF1β (red; A), phospo-TIF1β (red; B), PML (green) and DAPI (blue) and visualised by confocal microscopy. Colocalization of proteins is evident in the right-hand merge column (yellow).

4.3.6 TIF1β and phospho-TIF1β are not relocalized during Ad5 and Ad12 infection

Since both TIF1α and TIF1γ were relocalized into nuclear tracks upon Ad infection, the fate of TIF1β was also examined by immunofluorescence using the same method as described for Fig 4.6. In contrast to TIF1α and TIF1γ, TIF1β was not relocalized during Ad infection and instead remained pan-nuclear up to 18 hours post-Ad5 and -Ad12 infection (Fig 4.8A). To confirm this, a phospho-specific TIF1β antibody was used to stain HeLa cells since TIF1β is phosphorylated on serine 842 following Ad infection (presumably by ATM; see Chapter 3). Immunofluorescence revealed that while this modified protein was undetectable during mock infection, it was upregulated during Ad5 and Ad12 infection supporting the Western blot data from Chapter 3 (Fig 4.8B). However, despite this upregulation, phospho-TIF1β failed to colocalize with PML during infection and instead exhibited a granular, yet generally uniform staining pattern throughout the nucleus (Fig 4.8B). Taken together these data suggest that TIF1α, TIF1β and TIF1γ are differentially regulated in response to Ad infection.

4.3.7 Exogenous mutated TIF1γ exhibits restricted relocalization during Ad infection

During Ad5 infection E4ORF3 directly binds to the conserved N-terminal TRIM domain of TIF1α which possesses E3 ubiquitin ligase activity (Yondola and Hearing, 2007). Furthermore, the TRIM domain was found to be necessary and sufficient for Ad5E4ORF3 to relocalize TIF1α into PML-containing nuclear tracks. In order to determine if the same scenario was true for TIF1γ a transfection/infection approach was used.

HeLa cells were initially transfected with Flag-tagged expression vectors containing either full-length TIF1γ, or TIF1γ in which two cysteines at positions 125 and 128 were mutated to

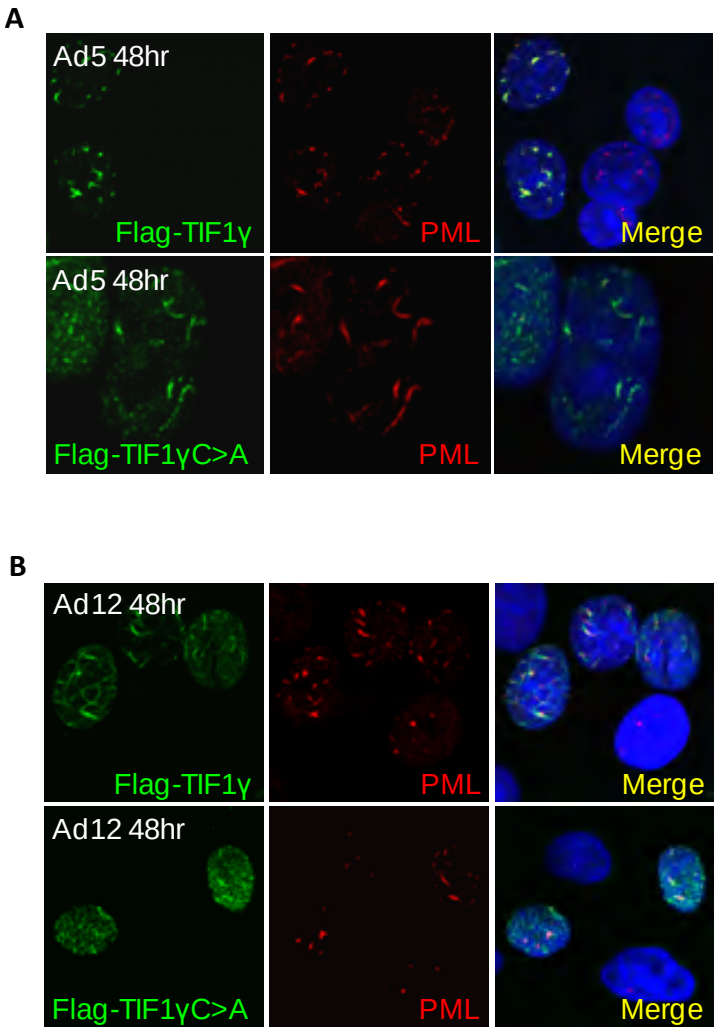


Fig 4.9A and B. Localization of exogenous *wt* TIF1γ and TIF1γC>A following adenovirus infection

HeLa cells were transfected with *wt* Flag-TIF1γ or Flag-TIF1γC>A plasmid DNA 48 hours prior to Ad5 (A) and Ad12 (B) infection. After a further 48 hours, cells were harvested and treated with a pre-extraction buffer before being fixed in 4% paraformaldehyde and stained for Flag (green), PML (red) and DAPI (blue). Images were visualised by confocal microscopy and colocalization of proteins is evident in the right-hand merge column (yellow).

alanines (TIF1γC>A). This point mutant bears an additional SfiI restriction site across the mutated cysteines which confers a loss of E3 ubiquitin ligase activity from the deletion of 37 N-terminal amino acids (Dupont *et al*., 2005). Forty-eight hours post-transfection cells were seeded onto glass slides for infection with Ad5 or Ad12, and after a further 48 hours they were harvested by treatment with a pre-extraction buffer and fixation in 4% PFA. Fixed cells were then stained for Flag, PML and DAPI and visualised by confocal microscopy. This experiment revealed that full-length TIF1γ formed clear nuclear tracks that colocalized with PML during Ad5 and Ad12 infection (Fig 4.9A and B). Interestingly however, the TIF1γC>A mutant was only partially relocalized following infection as the majority of TIF1γC>A remained in a pan nuclear state (Fig 4.9A and B). This defect was especially prominent following Ad12 infection where most of the nuclear tracks containing PML had no TIF1γC>A protein present.

4.3.8 E4ORF3 is required for Ad5- and Ad12-mediated TIF1γ degradation and relocalization

The requirement of specific Ad proteins for the relocalization and degradation of proteins varies depending on the serotype and the substrate. During Ad5 infection, Mre11, p53 and TIF1α are relocalized into nuclear tracks in an E4ORF3-dependent manner, and Mre11 and p53 are targeted for degradation through the action of E1B55K and E4ORF6 (Stracker *et al*., 2002, Evans and Hearing, 2005, Yondola and Hearing, 2007). In contrast, during Ad12 infection TOPBP1 is relocalized instead to VRCs and is then degraded in an E1B54K-independent E4ORF6-dependent manner (Blackford *et al*., 2010).

Given that TIF1γ relocalization during Ad infection is regulated in a similar manner to TIF1α, the role of E4ORF3 in Ad-mediated TIF1γ degradation and relocalization was investigated. This was explored, in the first instance, using a panel of Ad5 and Ad12 mutant viruses lacking distinct viral genes.

HeLa cells were thus mock-treated, infected with *wt* Ad5, or infected with the mutant Ad5 viruses *dl*1520 (ΔE1B55K), *dl*355 (ΔE4ORF6) or H5*pm*4155 (ΔE4ORF3/E4ORF6). After 24 and 48 hours cells were harvested and analysed by Western blotting. Infection with *dl*1520 and *dl*355 led to TIF1γ degradation in a manner indistinguishable from that seen with *wt* Ad5, although DNA ligase IV degradation was delayed (Fig 4.10A). Infection with H5*pm*4155 however, significantly inhibited the degradation of TIF1γ and DNA ligase IV degradation in comparison to the *wt* infection (Fig 4.10A). To investigate the effect of Ad5 protein expression on TIF1γ relocalization to nuclear tracks, HeLa cells were transfected with HA-tagged expression vectors containing Ad5E1B55K, Ad5E4ORF6 and Ad5E4ORF3. Cells were harvested after 48 hours post-transfection, processed for immunofluorescence as described above, and stained for TIF1γ, Ad5E1B55K (2A6), Ad5E4ORF6 (RSA3), Ad5E4ORF3 (6A11) and DAPI. Visualization by confocal microscopy revealed that expression of Ad5E1B55K or Ad5E4ORF6 alone was not sufficient to relocalize TIF1γ (Fig 4.10B). However, in cells expressing Ad5E4ORF3 alone, TIF1γ was found to colocalize with E4ORF3 in nuclear tracks (Fig 4.10B).

To further study the role of viral proteins in TIF1γ degradation, HeLa cells were transfected with E1B55K and/or E4ORF3/E4ORF6 Ad5 expression vectors. Cells were subsequently harvested and TIF1γ was analysed by Western blotting and immunofluorescence. Twenty-four hours post-cotransfection with Ad5E1B55K and Ad5E4ORF3, immunofluorescence revealed that TIF1γ was relocalized into nuclear track structures, and that by 48 hours TIF1γ

levels were completely depleted (Fig 4.11B). Furthermore, Western blot analysis revealed that TIF1γ levels were reduced 24 hours post-transfection with Ad5E4ORF3, but were not reduced following transfection with either Ad5E1B55K or Ad5E4ORF6 (Fig 4.11A). Cotransfection with Ad5E4ORF3 and Ad5E4ORF6 also had the capacity to relocalize TIF1γ into nuclear tracks (Fig 4.11B), however Western blotting revealed that TIF1γ protein levels were not affected by the coexpression of Ad5E1B55K and Ad5E4ORF6 (Fig 4.11A).

Given these findings, the consequence of Ad12 protein expression on TIF1γ degradation and relocalization was also examined by the use of mutant viruses and transfection of viral genes. Initially, HeLa cells were infected with *wt* Ad12 or two mutant Ad12 viruses, *dl*620 and *hr*703, neither of which express the E1B54K gene product. Levels of Ad substrates were analysed over a 48 hour time course by Western blotting, and results revealed that *dl*620 and *hr*703 retained the ability to degrade TIF1γ to the same extent seen during *wt* Ad12 infection (Fig 4.12A). Mre11, DNA ligase IV and p53 were used as positive controls, and as expected in the absence of E1B54K, p53 protein expression was increased and Mre11 and DNA ligase IV proteins were stabilized. To examine the requirements for Ad12E4ORF3 in TIF1γ degradation and relocalization during infection, HA-tagged expression vectors were transfected in to HeLa cells and subsequently analysed by Western blotting and immunofluorescence. Both Western blot analysis and confocal microscopy revealed that upon Ad12E4ORF3 expression, TIF1γ levels were reduced after 24 hours (Fig 4.12B and 4.12C). Confocal microscopy also revealed a more substantial reduction in TIF1γ protein levels 48 hours post-Ad12E4ORF3 transfection, whilst Ad12E1B54K expression had no effect (Fig 4.12C). Unlike the Ad5 transfections however, it is ambiguous as to whether TIF1γ colocalized with Ad12E4ORF3, or formed nuclear tracks, during transfection; although there was some evidence of this (Fig 4.12C). Akin to the Ad5 studies, expression of

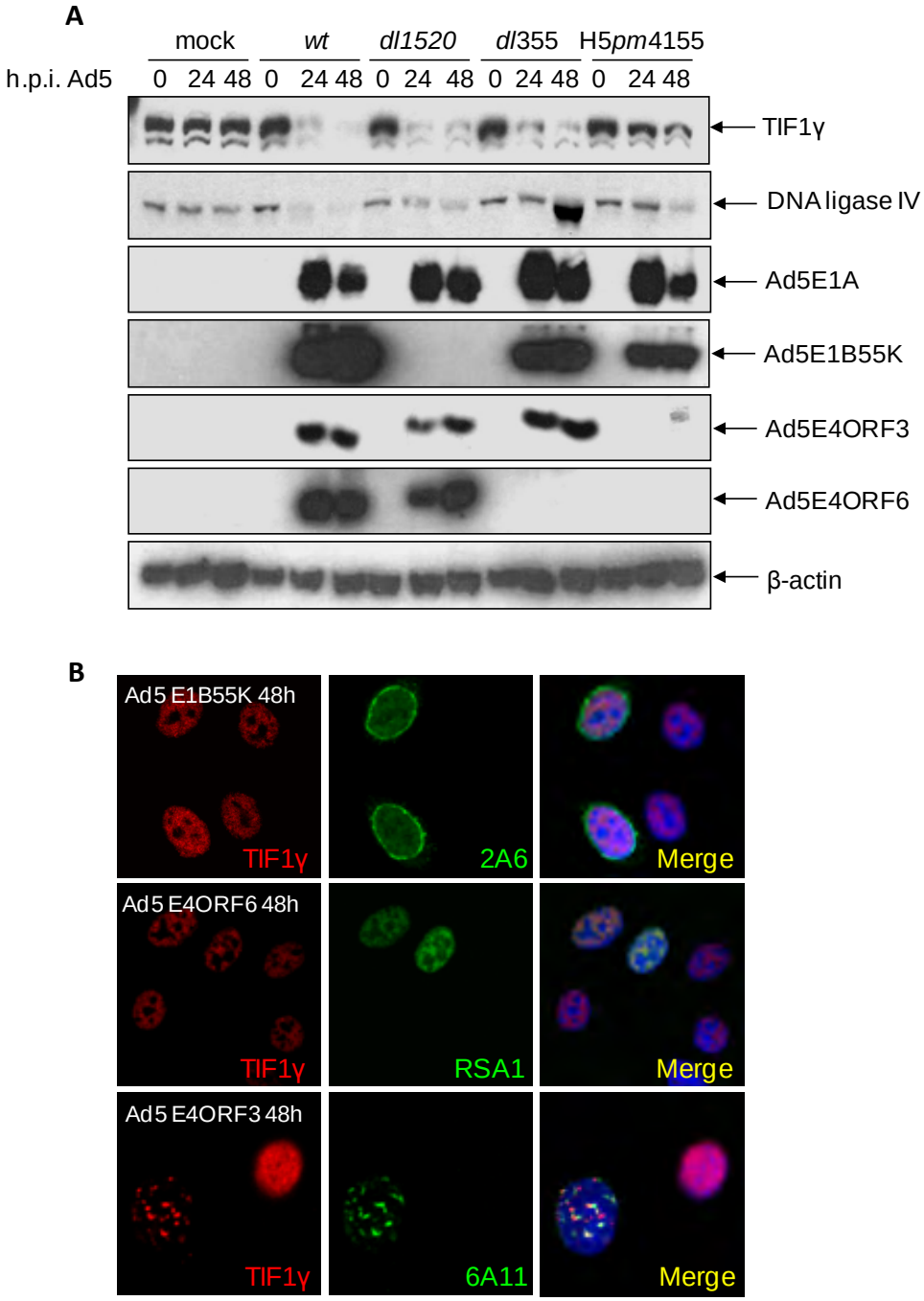


Fig 4.10A and B. E4ORF3 is required for Ad5-mediated TIF1γ degradation and relocalization

(A) HeLa cells were mock-treated, infected with *wt* Ad5 or infected with mutant Ad5 viruses (*dl*1520, *dl*355 and H5*pm*4155) and harvested at the indicated time points. Levels of protein expression was subsequently analysed by Western blotting. (B) HeLa cells were transfected with Ad5 HA-tagged E1B55K, E4ORF6 or E4ORF3 plasmid DNA and harvested after 48 hours. Cells were then treated with a pre-extraction buffer before being fixed in 4% paraformaldehyde and stained for TIF1γ (red), E1B55K(anti-2A6), E4ORF6 (anti-RSA3), E4ORF3 (anti-6A11; all green) and DAPI (blue). Images were visualised by confocal microscopy and colocalization of proteins is evident in the right-hand merge column (yellow).

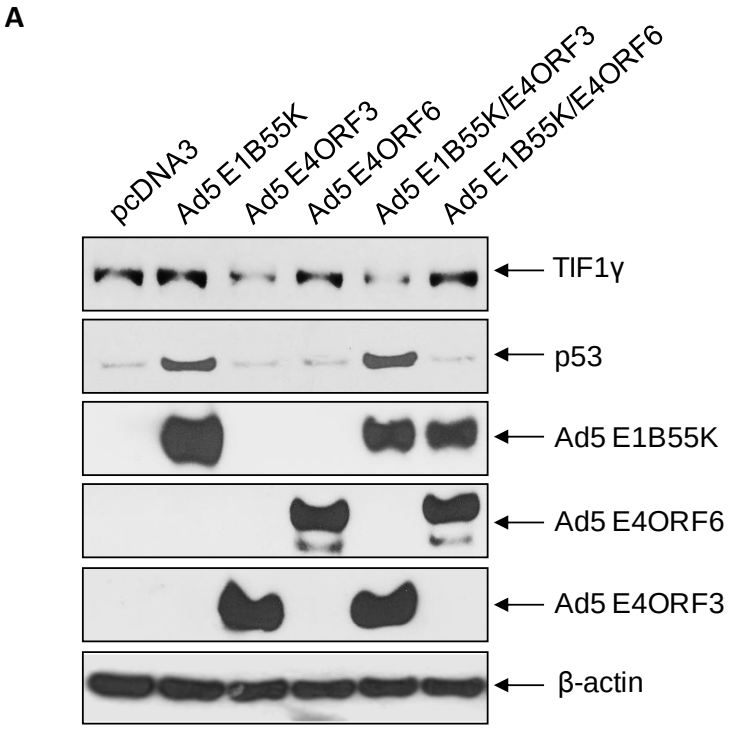
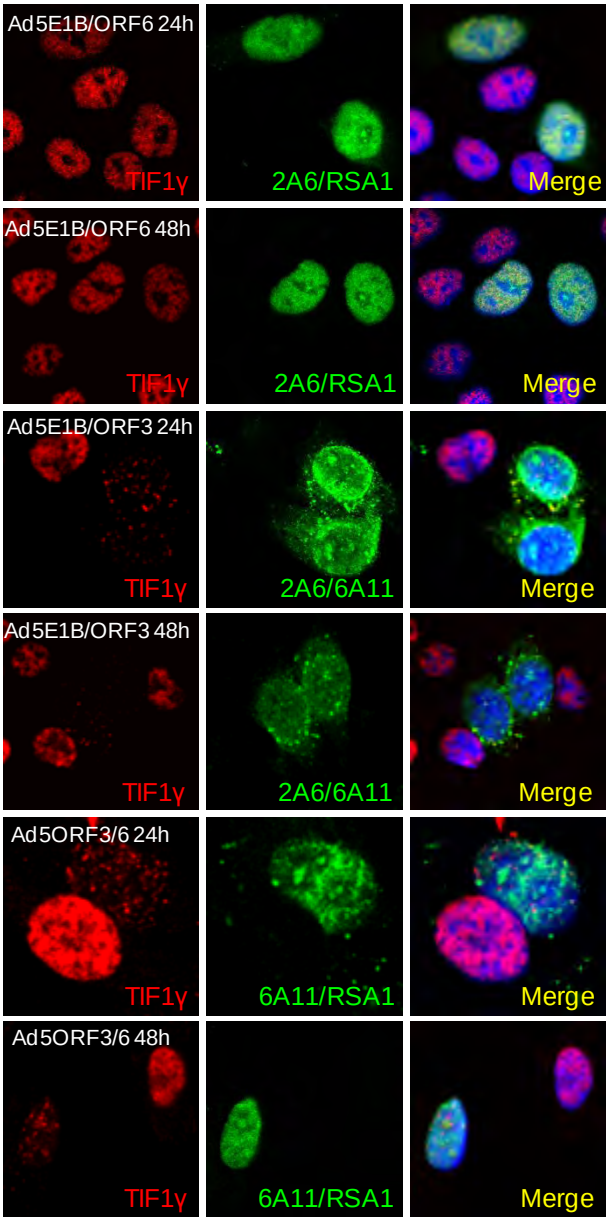


Fig 4.11A and B. Ad5-mediated TIF1γ degradation and relocalization is independent of E1B55K and E4ORF6

(A) HeLa cells were transfected or cotransfected with the indicated Ad5 HA-tagged E1B55K, E4ORF6 or E4ORF3 plasmid DNA and harvested after 24 hours. Levels of protein expression was analysed by SDS-PAGE and Western blotting. Transfection of pcDNA3 was used as a negative control. (B) Localization of these proteins was also studied by immunofluorescence. Cells were treated with a pre-extraction buffer before being fixed in 4% paraformaldehyde and stained for TIF1γ (red), 2A6 (E1B55K), RSA1 (E4ORF6) and 6A11 (E4ORF3 [all green]) and DAPI (blue). Images were visualised by confocal microscopy and colocalization of proteins is evident in the right-hand merge column (yellow).

B


Ad5E1B/ORF6 24h
TIF1γ
2A6/RSA1
Merge
Ad5E1B/ORF6 48h
TIF1γ
2A6/RSA1
Merge
Ad5E1B/ORF3 24h
TIF1γ
2A6/6A11
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Ad5E1B/ORF3 48h
TIF1γ
2A6/6A11
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TIF1γ
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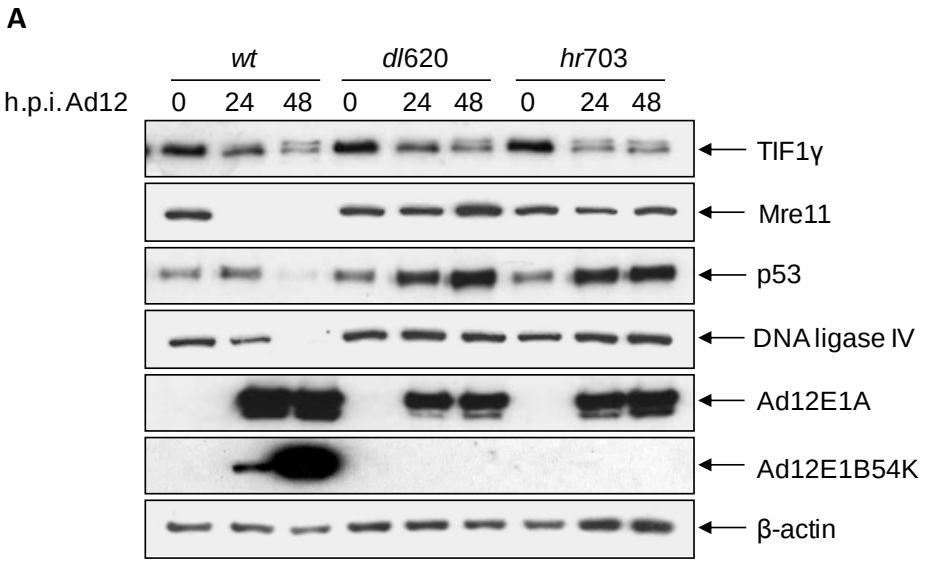
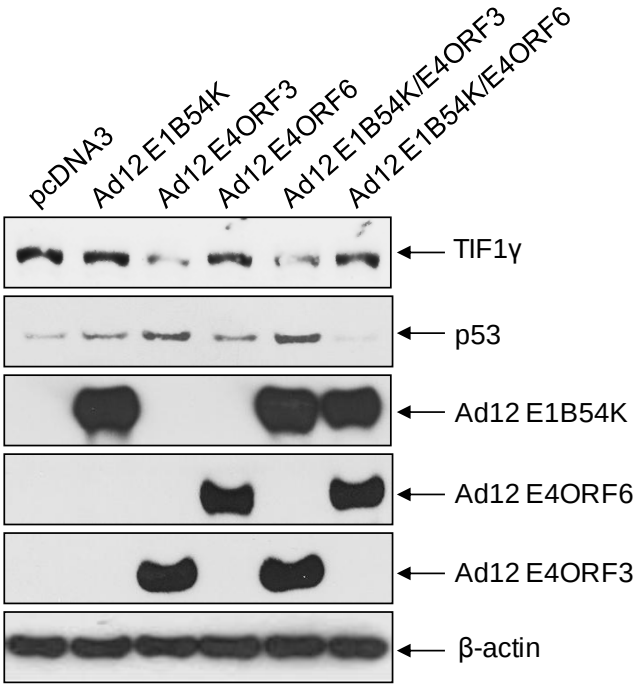


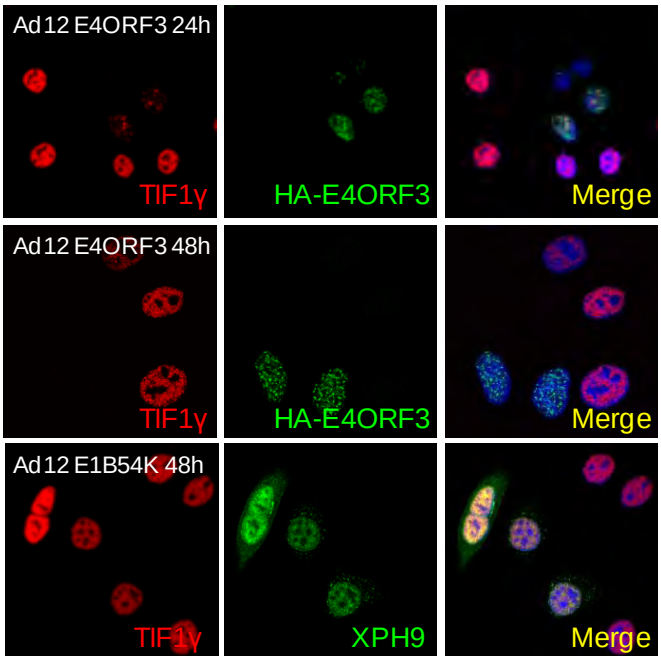
Fig 4.12A - C. E4ORF3 is required for Ad12-mediated TIF1γ degradation and relocalization

(A) HeLa cells were mock-treated, infected with *wt* Ad12 or infected with Ad12 mutants (*dl*620 and *hr*703) and harvested at the indicated times. Levels of protein expression was subsequently analysed by Western blotting. (B) HeLa cells were transfected or cotransfected with the indicated Ad12 HA-E1B55K, -E4ORF6 and –E4ORF3 plasmid DNA and harvested after 24 hours. Levels of protein expression was analysed by SDS-PAGE and Western blotting. Transfection of pcDNA3 was used as a negative control (C) HeLa cells were transfected with Ad12 HA-E1B54K and –E4ORF3 plasmid DNA and harvested after 24 hours. Cells were then treated with a pre-extraction buffer before being fixed in 4% paraformaldehyde and stained for TIF1γ (red), HA and XPH9 (E1B55K [both green]) and DAPI (blue). Images were visualised by confocal microscopy and colocalization of proteins is evident in the right-hand merge column (yellow).

B


pcDNA3
Ad12 E1B54K
Ad12 E4ORF3
Ad12 E4ORF6
Ad12 E1B54K/E4ORF3
Ad12 E1B54K/E4ORF6
TIF1γ
p53
Ad12 E1B54K
Ad12 E4ORF6
Ad12 E4ORF3
β-actin

C


Ad12 E4ORF3 24h
TIF1γ
HA-E4ORF3
Merge
Ad12 E4ORF3 48h
TIF1γ
HA-E4ORF3
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Ad12 E1B54K 48h
TIF1γ
XPH9
Merge

Ad12E1B54K and Ad12E4ORF6 either alone or in combination, had no effect upon TIF1γ protein levels, despite the ability of E1B54K/E4ORF6 to target p53 for degradation (Fig 4.12B). Taken together these data suggest that E4ORF3 is solely required to promote TIF1γ degradation during Ad5 and Ad12 infection.

4.3.9 Mass spectrometric determination of TIF1γ immunoprecipitates

In order to idenitify novel TIF1γ-binding proteins which may associate with the protein during Ad infection, further mass spectrometric analysis was carried out. To this end, HeLa cells were mock-treated or infected with Ad12 and harvested 24 hours post-infection before being immunoprecipitated with an antibody against TIF1γ. Immunoprecipitates were then incubated with Protein G-Sepharose beads, eluted by boiling in SDS sample buffer and separated by SDS-PAGE. Proteins were visualised and processed as described above. As a control, an anti-TIF1γ antibody was incubated in lysis buffer alone minus cell lysate. Also the proteasomal inhibitor MG132 was added to a separate Ad12 infection after 12 hours, with the hypothesis that by preventing TIF1γ degradation any protein-protein complexes formed would remain intact. The resulting Coomassie Brilliant Blue G-250-stained gel indicated the presence of several specific proteins not found in control lanes (Fig 4.13). The proteins identified in this experiment were grouped into four categories and are summarized in Table 4.2, along with the number of peptides detected. Firstly, the presence of the three TIF1 family members in both mock-treated and Ad12-infected cells supports the efficacy of the experiment. Interestingly, several samples also identified a putative serine-proline (S/P) phosphorylation site in the TIF1γ peptide sequence QSGLSSLVNGKS*PIR. This was only

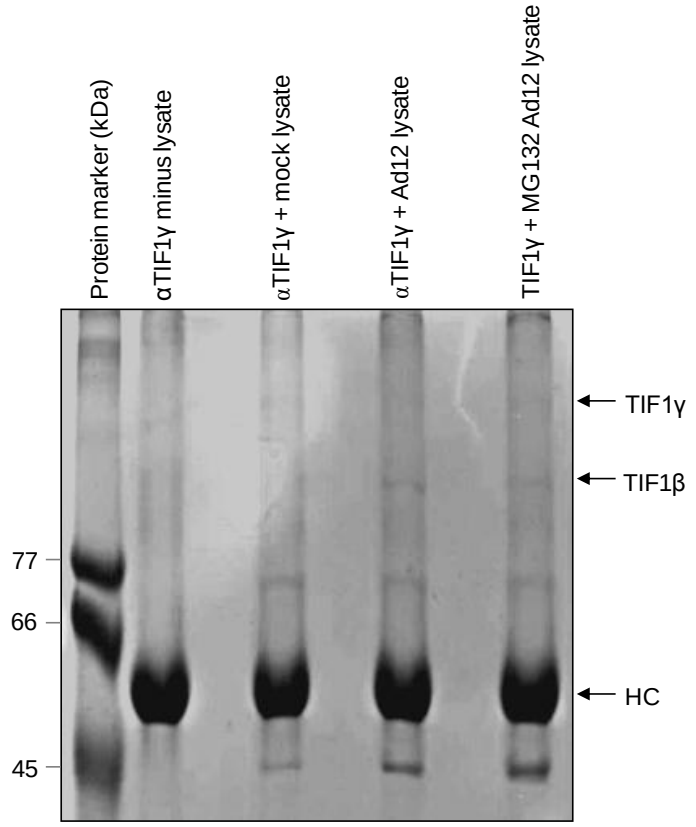

Fig 4.13. Separation of TIF1γ immunoprecipitates by SDS-PAGE

HeLa cells were mock-treated or infected with Ad12. After 12 hours the proteasomal inhibitor MG132 was added to indicated cells, and after a further 12 hours all samples were harvested in NETN lysis buffer. TIF1γ was immunoprecipitated from 5mg of cell lysate and bound proteins were incubated with Protein G-Sepharose beads which were subsequently washed and eluted. Eluted proteins were resolved by SDS-PAGE and stained with Coomassie blue G125. Some identified proteins are indicated by arrows and HC donates the antibody heavy chain.

Table 4.2. Summary of TIF1γ interacting proteins identified by mass spectrometric analysis

Protein	MW (kDa)	Number of peptides		
		Mock	Ad12	Ad12 +MG132
TIF1 family members				
TIF1γ	122	29	22	27
TIF1α	117	19	14	16
TIF1β	89	-	4	-
Known adenovirus substrates				
p53	53	-	3	-
PML	70	-	3	-
DNA ligase IV	96	-	2	-
Ubiquitin pathways components				
CUL3	89	-	2	-
CUL9	281	-	4	-
USP9	289	4	3	-
DNA damage response proteins				
ATR	303	-	6	14
Chk1	54	-	3	-

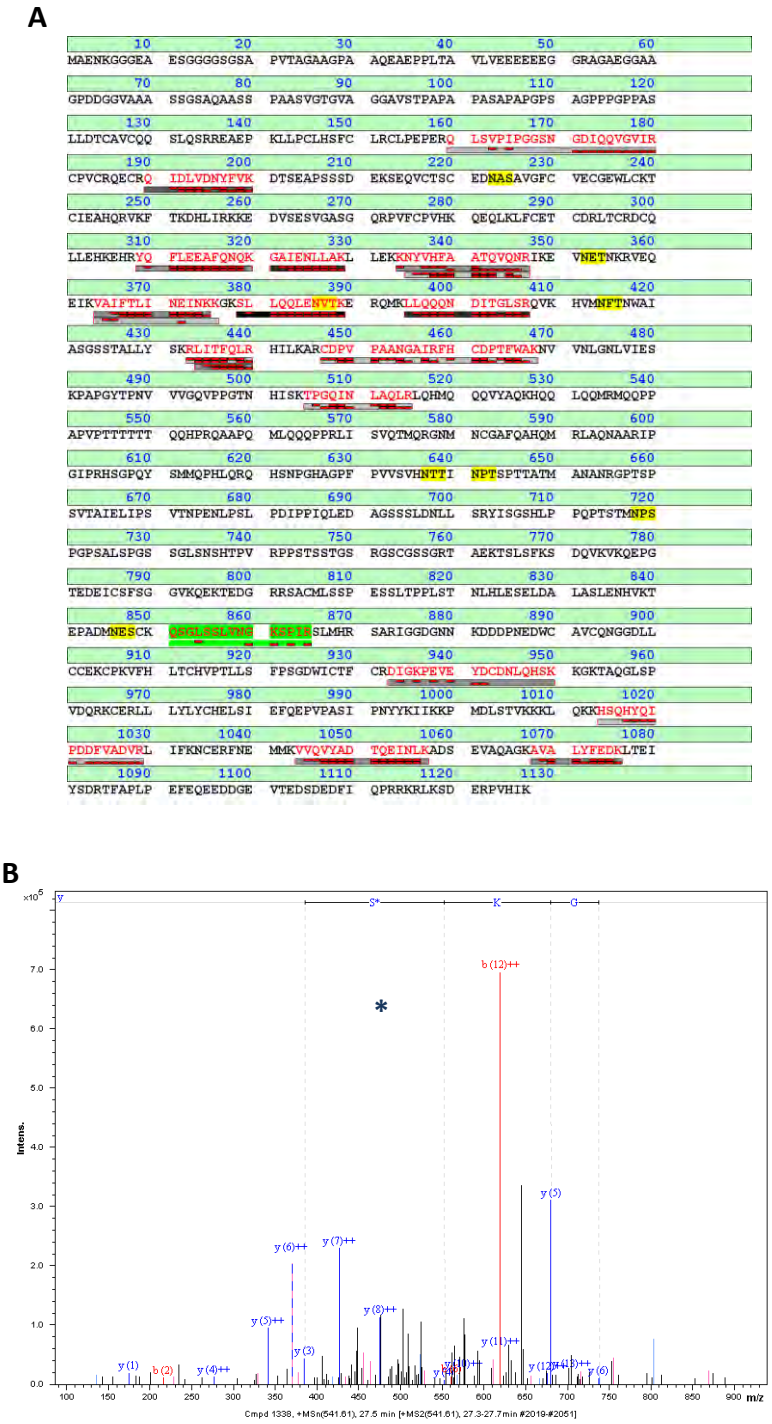

Fig 4.14A and B. ETD-mass spectrometric determination of TIF1γ and phospho-TIF1γ peptides isolated from TIF1γ immunoprecipitates

(A) Fifteen TIF1γ peptides (red) and one putative phosho-TIF1γ peptide (green) were identified by mass spectrometry from TIF1γ immunoprecipitates (putative glycosylation sites are highlighted in yellow). (B) A mass spectrogram illustrating the ions identified from the phospho-TIF1γ peptide QSGLSSLVNGKS#PIR. This peptide generated a Mascot score of 38.76 (* indicates the specific putative phosphorylated residue).

identified in Ad12-infected HeLa cells treated with or without MG132, and not in mock-treated cells. One example of this from Ad12-infected cells is shown in Figure 4.14A, where 15 TIF1γ peptides (highlighted in red) were identified including the putative S/P phosphorylation site (highlighted in green), which gave a significant Mascot score of 38.76 (Fig 4.14A). The mass spectrogram of this individual phospho-peptide can be seen in Figure 4.15B where the putative phosphorylation site is marked with an asterisk.

The second group of peptides identified belong to proteins already confirmed to interact with Ad proteins, further implicating a role for TIF1γ during infection. These proteins included p53, DNA ligase IV and PML. Thirdly, three proteins involved in ubiquitylation pathways were identified. These were CUL3 and CUL9 which are cullin proteins that nucleate CRLs, and the USP9 DUB which was also identified as a potential Ad12E1B54K-interacting protein (Table 4.1).

The final group of proteins identified from TIF1γ immunoprecipitates are involved in the DDR. Six peptides from the ATR kinase were identified during Ad12 infection, while interestingly three peptides from its substrate Chk1 were also identified. This is in addition to p53, PML and DNA ligase IV which also function in DDR pathways.

4.3.10 TIF1γ interacts with ATR

One of the most interesting findings from this mass spectrometric analysis was the putative association between TIF1γ and ATR during Ad infection. In order to confirm this interaction, TIF1γ was immunoprecipitated from mock-treated and Ad12-infected HeLa cells and immunoprecipitates were analysed for ATR binding using a specific anti-ATR antibody. Results indicated that TIF1γ interacts with ATR in both mock-treated and Ad12-infected

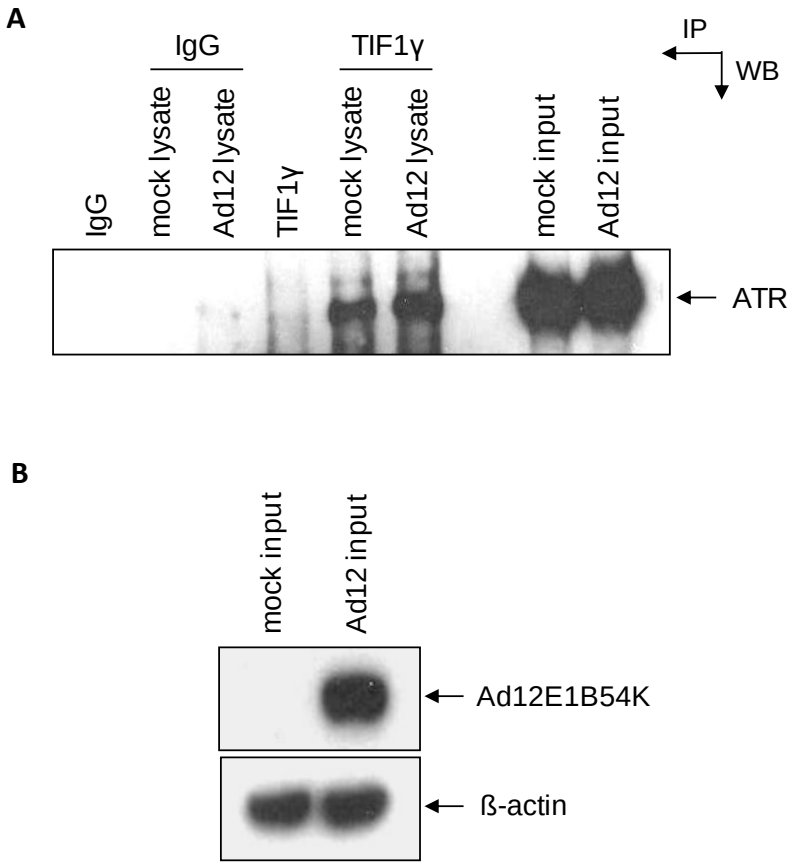

Fig 4.15A and B. TIF1γ interacts with ATR in Ad12-infected and uninfected cells

TIF1γ was immunoprecipitated from 4mg of mock and Ad12-infected HeLa lysate. After incubation with Protein G-Sepharose beads, samples were washed, eluted and resolved by SDS-PAGE. ATR co-immunoprecipitates were detected by Western blotting (A). Mock and Ad12-infected HeLa lysates from (A) were also analysed by Western blotting for the expression of Ad12E1B54K and β-actin as a loading control (B).

cells, with perhaps a slightly greater avidity during infection (Fig 4.15A). Western blots for Ad12E1B54K were also carried out to demonstrate infection (Fig 4.15B).

4.3.11 TIF1γ expression is unaffected by UVA-irradiation

Given the novel interaction revealed between TIF1γ and ATR, the potential role of TIF1γ in the DDR was explored. Thus, HeLa cells were mock-treated or treated with 25 J/m^2 of UVA-irradiation to activate ATR kinase activity through the generation of ssDNA. Over a period of 8 hours cells were harvested and analysed by Western blotting. Results revealed that although Chk1 became phosphorylated at serine residue 345 one hour post-UVA treatment as expected, TIF1γ protein expression remained unaffected by UVA exposure (Fig 4.16).

Proteins involved in the DDR are often relocalized into discrete nuclear foci following the induction of DNA damage. In order to determine if TIF1γ is similarly relocalized during DNA damage, DNA breaks were experimentally introduced into HeLa cells by laser microirradiation. Briefly, cells seeded in specialised chambered coverglass dishes were sensitized with a minor groove DNA-binding dye to facilitate the introduction of DNA breaks. They were then placed into a heated chamber mounted on a LSM 510 META confocal microscope and irradiated using a 364 nm UVA laser. Following DNA damage induction, immunofluorescence was carried out and cells were visualised by confocal microscopy. H2AX phosphorylation was used as a positive indicator for the presence of DNA breaks, and as such γH2AX appeared in distinct "stripes" caused by the UVA laser (Figure 4.17). TIF1γ failed to colocalize with γH2AX in UVA-treated cells and was instead distributed in its customary pan-nuclear fashion.

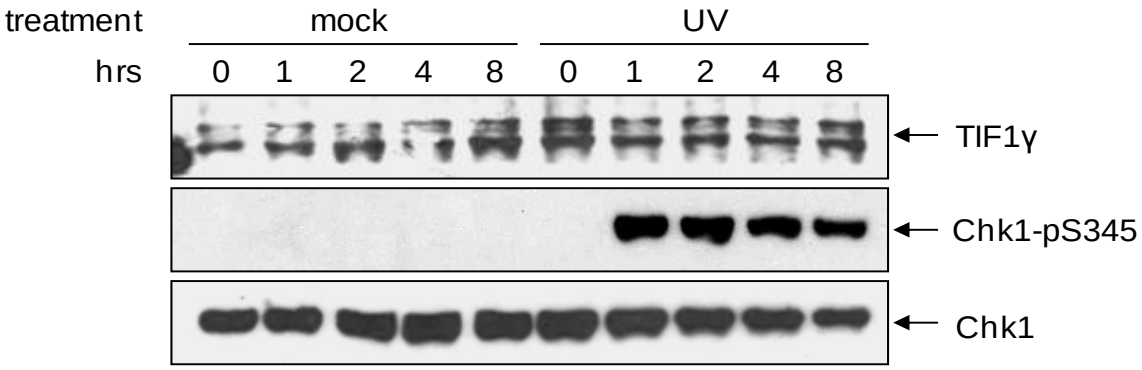

Fig 4.16. TIF1γ protein expression is not altered following UVA treatment

HeLa cells were either mock-treated or treated with 25 J/m^2 UVA and harvested at the indicated times. TIF1γ expression was analysed by Western blotting along with antibodies against Chk1-pS345 and Chk1 as controls.

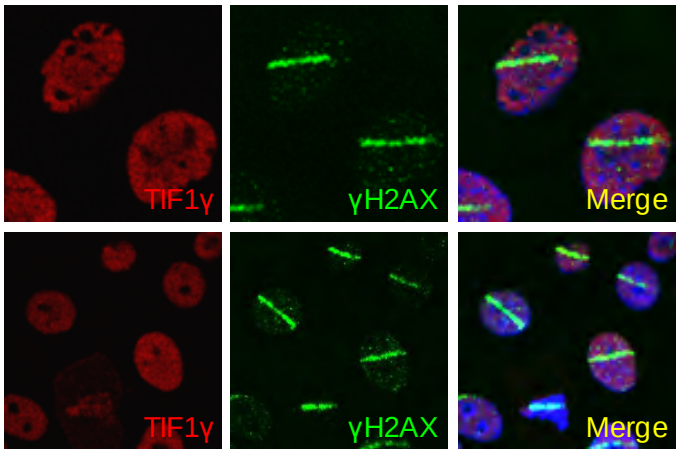

Fig 4.17. TIF1γ is not relocalized to DNA damage sites following UVA laser microirradiation

HeLa cells were microirradiated with a 364 nm UVA laser before being treated with a pre-extraction buffer and fixed in 4% paraformaldehyde. Immunofluorescence was carried out using antibodies against γH2AX (green) and TIF1γ (red). DAPI (blue) was used to stain DNA, and cells were visualised by confocal microscopy.

4.4 DISCUSSION

The application of mass spectrometry has previously identified the Ad substrate integrin alpha 3 as well as CUL5, RBX1 and Elongins B and C proteins which are involved in the ubiquitin-proteasome pathways utilised by Ad (Querido *et al.*, 2001, Harada *et al.*, 2002, Dallaire *et al.*, 2009a). Data presented in this Chapter describe the successful use of proteomics as a method to identify novel Ad12E1B54K- and TIF1γ-interacting proteins. The initial mass spectrometry experiment to identify Ad12E1B54K-interacting proteins was given credence as an appropriate technique following the identification of known Ad12E1B54K-interacting proteins, and the subsequent validation of novel interacting proteins, such as TIF1γ and TIF1β by co-immunoprecipitation (Fig 4.1-4.3). Since three out of the four known TIF1 family members were identified in this single screen it was reasonable to speculate that they function within the same pathway during Ad infection, although further evidence questioned this possibility. Indeed, confocal microscopy revealed that while TIF1γ and TIF1α were relocalized into PML-containing nuclear tracks, and TIF1γ was degraded during Ad infection, TIF1β protein levels and distribution remained unaffected (Fig 4.5-4.8). Moreover, despite their overall structural relatedness, and the fact that TIF1α, TIF1β and TIF1γ act as transcriptional repressors when tethered to DNA, these proteins have previously demonstrated significant functional diversity (Le Douarin *et al.*, 1996, Venturini *et al.*, 1999).

A previous study demonstrated that Ad5E4ORF3 binds and reorganizes TIF1α during infection, but reported unpublished data to show that TIF1α protein levels were unaffected (Yondola and Hearing, 2007). Consistent with these findings, work presented in this Chapter demonstrates that TIF1α is relocalized by E4ORF3 into nuclear tracks during Ad5 and Ad12 infection. Amongst other things, this relocalization is suggested to facilitate the ability of TIF1α to transactivate the glucocorticoid receptor (Teyssier *et al.*, 2006, Yondola and

Hearing, 2007). This hormone signalling pathway may be beneficial to adenoviruses since E4ORF3 has been shown to activate the glucocorticoid-responsive promoter (Wienzek and Dobbelstein, 2001).

In addition to TIF1α, these data strongly imply that TIF1γ is relocalized during Ad5 and Ad12 infection, and is likely to be targeted for degradation during infection with Ad3, Ad5, Ad7, Ad11 and Ad12 serotypes, which represent four out of the seven Ad groups. Although the precise mechanism of TIF1γ depletion has not been addressed in this Chapter it is feasible that, like with other Ad substrates, it is driven by ubiquitin-mediated proteasome-dependent degradation. Mass spectrometry data from Ad12E1B54K (where TIF1γ was initially detected) and TIF1γ immunoprecipitates identified the E3 ubiquitin ligase complex scaffold proteins CUL3, CUL7 and CUL9 as potential interactors (Table 4.1 and 4.2). Members of the cullin family have previously been implicated in the degradation of Ad substrates, for example CUL2- and CUL5-based E3 ubiquitin ligases are hijacked to induce p53 degradation by Ad12 and Ad5 E1B55K/E4ORF6 complexes, respectively (Querido *et al*., 2001, Blackford *et al*., 2010). Data presented in Chapter 5 will further examine the roles of CRLs in the degradation of TIF1γ during Ad infection.

The use of mutant viruses in this study has helped decipher the mechanism behind Ad-mediated TIF1γ regulation. During infection with Ad5E1B55K, Ad5E4ORF6 and Ad12E1B54K deletion mutants, TIF1γ was degraded at the same rate seen with the *wt* viruses (Fig 4.10A and Fig 4.12A). Although a mutant Ad12 virus lacking the E4ORF3 protein has yet to be generated, infection with H5*pm*4155 (Ad5ΔE4ORF3/E4ORF6) lead to a significant stabilization of TIF1γ, suggesting a direct role for Ad5E4ORF3 in promoting the degradation of TIF1γ. Data from Chapter 3 demonstrate that H5*pm*4155 infection results in the formation of multimeric Ad DNA. This is presumably due to the persistence of Mre11 nuclease activity

and it is unlikely that TIF1γ contributes to concatemer formation (Stracker *et al*., 2002). Transfection studies with Ad5 and Ad12 DNA expression vectors also confirmed that the presence of E4ORF3 was sufficient to promote the loss of TIF1γ 24 hours post-transfection, while in the same experiment the presence of E1B55K and E4ORF6 did not affect TIF1γ degradation (Fig 4.11A and 4.12B). Futhermore, E1B55K/E4ORF6 cotransfections had a negligible affect on TIF1γ protein levels confirming that TIF1γ regulation by adenoviruses is distinct from that of other substrates (Querido *et al*., 2001, Stracker *et al*., 2002, Baker *et al*., 2007, Dallaire *et al*., 2009b, Blackford *et al*., 2010, Orazio *et al*., 2011).

TIF1γ localization during Ad infection was determined by studying both the endogenous and exogenously expressed protein (Fig 4.6 and 4.9). Confocal microscopy revealed clear colocalization of TIF1γ and TIF1α proteins with PML in nuclear tracks, and this was conserved during Ad5 and Ad12 infection. On close inspection it can be noticed that the overlap between TIF1γ and TIF1α with PML staining is incomplete and probably arises because, like Mre11, their redistribution are separable events (Stracker *et al*., 2005). The significance of relocalizing proteins into nuclear tracks during Ad infection remains ambiguous (Geoffroy and Chelbi-Alix, 2011, Greer *et al*., 2011). Is it simply a mechanism by which proteins are sequestered away from sites of viral replication? Or do these structures harbour the ubiquitin-proteasome machinery necessary for substrate degradation? Multifaceted proteins such as TIF1γ could even perform important pro- or antiviral functions in these tracks such as transcriptional repression of cellular genes, activation of viral late gene expression or ubiquitylation.

Further analysis of cells transfected with viral DNA revealed that TIF1γ relocalization into nuclear tracks was also dependent on E4ORF3 expression (Fig 4.10-4.12). Indeed, during both Ad infection and viral gene expression studies, E4ORF3 was independently sufficient

and necessary to relocalize TIF1γ. Taken together, these data indicate that during Ad infection E4ORF3 uniquely functions to promote the degradation and relocalization of TIF1γ, however given the mass spectrometry and co-immunoprecipitation data, E1B55K may also have a non-essential role (Figs 4.1 and 4.2). E1B55K and E4ORF3 have previously been shown to functionally associate with each other within a small cellular pool separate from E1B55K/E4ORF6 complexes (Leppard and Everett, 1999). If this were the case it is likely that E4ORF3 and E1B55K interact with TIF1γ independently and have distinct roles in regulating TIF1γ, for instance E1B55K binding may modulate its transcriptional activity while E4ORF3 may solely be responsible for inducing its degradation. In addition to inducing the relocalization of cellular substrates E4ORF3 is also implicated in viral DNA replication, late mRNA splicing and late gene expression. Therefore studying these processes may provide clues as to the importance of TIF1γ degradation.

Ad5E4ORF3 has previously been shown to specifically target the N-terminal TRIM domain of TIF1α, which is conserved among TRIM family members (Yondola and Hearing, 2007). The importance of the TRIM domain in TIF1γ relocalization was studied here using a TIF1γC>A mutant construct which is catalytically inactive and unable to induce the formation of ubiquitin-conjugated products of SMAD4 (Dupont *et al*., 2005). Figure 4.10 demonstrates that this TIF1γ mutant was also partially defective in localizing to nuclear tracks during Ad12 (and to a lesser extent Ad5) infection when compared to the *wt* virus, indicating that E4ORF3 may interact with the TRIM domain of TIF1γ (Fig 4.9). The difference between Ad5 and Ad12 may reflect differences in E4ORF3 coding sequences, and it is possible that as with other Ad substrates these serotypes regulate TIF1γ in distinct fashions.

Mass spectrometric analysis was also carried out on TIF1γ co-immunoprecipitates from Ad12-infected cells, and a number of potential interacting proteins were identified (Table 4.2). Interestingly, p53, DNA ligase IV and PML were all identified giving credibility to this study since they are all relocalized along with TIF1γ into nuclear tracks by E4ORF3. Peptides from the protein USP9 were also detected in this screen, both before and during infection. This DUB opposes the activity of TIF1γ in the TGF-β signalling pathway which plays essential roles in growth and development (Dupont *et al*., 2009). Since these USP9 peptides were identified both before and after Ad12 infection, and no complementing experiments were carried out, it is difficult to speculate whether this is of consequence to the virus however it was also detected in immunocomplexes with Ad12E1B54K.

Other peptides of interest belonged to ATR and Chk1 proteins, which are principal players in the cellular response to DNA damage (Table 4.2). ATR is a PIKK which responds primarily to ssDNA breaks and is crucial for maintaining stability during S phase of the cell cycle (Zou, 2007, Cimprich and Cortez, 2008). Following activation, ATR phosphorylates Chk1 which acts as a transducer to trigger cell cycle arrest (Zhao and Piwnica-Worms, 2001). Interestingly ATR, along with its cofactor protein ATRIP, relocalize to VRCs during Ad5 and Ad12 infection, although this does not initiate a DDR with regards to Chk1 phosphorylation (Blackford *et al*., 2008, Carson *et al*., 2009). Results from Chapter 3 demonstrate that other serotypes (Ad3, Ad4, Ad7 and Ad11) are capable of inducing Chk1 phosphorylation and suggest that it is not detrimental to their replication. However, ATR regulation and ATR and Chk1 localization during these infections is currently unknown.

Ad proteins have previously been shown to impede cellular responses to DNA damaging reagents (Carson *et al*., 2009). Indeed, in comparison to control cells, cells expressing Ad5E4ORF3 which were microirradiated with a UVA laser showed a reduced localization of

Nbs1 to sites of DNA damage, and the protein was instead immobilized in nuclear tracks (Carson *et al*., 2009). A similar occurrence was seen in Ad5E4ORF3 expressing cells treated with HU where ATR signalling was found to be abrogated (Carson *et al*., 2009). In the current study co-immunoprecipitations revealed that TIF1γ and ATR interact with each other *in vivo*, and interestingly, this association was detected in both infected and non-infected cells (Fig 4.15). Combined with the mass spectrometry results summarized in Tables 4.1 and 4.2, this suggests a possible role for TIF1γ in the DDR. Contrary to this hypothesis however, TIF1γ protein expression was not affected by UVA damage, nor was it relocalized to sites of DNA damage following UVA microirradiation (Fig 4.16 and 4.17). Despite these results it is still plausible that TIF1γ may have an as yet unidentified role in the DDR.

Results from Chapter 3 revealed that the DDR protein TIF1β is phosphorylated during infection with all serotypes studied, presumably as a consequence of ATM kinase activity. In this Chapter, data indicate that TIF1β is unlikely to be an Ad substrate, although recent research has implicated it in controlling the switch from viral latency to lytic replication during KSHV infection (Cheng *et al*., 2009).

Several other TRIM family members that display antiviral activities have previously been identified, including TRIM1, TRIM5α, TRIM19 (PML), TRIM22 and TRIM30 (Nisole *et al*., 2005). The most studied member of this family, PML, interferes with the replicative cycle of many DNA and RNA viruses, and similar to the disruption of PODs by E4ORF3, its integrity is also affected by viral proteins from HCMV and EBV DNA viruses (Adamson and Kenney, 2001, Kang *et al*., 2006, Salsman *et al*., 2011). Retroviruses are also limited by TRIM proteins, for example human TRIM5α restricts MLV by targeting the capsid and other viral components for proteasomal degradation (Yap *et al*., 2004, Maillard *et al*., 2010). Along with

other publications this suggests that TRIM family members are a widespread class of proteins involved in the cellular antiviral response (Nisole *et al*., 2005).

In addition to identifying novel interacting proteins, the AmaZon ion trap mass spectrometer is able to preserve post-translational modifications such as the addition of phosphate groups to an amino acid side chain (Sobott *et al*., 2009). This technology enabled an advanced analysis of Ad12-infected TIF1γ immunoprecipitates and lead to the identification of a putative S/P phosphorylation site in the TIF1γ peptide sequence QSGLSSLVNGKS*PIR (Fig 4.14). Located at Ser862, this residue lies between the N-terminal TRIM domain and the C-terminal PHD finger, in an unconserved region previously known to encompass protein binding sites for SMAD2 and SMAD3 (He *et al*., 2006). Furthermore, the S/P site is just 28 residues away from the PHD domain which, although has an unknown function for TIF1γ, is required for TIF1β SUMOylation (Mascle *et al*., 2007). The likelihood of this site being phosphorylated during Ad infection is supported by a TIF1γ band shift seen during Ad12 infection (Fig 4.5A), suggesting that the slower migrating protein is post-translationally modified (although other modifications besides phosphorylation cannot be ruled out).

This specific Ser862 residue has previously been identified, but not studied, during a widespread screen examining proteins whose phosphorylation status is cell cycle-regulated (Dephoure *et al*., 2008). This publication identified the putative S/P phosphorylation site in cells arrested in G_1 and mitotic phases of the cell cycle, although cells arrested in other phases were not analysed. Recent work in our laboratory using bromodeoxyuridine (BrdU) incorporation and confocal microscopy, suggests that RNAi of the *TIF1γ* gene arrests HeLa cells in S and M phases of the cell cycle (Sedgwick and Turnell, unpublished observations). Therefore if TIF1γ has a role in cell cycle regulation adenoviruses may exploit this by

promoting its degradation in a phosphorylation-dependent manner. On the other hand, TIF1γ phosphorylation may just be a result of the Ad-induced S phase.

Although it was beyond the scope of this study, it will be interesting to validate this S/P phosphorylation site. Multiple serine kinases are activated during the cell cycle and by DNA damaging events, thus if Ser862 is confirmed as a *bona fide* phosphorylation site there would be many protein kinase candidates. Considering the similarity in peptide sequence, one likely candidate would be a CDK which are known to target [ST]PX[RK] canonical motifs (Songyang *et al*., 1994, Holmes and Soloman, 1996). Furthermore, E1A has previously been shown to associate with CDK complexes through interactions with the pRB family of proteins (Herrmann *et al*., 1991, Faha *et al*., 1993). KSHV viral protein kinases have been shown to phosphorylate TIF1 proteins during infection, therefore novel Ad protein kinases cannot be discounted (Cheng *et al*., 2009). Finally, TIF1 family members are often described as atypical protein kinases due to the ability of TIF1α and TIF1β to phosphorylate themselves, and other substrates, through an undefined kinase domain (Fraser *et al*., 1998, Nielsen *et al*., 1999). Due to the high degree of sequence conservation within the TIF1 family, TIF1γ is also suspected to possess intrinsic protein kinase activity, so it is possible that Ad infection induces TIF1γ autophosphorylation of Ser862.

Results described in this Chapter suggest that TIF1γ protein stability and localization is affected by Ad E4ORF3 expression in a distinct manner from other substrates, although the purpose and functional relevance of this regulation remains elusive. Its conserved nature may reflect the importance of targeting TIF1γ and indicates a potential role for this protein in the cellular antiviral response. Although data presented in Figure 4.5 suggest that TIF1γ depletion by RNAi does not affect the ability of Ad12 to degrade Mre11, future experiments could analyse other Ad substrates and functions since TIF1γ has the potential to act as an E3

ubiquitin ligase. In addition, further analysis of the TIF1γC>A mutant, and possibly the generation of a mutant encompassing the putative S/P phosphorylation site, may shed light on the significance of TIF1γ degradation and its possible function during Ad infection.

CHAPTER 5

REGULATION OF TIF1γ AND MRE11 BY E3 UBIQUITIN LIGASE COMPLEXES DURING ADENOVIRUS INFECTION

5.1 INTRODUCTION

Post-translational ubiquitin modifications mediate a multitude of cellular regulatory pathways including cell cycle progression, cellular differentiation, apoptosis and the DDR (Welchman *et al*., 2005). In this regard ubiquitylation regulates protein function through proteolytic and non-proteolytic mechanisms (Wilkinson *et al*., 2000, Glickman and Ciechanover, 2002).

The ubiquitylation process involves at least three types of enzyme: initially, an E1 enzyme interacts with, and activates, free ubiquitin in an ATP-dependent process; secondly, an E2 enzyme conjugates to the ubiquitin; and finally an E3 ubiquitin ligase enzyme catalyses the transfer of ubiquitin from the E2 to the protein substrate (Hershko *et al*., 1983, Welchman *et al*., 2005). Two main classes of E3 ubiquitin ligase have been identified based on the presence of either a HECT or RING domain (Al-Hakim *et al*., 2010). CRLs are large multi-subunit RING ligase complexes characterized by the presence of a central globular cullin subunit which acts as a scaffold and linker protein (Zheng *et al*., 2002). There are seven *bona fide* members of the highly conserved cullin family (CUL1, 2, 3, 4A, 4B, 5 and 7; Petroski and Deshaies, 2005), as well as several proteins which contain a cullin-homology domain (APC2 and CUL9; Yu *et al*., 1998, Skaar *et al*., 2007).

During Ad infection it is well established that E1B55K and E4ORF6 assemble into a complex that targets cellular proteins for degradation via the 26S proteasome (Querido *et al*., 2001). It has been determined, in part, that adenoviruses utilise CUL2- or CUL5-based E3 ubiquitin ligase complexes to promote the degradation of substrates; CUL2- and CUL5-based complexes assemble in a similar fashion to one another and comprise RBX1 RING domains and Elongins B and C, which determine substrate specificity at the N-terminus (Querido *et al*., 2001, Harada *et al*., 2002, Baker *et al*., 2007, Mahrour *et al*., 2008, Blackford *et al*., 2010). In most instances it accepted that E1B55K serves as the substrate recognition

component, while E4ORF6 recruits the cellular CRL via a direct interaction between Elongins B and C and one of its three BC boxes (Harada *et al*., 2002, Blanchette *et al*., 2004, Cheng *et al*., 2007, Luo *et al*., 2007). As with all cullin-based E3 ubiquitin ligases, the activity of these complexes is regulated through the covalent attachment of the ubiquitin-like polypeptide NEDD8 in a process known as neddylation (Pintard *et al*., 2003). This modification facilitates conformational changes within the cullin and RBX1 domains which are required to promote ubiquitylation, although little is known of how adenoviruses control this process (Duda *et al*., 2008).

Based on several reports, there seems to be a degree of heterogeneity in the composition of E1B55K/E4ORF6-containing CRLs between both virus serotypes and substrates, such that CUL2- and CUL5-based complexes are utilised to degrade p53 by Ad12 and Ad5 viruses, respectively (Querido *et al*., 2001, Blackford *et al*., 2010, Cheng *et al*., 2011). In addition to the diversity seen with p53, the Ad12-induced degradation of TOPBP1 utilises a CUL2-based E3 ubiquitin ligase complex where E4ORF6 probably acts as the adaptor protein by binding to substrates in the absence of E1B55K (Blackford *et al*., 2010). Previous reports also suggest that Mre11 and DNA ligase IV are degraded via a CUL5-based E3 ubiquitin ligase during Ad5 infection (Querido *et al*., 2001, Baker *et al*., 2007, Dallaire *et al*., 2009b), however preliminary data from our laboratory suggest that this is not the case (Forrester *et al*., 2011). Given these findings, and the identification of potentially novel E1B55K- and TIF1γ-interacting proteins as discussed in Chapter 4, it is possible that other E3 ubiquitin ligase complexes may be important for the degradation of Ad substrates.

5.2 AIMS

The specific aims of this Chapter were to further investigate the regulation of TIF1γ during Ad infection and to determine the underlying mechanisms involved in targeting known and novel Ad substrates for degradation by:

a) Verifying TIF1γ as a *bona fide* Ad substrate.

b) Exploring potential roles of cullin-based E3 ubiquitin ligases during Ad infection.

c) Investigating the roles of other E3 ubiquitin ligases during Ad infection.

d) Analysing the importance of ATR and CDK kinase activity in the degradation of Ad substrates.

5.3 RESULTS

5.3.1 TIF1γ is degraded in a proteasome-dependent manner during Ad5 and Ad12 infection

Results described in Chapter 4 demonstrate that the protein levels of the cellular transcriptional regulator TIF1γ are reduced following infection with a panel of Ad serotypes. To ascertain if this depletion is due to ubiquitin-mediated proteasome-dependent degradation, experiments were carried out using the potent cell-permeable proteasome inhibitor MG132. MG132 is a peptide aldehyde that can enter mammalian cells and block the proteolytic activity of the 26S proteasome by binding to the active sites of its 20S catalytic component (Zhang *et al*., 2009).

Thus, HeLa cells were first infected with Ad5 or Ad12 before being treated after 2 hours with either MG132 (10 μM in DSMO) or DMSO alone which served as a negative control. Cells were then harvested and analysed by Western blotting. Results indicate that while TIF1γ and DNA ligase IV protein levels were reduced 24 hours post Ad5- and Ad12-infection in cells treated with DMSO alone, both proteins were stabilized in the presence of MG132 (Fig 5.1A and B). This confirms that TIF1γ, like other cellular substrates including DNA ligase IV, Mre11 and p53, is targeted for ubiquitin-mediated proteasome-dependent degradation during Ad5 and Ad12 infection.

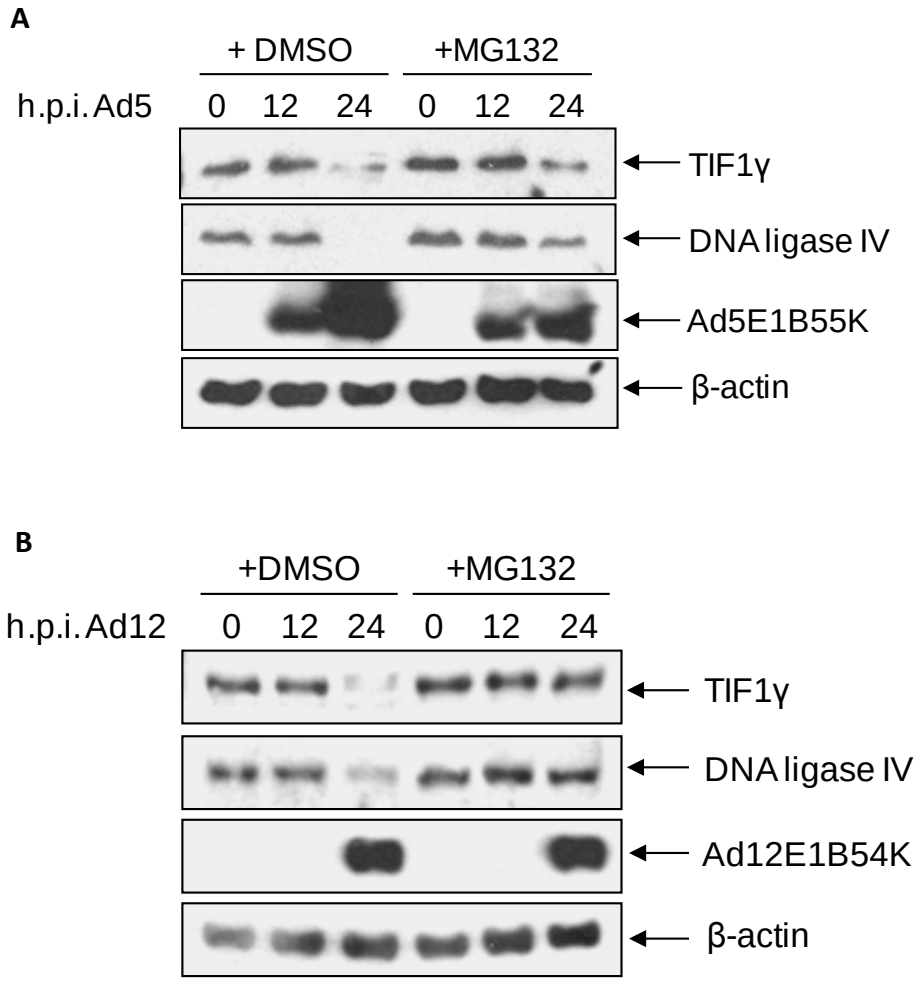

Fig 5.1 A and B. TIF1γ is degraded in a proteasome-dependent manner by Ad5 and Ad12

HeLa cells were infected with Ad5 (A) or Ad12 (B) in the presence or absence of 10 μM MG132 made up in DMSO which was added 2 hours after infection. Cells were harvested at the indicated time points and analysed by Western blotting using DNA ligase IV as a positive control and ß-actin as a loading control.

5.3.2 CUL2- and CUL5-based E3 ubiquitin ligase complexes are not required for TIF1γ, Mre11 or DNA ligase IV degradation during Ad5 or Ad12 infection

As discussed in the introduction to this Chapter, Ad proteins can hijack CRLs to target cellular proteins for proteasomal degradation (Querido *et al*., 2001, Harada *et al*., 2002, Blanchette *et al*., 2004, Blackford *et al*., 2010). To determine if adenoviruses similarly utilise CUL2 or CUL5 to promote the degradation of TIF1γ during Ad infection a series of RNAi experiments were carried out to silence their gene expression.

HeLa cells were therefore transfected with specific CUL2 and CUL5 siRNA oligonucleotides to specifically knockdown the expression of CUL2 and CUL5 proteins, or alternatively treated with a non-silencing siRNA to serve as a control. Twenty-four hours post-transfection, cells were infected with Ad5 or Ad12 viruses and harvested at the appropriate time points. Consistent with other studies, depletion of CUL5 during Ad infection inhibited p53 degradation, however it had no effect on TIF1γ, DNA ligase IV or Mre11 degradation (Fig 5.2A). CUL2 knockdown had no effect the degradation of any substrates during Ad5 infection, however during Ad12 infection it appropriately stabilized p53 and TOPBP1 protein expression (Fig 5.2; Blackford *et al*., 2010). CUL5 knockdowns also failed to inhibit the degradation of TIF1γ, DNA ligase IV or Mre11 during Ad12 infection (Fig 5.2B). Taken together these data suggest that adenoviruses do not utilise CUL2- or CUL5-based E3 ubiquitin ligase complexes in order to promote the degradation of TIF1γ, DNA ligase IV or Mre11 during infection.

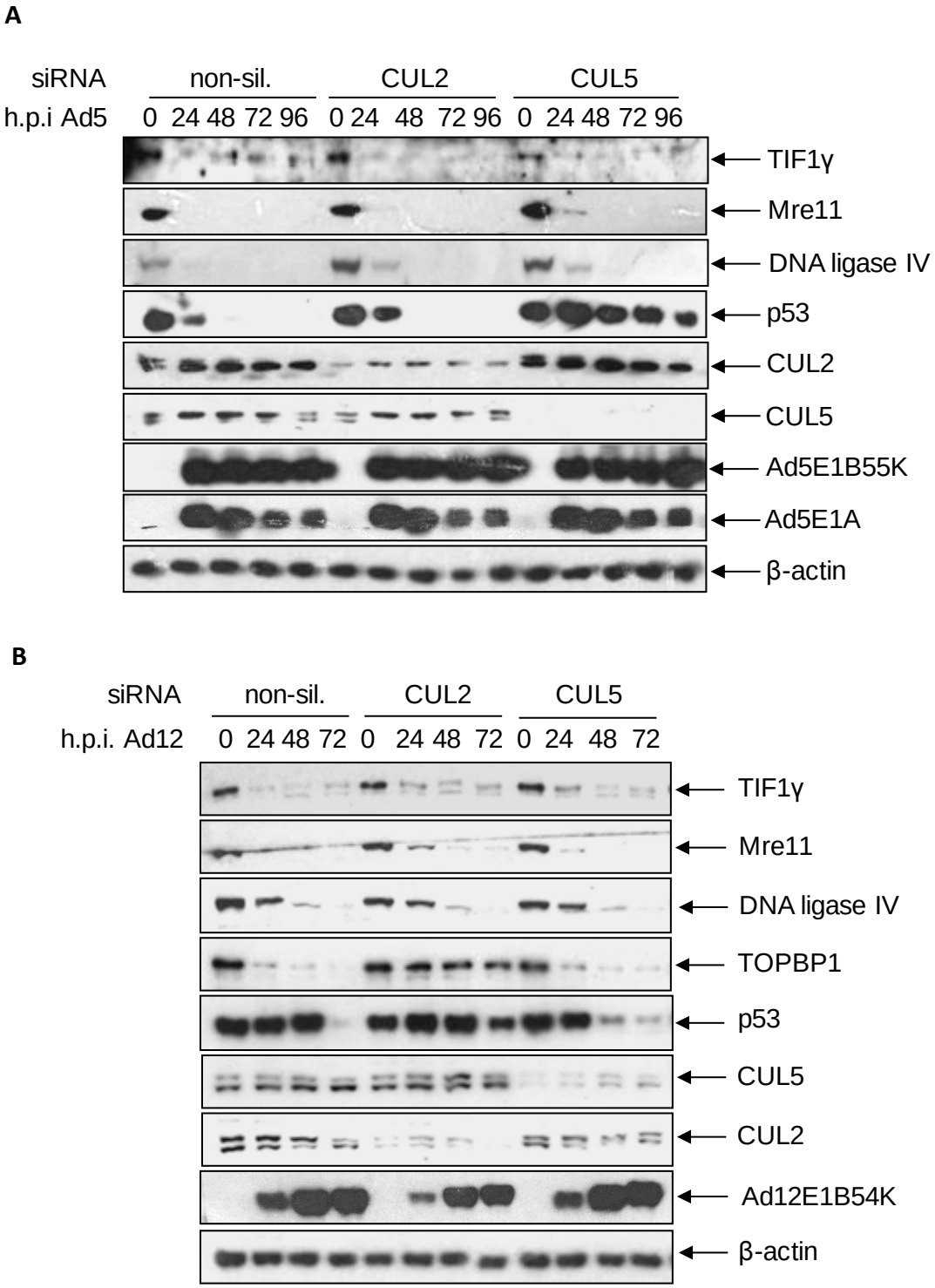

Fig 5.2 A and B. Degradation of TIF1γ, Mre11 and DNA ligase IV during Ad5 and Ad12 infection is CUL2- and CUL5-independent

HeLa cells were transfected with non-silencing siRNA or siRNA targeting CUL2 or CUL5 before being infected after 24 hours with Ad5 (A) and Ad12 (B). Cells were harvested and prepared for Western blotting at the indicated time points post-infection.

5.3.3 CUL1- and CUL3-based E3 ubiquitin ligase complexes are not required for Mre11 degradation during Ad5 infection

In order to ascertain whether other CRLs are required for the degradation of these substrates, systematic siRNA knockdowns of individual cullin family members were carried out and the stability of these proteins following Ad infection were subsequently assessed. Given that CUL1 is known to interact with E1A (Isobe *et al*., 2009), and that CUL3 was identified as a putative TIF1γ-interacting protein in Chapter 4, initially the possible usage of these cullins in Ad-substrate degradation were determined. The impact of CUL1 and CUL3 depletion on the Ad5-induced degradation of Mre11 was analysed in H1299 CUL5 knockdown, and H1299 control (pcDNA3) cells which were transfected with specific CUL1 or CUL3 siRNA oligonucleotides. H1299 CUL5 knockdown cells were utilised to rule out any functional redundancies that may exist between CUL1/CUL3 and CUL5. Western blotting revealed that although CUL1 and CUL3 knockdown was achieved, this had no effect on Mre11 degradation at 24 or 48 hours post-Ad5 infection (Fig 5.3). In addition to eliminating a role for CUL1 and CUL3 in Ad-mediated degradation of target substrates these data also support the notion that CUL5 is dispensable for Mre11 degradation.

5.3.4 CUL4A- and CUL4B-based E3 ubiquitin ligase complexes are not required for TIF1γ or Mre11 degradation during Ad5 infection

Next, the possible usage of CUL4A- or CUL4B-based E3 ubiquitin ligase complexes in Ad-substrate degradation was investigated. CUL4A has been implicated in the degradation of substrates from other viruses besides Ad such as HIV-1 and hepatitis B (Transey and Margottin-Goguet, 2009, Ahn *et al*., 2010, Belzile *et al*., 2010, Li *et al*., 2010). To determine

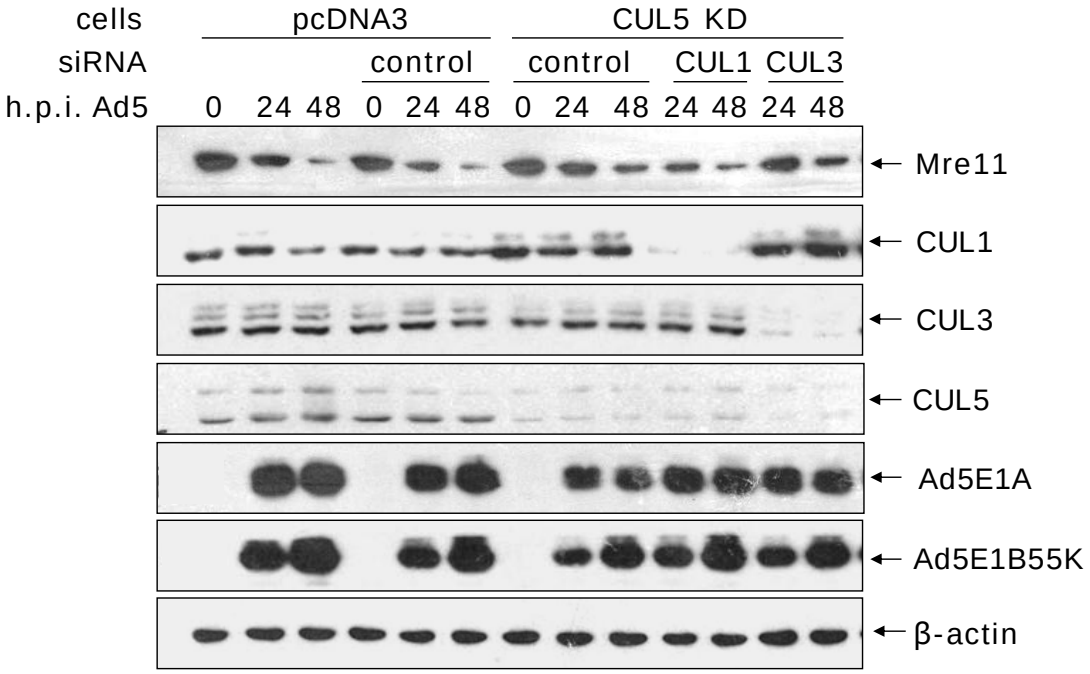

Fig 5.3. Degradation of Mre11 during Ad5 infection is CUL1-, CUL3- and CUL5-independent

H1299 CUL5 knockdown cells and H1299 control cells (pcDNA3) were transfected with non-silencing siRNA or siRNA targeting CUL1 or CUL3 before being infected after 24 hours with Ad5. Cells were harvested and prepared for Western blotting at the indicated time points post-infection.

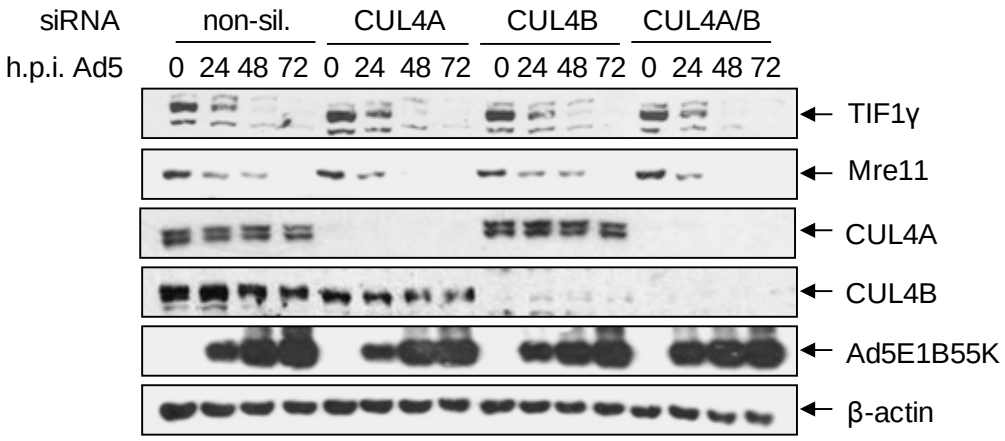

Fig 5.4. Degradation of TIF1γ and Mre11 during Ad5 and Ad12 infection is CUL4A- and CUL4B-independent

HeLa cells were transfected with non-silencing siRNA or siRNA targeting CUL4A and/or CUL4B before being infected after 24 hours with Ad5. Cells were harvested and prepared for Western blotting at the indicated time points post-infection.

if CUL4A, or its closely related ancestor CUL4B, is involved in the degradation of Ad substrates, transfections were carried out using CUL4A- and CUL4B-specific siRNA.

Following the transfection of CUL4A and/or CUL4B siRNA oligonucleotides, HeLa cells were infected with Ad5 and harvested over a period of 72 hours. Western blotting revealed that the depletion of these cullins was unable to inhibit, or delay, the degradation of TIF1γ or Mre11 during Ad5 infection (Fig 5.4). To allow for any functional redundancies between the CUL4A and CUL4B proteins, cotransfections were also carried out with CUL4A and CUL4B siRNAs; however this co-depletion also had no effect on TIF1γ or Mre11 degradation during Ad5 infection (Fig 5.4).

5.3.5 CUL7-based E3 ubiquitin ligase complexes are not required for TIF1γ or Mre11 degradation during Ad4, Ad5 or Ad12 infection

The final member of the cullin family to be considered for inducing Ad-mediated substrate degradation was CUL7 which was identified, along with TIF1γ, in immunocomplexes with Ad12E1B54K in Chapter 4. Thus, HeLa cells were initially transfected with CUL7-specific siRNA before being infected with Ad4, Ad5 and Ad12 serotypes. Western blotting revealed that CUL7 knockdown had no effect on TIF1γ or Mre11 degradation which was evident 24 hours post-Ad4, -Ad5 and -Ad12 infection (Fig 5.5A-C).

Taken in their entirety, these data suggest that adenoviruses do not rely solely upon CRLs in order to promote substrate degradation during infection.

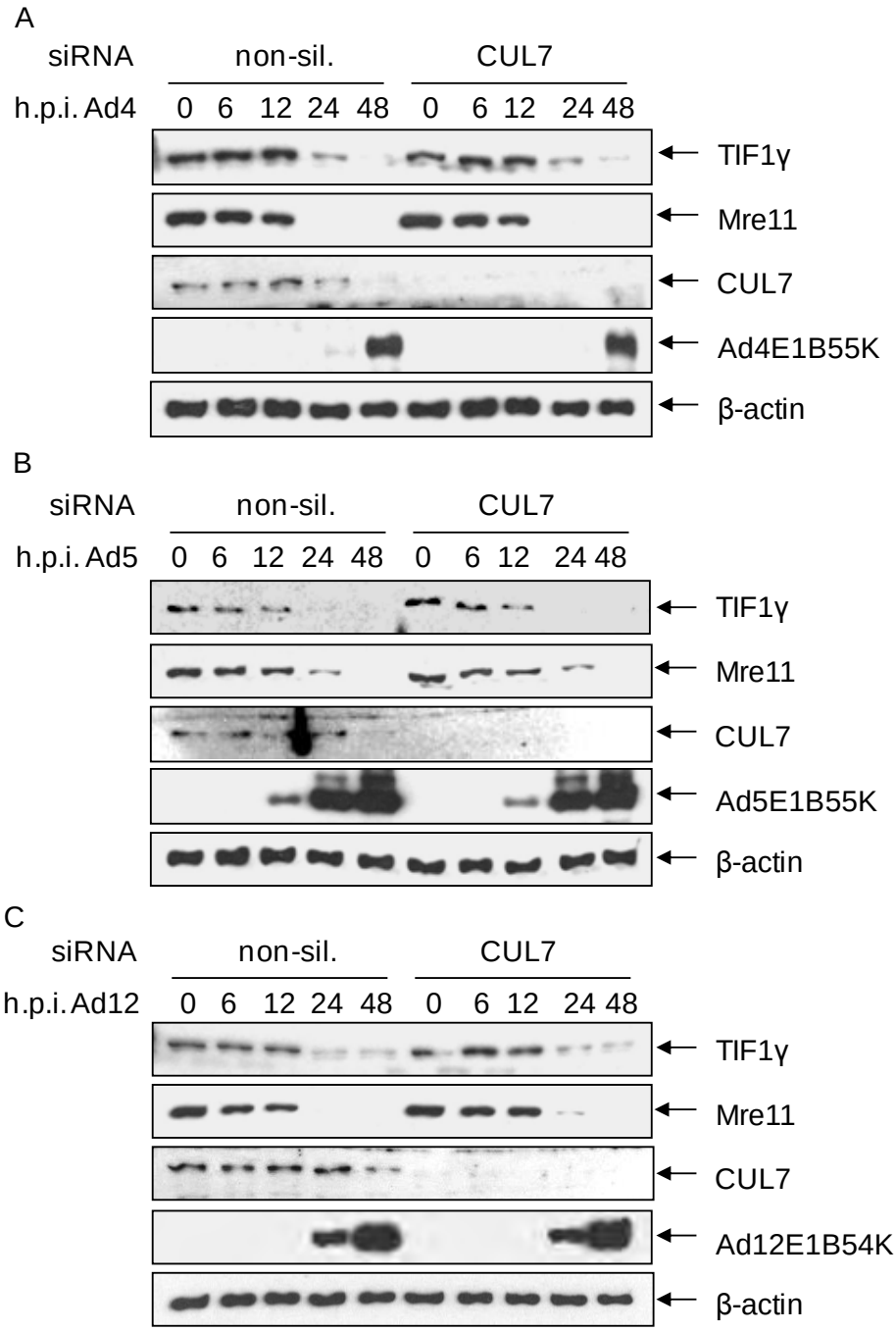

Fig 5.5A-C. Degradation of TIF1γ and Mre11 during Ad4, Ad5 and Ad12 infection is CUL7-independent

HeLa cells were transfected with non-silencing siRNA or siRNA targeting CUL7 before being infected after 24 hours with Ad4 (A), Ad5 (B) or Ad12 (C). Cells were harvested and prepared for Western blotting at the indicated time points post-infection.

5.3.6 TIF1α is not required for TIF1γ degradation during Ad5 or Ad12 infection

Given that the data so far presented in this Chapter suggests that Ad-mediated TIF1γ degradation is not driven by CRLs containing CUL1-7 proteins, the potential requirement of non-cullin-based E3 ubiquitin complexes was investigated. One potential candidate was TIF1α which has a close relationship with TIF1γ and was also identified in Chapter 4 as putative Ad12E1B54K-interacting protein.

To examine this further, HeLa cells were transfected with TIF1α-specific siRNA before being infected with Ad5 or Ad12 and harvested. Western blot analysis demonstrated that TIF1α knockdown had no effect on TIF1γ degradation during Ad5 infection (Fig 5.6A), or TIF1γ or Mre11 degradation during Ad12 infection (Fig 5.6B).

5.3.7 TIF1γ degradation during Ad5 and Ad12 infection is independent of ATR kinase activity

ETD mass spectrometric analysis of TIF1γ immunoprecipitates described in Chapter 4 identified a putative S/P phosphorylation site within the central unconserved region of the TIF1γ protein that was phosphorylated in response to Ad infection. The same screen also identified the serine/threonine-protein kinase ATR which was shown to interact with TIF1γ *in vivo*. ATR is activated in response to DNA damage (and during infection with certain Ad serotypes), however its catalytic activity can be inhibited by the radiosensitizing agent caffeine (Hall-Jackson *et al*., 1999, Sarkaria *et al*., 1999).

In some instances phosphorylation events are known to stimulate ubiquitin-mediated degradation (Koepp *et al*., 2001, Lin *et al*., 2006); therefore to determine if ATR kinase

activity has a role in TIF1γ degradation during Ad infection, HeLa cells were first infected with Ad5 or Ad12 and subsequently incubated with caffeine (5 mM in DMSO), or DMSO alone as a negative control. Western blotting revealed that TIF1γ was still degraded during Ad5 and Ad12 infection in cells treated with caffeine, whereas H2AX was appropriately phosphorylated to a lesser extent confirming that ATR kinase activity was inhibited (Fig 5.7A and B). Noticeably, TIF1γ protein levels were generally reduced in the presence of the drug, even in mock treated cells, suggesting that ATR, or another caffeine-sensitive kinase, may stabilize TIF1γ protein levels (Fig 5.7A and B).

5.3.8 TIF1γ degradation during Ad5 infection is independent of CDK kinase activity

Interestingly, the putative S/P TIF1γ phosphorylation site identified in Chapter 4 was flanked by similar residues to those found in canonical CDK-target motifs (Songyang *et al*., 1994, Holmes and Soloman, 1996). Therefore the significance of CDK activity on Ad5-induced TIF1γ degradation was examined using the purine analogue roscovitine, which is a potent and selective inhibitor of CDK phosphorylation (Meijer *et al*., 2007).

HeLa cells were first infected with Ad5 and subsequently incubated with roscovitine (20 μM in DMSO) to inhibit CDK kinase activity, or DMSO alone as a negative control. Western blot analysis revealed that TIF1γ degradation was not inhibited in the presence of roscovitine during Ad5 infection (Fig 5.8). Due to the effect of Ad infection on cell cycle progression, a suitable positive control was not able to be included in this experiment. Taken together these data suggest that ATR and CDKs do not promote TIF1γ degradation during Ad infection.

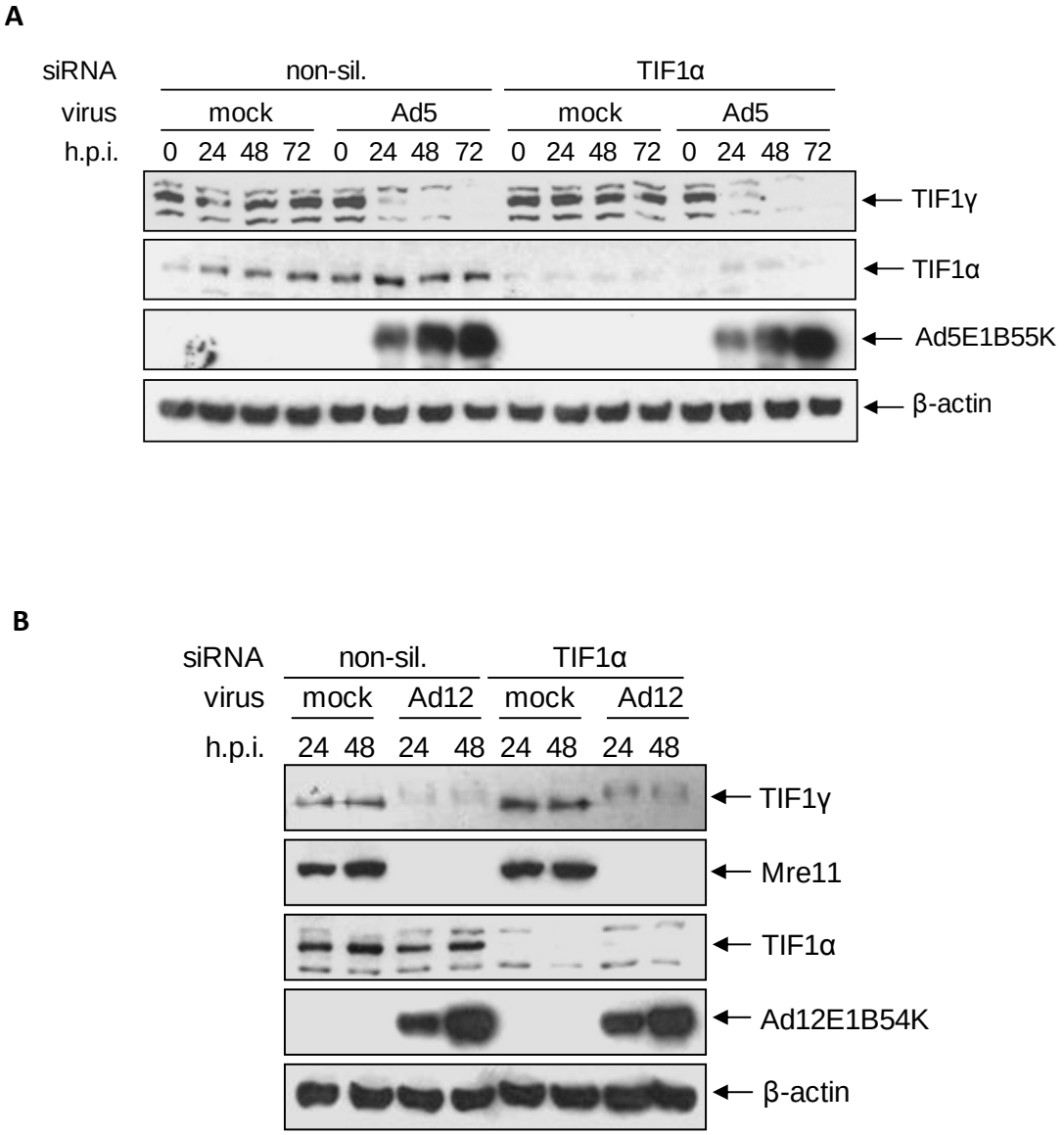

Fig 5.6A and B. Degradation of TIF1γ during Ad5 and Ad12 infection is TIF1α-independent

HeLa cells were transfected with non-silencing siRNA or siRNA targeting TIF1α before being infected after 24 hours with Ad5 or Ad12. Cells were harvested and prepared for Western blotting at the indicated time points post-infection.

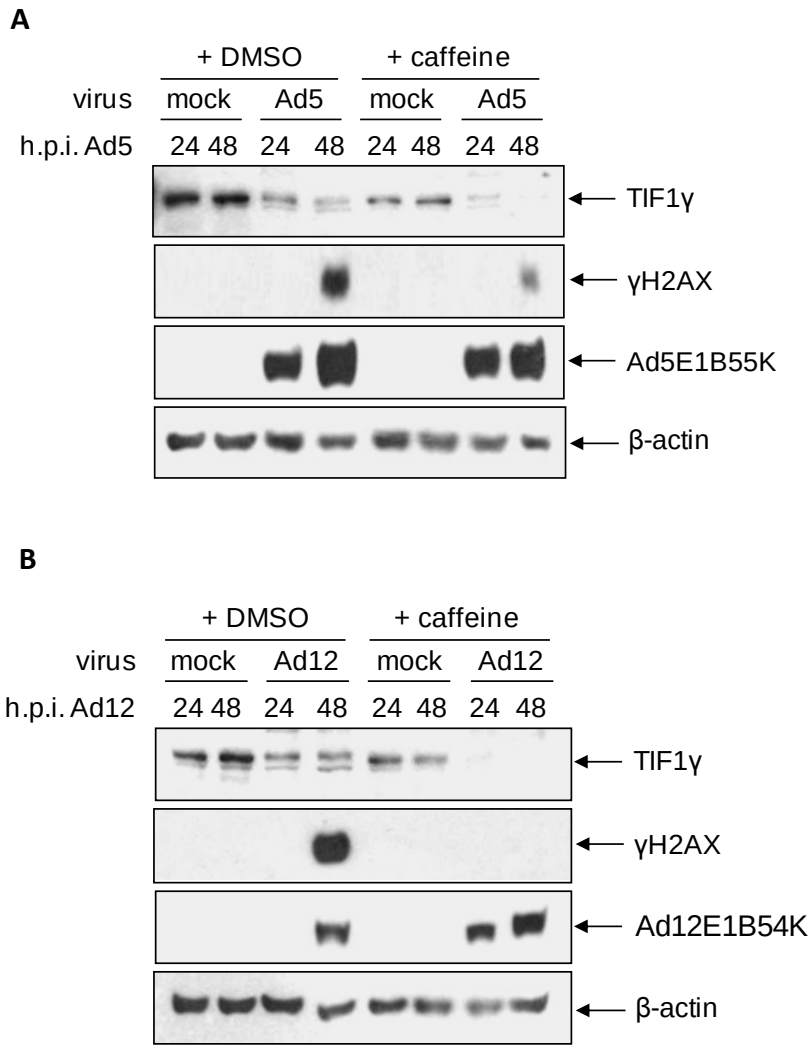

Fig 5.7 A and B. Degradation of TIF1γ during Ad5 and Ad12 infection is independent of putative ATR phosphorylation

HeLa cells were infected with Ad5 (A) or Ad12 (B) in the presence or absence of 5 mM caffeine made up in DMSO which was added 2 hours after infection. Cells were harvested after 24 and 48 hours and prepared for Western blotting. H2AX phosphorylation was used as a positive control.

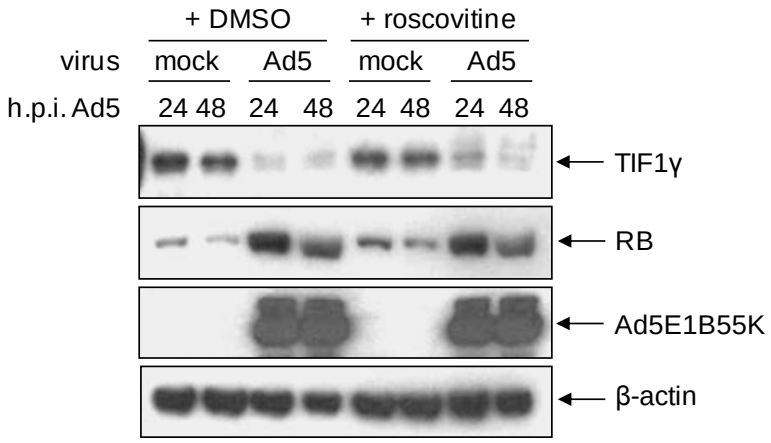

Fig 5.8. Degradation of TIF1γ during Ad5 infection is independent of putative CDK phosphorylation

HeLa cells were infected with Ad5 in the presence or absence of 20 μM roscovitine made up in DMSO which was added 2 hours after infection. Cells were harvested after 24 and 48 hours and prepared for Western blotting.

5.4 DISCUSSION

In conjunction with observations described in Chapter 4, data presented in this Chapter support the notion that TIF1γ is targeted for ubiquitin-mediated proteasome-dependent degradation during Ad infection (Fig 5.1). Based on these findings, putative TIF1γ ubiquitylation and phosphorylation modification events were investigated during Ad infection. The role of CRLs in TIF1γ degradation were considered since CUL2 and CUL5 have previously been implicated in Ad-dependent substrate proteolysis (Querido *et al*., 2001, Harada *et al*., 2002, Baker *et al*., 2007, Blackford *et al*., 2010).

RNAi was utilised to interfere with the gene expression of most cullin proteins, and Western blotting demonstrated a significant level of knockdown was achieved in all the experiments reported here. However results revealed that during both Ad5 and Ad12 infection, CUL2 and CUL5 depletion had no effect on Mre11, DNA ligase IV or TIF1γ degradation, which progressed unabated (Fig 5.2); indeed, Mre11 was also degraded during Ad5 infection in H1299 CUL5 knockdown cells (Fig 5.3). As anticipated, p53 was not degraded during Ad5 and Ad12 infection when cells were transfected with CUL5 and CUL2 siRNA, respectively. These data contradict current assumptions that Mre11 is degraded by a CUL5-based E3 ubiquitin ligase complex; however even in these previous investigations the evidence is questionable. For example, Mre11 and integrin alpha 3 are only slightly stabilized in Ad5-infected H1299 CUL5 knockdown cells, and E1B55K/E4ORF6 protein expression levels varied appreciably from control samples (Querido *et al*., 2001, Dallaire *et al*., 2009b).

A recent publication also suggests that adenoviruses exclusively utilise CUL2- or CUL5-based E3 ubiquitin ligase complexes to degrade substrate proteins, which is in contrast to data presented here (Cheng *et al*., 2011). Cheng and colleagues reported that tagged E4ORF6 plasmid constructs for Ad4, Ad5, Ad9 and Ad34 co-immunoprecipitated with CUL5, while

constructs for Ad12, Ad16 and Ad40 co-immunoprecipitated with CUL2. Degradation assays using plasmid DNA also showed that in the presence of Ad5 and Ad12 E1B55K/E4ORF6 proteins, H1299 CUL5 and CUL2 knockdown cells, respectively, were unable to degrade Mre11 (although the expression of Ad5E1B55K was significantly less than in control cells; Cheng *et al*., 2011). Several discrepancies from this study have already been reported in Chapter 3 regarding substrate specificities. Reasons for these differences remain unknown at present, although in contrast to previous reports, the work described here utilised HeLa cells in the context of a full infection, and it is possible that the presence of other viral gene products may be significant. It should also be noted that the expression of a dominant-negative CUL5 mutant protein has been shown to inhibit the degradation of DNA ligase IV during Ad5 infection (Baker *et al*., 2007), however, in the current study Ad5- and Ad12-mediated degradation of DNA ligase IV was not inhibited when CUL5 or CUL2 protein expression was depleted by siRNA (Fig 5.2).

CUL2- and CUL5-based E3 ubiquitin ligase complexes are functionally redundant for oogenesis and activation of the mitogen-activated protein (MAP) kinase, MPK-1, in *Caenorhabditis elegans* (Sasagawa *et al*., 2007). Therefore to accommodate this possibility during Ad-mediated proteolysis of Mre11, DNA ligase IV and TIF1γ, future experiments could be carried out where cells are cotransfected with siRNAs targeting both CUL2 and CUL5.

There are two possible shortcomings in the current data which must be considered, as there can often be limitations and weaknesses in experimental procedures. Firstly, all gene depletions carried out on cullin and TIF1α proteins in this Chapter were achieved though siRNA-mediated knockdown which, unlike stable conditional "knockout" models, may not completely silence gene expression in 100% of the cell population. Therefore, despite the

strong overall reduction gained from RNAi, residual expression of these proteins may account for the degradation of Mre11 and TIF1γ seen during Ad infection. With siRNA there is also the possibility that it will generate "off-target" effects within the cell (Jackson and Linsley, 2010). Secondly, it must be considered that E3 ubiquitin ligase complexes may have different affinities for different substrates. For example, while siRNA knockdown of CUL2 was sufficient to stabilize p53 and TOPBP1 protein expression during Ad12 infection, residual levels of functioning CUL2 complexes may have higher affinities for other proteins such as Mre11 and TIF1γ causing them to be preferentially targeted for degradation.

This study also investigated the role of CUL1-, CUL3-, CUL4A-, CUL4B- and CUL7-based E3 ubiquitin ligase complexes in Ad-mediated degradation of target substrates. Some of these scaffold proteins are already reported to be exploited by a range of viruses known to interact with the ubiquitin-proteasome system to promote viral replication and circumvent immune responses. It is often suggested that the hijack of such complexes is achieved by viral proteins mimicking cellular adaptors. Indeed, the EBV encoded protein BPLF1 has recently been found to bind directly to and deneddylate CUL1 and CUL4A (Gastaldello *et al*., 2010). This inactivation leads to the stabilization of CDT1 (chromatin licensing and DNA replication factor 1) which induces a deregulated S phase-like environment that is permissive for viral DNA replication (Gastaldello *et al*., 2010). Cellular transformation by SV40 TAg is also dependent on TAg binding to a cellular CUL7-based E3 ubiquitin ligase complex, while this same complex aids viral replication by inducing the proteolysis of MRN to manipulate the cellular DDR (Ali *et al*., 2004, Zhao *et al*., 2008). Recently, cullins have also been implicated in HIV-1 cell cycle regulation. In this instance, the HIV-1 Vpr protein manipulates DDB1-CUL4A E3 ubiquitin ligase complex activity to induce G_2/M arrest and degrade its cellular substrates (Transey and Margottin-Goguet, 2009, Ahn *et al*., 2010, Belzile *et al*., 2010).

CUL4-contining E3 ubiquitin ligase complexes are also hijacked by other viral proteins such as paramyxovirus SV5, hepatitis B virus X (HBX) protein and woodchuck hepatitis B virus X (WHX) protein to facilitate viral replication (Li *et al*., 2010).

In contrast, viruses can also interfere with CRLs to reduce the degradation of target proteins. Indeed, to aid unregulated proliferation, Ad E1A directly binds CUL1 to attenuate the function of the SCF^{Fbw7} E3 ubiquitin ligase complex (Isobe *et al*., 2009). It was therefore unlikely that CUL1 complexes would be utilised by Ad E1B55K/E4ORF6 proteins to ubiquitylate cellular substrates, and this was the conclusion drawn from knockdown experiments examining Mre11 degradation during Ad5 infection (Fig 5.3). Mre11 and TIF1γ degradation during Ad infection was also unaffected by the siRNA-mediated knockdown of CUL3, CUL4A, CUL4B or CUL7 (Fig 5.3-5.5).

Cullin activity is regulated through the covalent attachment of the ubiquitin-like polypeptide, NEDD8, and neddylated versions of these proteins can be detected as slower migrating bands during SDS-PAGE and Western blotting (Pintard *et al*., 2003, Duda *et al*., 2008). Thus it was seen that CUL5 and CUL2 proteins became neddylated during Ad5 and Ad12 infection, respectively (Fig 5.2), presumably for the degradation of p53 and TOPBP1 (Blackford *et al*., 2010). However for all the other cullin proteins examined, banding patterns were relatively uniform during Ad infection suggesting a lack of activation (Fig 5.3-5.5). Taken together these data suggest that Mre11 and TIF1γ are not targeted by CRLs during Ad5 or Ad12 infection.

Another candidate for directing E3 ubiquitin ligase activity towards cellular Ad substrates was TIF1α. This protein contains an N-terminal RING-finger motif and was identified in Chapter 4 as a binding partner of Ad12E1B54K. In addition, TIF1α is relocalized into PML-containing nuclear tracks by E4ORF3 during Ad infection where its function remains

unknown, although it is not targeted for degradation like its close relative TIF1γ (Chapter 4; Yondola and Hearing, 2007). Despite these features, siRNA-mediated knockdown of TIF1α failed to rescue Mre11 or TIF1γ degradation which was still apparent 24 hours post-Ad5 and -Ad12 infection (Fig 5.6).

Given that TIF1γ is a *bona fide* E3 ubiquitin ligase itself, it may also be capable of autoubiquitylating itself to regulate its own degradation during Ad infection. Indeed, this has recently been reported for the first time where PHD/BRD-dependent TIF1γ histone binding induced autoubiquitylation of its TRIM domain, in the presence or absence of TGF-β signalling (Agricola *et al.*, 2011). Data from Chapter 4 suggest that Ad-mediated TIF1γ relocalization, and possibly degradation, is dependent the interaction between its TRIM domain and E4ORF3. Therefore if TIF1γ does undergo autoubiquitylation, this may explain why the TIF1γC>A point mutant with no E3 ubiquitin ligase activity is less readily relocalized into E4ORF3-containing nuclear tracks during Ad12 infection (Fig 4.9B), and suggest that E4ORF3 may activate this putative TIF1γ ligase activity. It will therefore be interesting to investigate whether the TIF1γC>A mutant is able to be degraded during Ad infection, however as E4ORF3 is likely to interact with the TRIM domain of TIF1γ it will be difficult to discriminate between E4ORF3 recruiting TIF1γ to nuclear tracks, and cellular E3 ubiquitin ligase complexes and E4ORF3 functioning to directly activate intrinsic E3 ubiquitin ligase activity.

Results from Chapter 4 suggested that TIF1γ is phosphorylated during Ad infection at Ser862. Phosphorylation events are also known to promote the destruction of proteins via the ubiquitin-proteasome pathway (Koepp *et al.*, 2001, Lin *et al.*, 2006), therefore in the current Chapter the potential role of two classes of protein kinases were investigated; ATR, a serine/threonine kinase activated in response to DNA damage, and CDKs which are central

regulators of the cell cycle. CDKs phosphorylate their substrates on serine or threonine residues which are followed by a proline at position +1 and an arginine or lysine at position +3 (Songyang *et al*., 1994). This arrangement is analogous to the S*PIR sequence identified in Chapter 4. Unfortunately, both ATR and CDK chemical inhibitors failed to significantly inhibit TIF1γ degradation in Ad-infected cells (Fig 5.7 and 5.8). This suggests that either phosphorylation is inconsequential for TIF1γ degradation, or that Ser862 is phosphorylated by another cellular kinase. The future development of a phospho-specific antibody or the mutagenesis of Ser862 may help to determine the importance of this putative modification. Data presented in Chapter 4 revealed that TIF1γ degradation is conserved amongst a selection of Ad serotypes from species A, B1, B2 and C. Out of these viruses, Ad3, Ad7 and Ad11 (unlike Ad5 and Ad12) have been shown to activate ATR kinase activity towards Chk1 during infection (Chapter 3), therefore it will also be interesting to investigate the effect caffeine has on the ability of these viruses to degrade TIF1γ.

In addition to the E3 ubiquitin ligases mentioned here, several other proteins with known or putative ligase activity were also examined. These included CUL9, UBR5, CtIP, CBP, p300 and APC3; however siRNA-mediated knockdown of all of these genes failed to impede Mre11 degradation during Ad infection (data not shown).

Given the diversity of p53 regulation seen between adenoviruses, it is likely that Mre11, DNA ligase IV and TIF1γ are differentially regulated during infection by other Ad serotypes. To our knowledge, the only similarity between adenoviruses and cullin specificity is that both Ad4 and Ad5 utilise a CUL5-based E3 ubiquitin ligase to target p53 for degradation (Fig 5.2A and data not shown). At present, it is not understood how CRL selection occurs by E4ORF6 since E4ORF6 sequences do not contain the CUL2- or CUL5-consensus motifs that are used by cellular proteins (Luo *et al*., 2007, Mahrour *et al*., 2008). This suggests that CRLs

are hijacked by viral proteins during Ad5 and Ad12 infection via a non-canonical process or binding sequence.

Data from Chapter 4 strongly imply that TIF1γ protein stability is affected by Ad E4ORF3 expression in a manner distinct from other substrates, therefore it is likely that TIF1γ and Mre11 are differentially regulated during Ad infection. This is supported by the fact that, while all serotypes studied were capable of degrading TIF1γ (and DNA ligase IV), only some degraded Mre11. Although the present study failed to identify the specific E3 ubiquitin ligase complex required for degrading these proteins, there are many more candidates yet to be investigated, some of which were identified by mass spectrometry in Chapter 4. It should also be noted that ubiquitylation has not actually been demonstrated on any of the MRN components, and since the absence of one member is sufficient to destabilize the entire complex the direct target is still yet to be determined.

In conclusion, it appears that the Ad-mediated degradation of target substrates is more complex than first described, and that more work is needed to fully determine the relationship between adenoviruses and the ubiquitin-proteasome pathway.

CHAPTER 6

FINAL DISCUSSION AND FUTURE PERSPECTIVES

6.1 FUTURE PERSPECTIVES OF SEROTYPE-SPECIFIC INACTIVATION OF THE DDR

Previously, it had been generally assumed that the relationship between adenoviruses and the DDR was, with some exceptions, broadly similar amongst different serotypes (Stracker *et al*., 2005, Cheng *et al*., 2011). However, recent observations from our laboratory showing that Ad5 and Ad12 differentially inactivate ATR (Blackford *et al*., 2010) prompted the current study into how adenoviruses from other Ad species interact with the cellular DDR.

Data presented in Chapter 3 revealed that representative Ad serotypes from species A to E are all capable of activating a subset of the DDR, but there was considerable diversity regarding how these serotypes abrogated signal transduction pathways in order to prevent checkpoint activation and apoptosis. Interestingly, out the four cellular Ad substrates examined, only DNA ligase IV protein levels were reduced following infection with all viral serotypes tested. Presumably DNA ligase IV degradation prevents the illegitimate repair of viral genomes by NHEJ, as previously reported (Baker *et al*., 2007, Jayaram *et al*., 2008). It will be interesting to ascertain by immunofluorescence if DNA ligase IV is relocalized into nuclear tracks prior to its degradation though the development a suitable antibody, or by using a tagged construct to detect it exogenously. Relocalization is the fate for most Ad substrates, therefore this will be especially interesting to determine for the species B serotypes, Ad3, Ad7 and Ad11, which are unable to degrade or relocalize Mre11, p53 or TOPBP1.

In addition to DNA ligase IV degradation, it is also possible that species B serotypes have evolved novel mechanisms to circumvent the DDR, especially since the E4ORF6 BC boxes from these viruses are highly homologous to those of Ad5 and Ad12 and may therefore have the ability to interact with cellular proteins (Cheng *et al*., 2007). The development of mutant viruses and antibodies against these less studied serotypes may help to decipher these

theories, and to determine whether their E1B55K and E4ORF6 proteins are responsible for inducing the ubiquitin-mediated proteasome-dependent degradation of DNA ligase IV.

The levels of p53 following infection were also examined in Chapter 3 and it was discovered that during Ad3, Ad7, Ad9 and Ad11 infection p53 protein levels were increased substantially (presumably through the action of E1A); however in this instance p53 was transcriptionally inactive. As p53 regulation is extremely complex, and MDM2 protein levels were reduced during these infections, further studies are needed to determine the mechanism behind p53-MDM2 inactivation and its significance to the virus. One possibility is that this transcriptional inactivation is caused by an epigenetic mechanism that occurs irrespective of protein degradation. Indeed, a recent study revealed that during Ad5 infection E4ORF3 induces *de novo* H3K9me3 methylated heterochromatin formation at p53 target promoters in order to prevent p53 binding to promoter sites (Soria *et al*., 2010).

In agreement with previous reports, the studies presented in Chapter 3 of this thesis determined that Chk1 was not activated during Ad5 or Ad12 infection, however, during Ad3, Ad7 and Ad11 infection Chk1 was phosphorylated. This correlates with the inability of species B viruses to degrade or relocalize the MRN complex, and suggests that a degree of ATR activation is tolerated by these viruses as no indication of apoptosis was seen. As Chk1 regulates the S phase checkpoint through CDC25A degradation, and since E1A has been shown to increase CDC25A phosphatase activity, it will be interesting in future studies to take a closer look at Chk1/CDC25A pathway components in cells infected with species B viruses (Spitkovsky *et al*., 1996, Grand *et al*., 1998, Xiao *et al*., 2003). Furthermore, phospho-specific antibodies could be utilised to determine the localization of activated Chk1 during Ad3, Ad7 and Ad11 infection, as sequestration by viral proteins may impede signal transduction. It is also possible that Chk1 activation by species B adenoviruses could be akin

to HIV-1 and SV40 infections where activated DDR pathways are utilised to promote viral genome replication (Lai *et al*., 2005, Boichuk *et al*., 2010, Rohaly *et al*., 2010). Indeed, previous reports have suggested that certain undefined aspects of the species B Ad life cycle differ from canonical pathways utilised by other serotypes (Hall *et al*., 2010). These include virus disassembly, virus release, replication efficiency and utilising the human membrane cofactor CD46 receptor rather than the CAR receptor for cellular attachment (Kawakami *et al*., 2003, Sirena *et al*., 2004, Hall *et al*., 2010).

Immunofluorescence and Western blotting techniques employed in Chapter 3 further investigated the relationship between protein relocalization and degradation. For Mre11, these data indicate that there is not always an obvious correlation between these events since during Ad12 infection Mre11 is degraded but resides in VRCs, while during Ad9 infection it is relocalized into nuclear tracks but not degraded. At present the purpose of protein relocalization during Ad infection is not fully understood and more work is needed to understand why adenoviruses have different effects on the cellular DDR.

6.2 A ROLE FOR TIF1γ IN THE CELLULAR DEFENCE TO AD INFECTION

This study endeavoured to identify novel Ad-interacting proteins via mass spectrometric analysis of anti-Ad12E1B54K immunoprecipitates. Using this approach, the transcriptional regulator TIF1γ was identified as a *bona fide* E1B55K-interacting protein which is targeted for ubiquitin-mediated proteasome-dependent degradation during Ad3, Ad5, Ad7, Ad11 and Ad12 infection.

The most interesting aspect of this interaction is the unique mechanism by which TIF1γ is degraded by adenoviruses. Indeed, unlike other Ad substrates TIF1γ is both degraded and

relocalized in an E1B55K/E4ORF6-independent E4ORF3-dependent manner representing a novel way in which adenoviruses manipulate the cellular environment. Although the relationship is yet to be completely defined, future co-immunoprecipitation and *in vitro* GST (glutathione S-transferase)-pulldown binding assays, should help to decipher the nature of the TIF1γ-E4ORF3 interaction. Ad infections could also be carried out in cells transfected with the E3 ubiquitin ligase dead TIF1γC>A mutant to determine if, along with restricted relocalization, this mutant protein is unable to be degraded by E4ORF3. This could also be addressed by transfecting cells with a TIF1γ mutant expressing only the N-terminal TRIM domain and seeing if this is sufficient for E4ORF3 to target the protein; data indicate that Ad5E4ORF3 interacts with TIF1α through its TRIM domain (Yondola and Hearing, 2007). In addition to proteolytic degradation, TIF1γ (akin to p53) function might be modulated by E4ORF3-dependent methylation of TIF1γ-target promoters (Soria *et al*., 2010). Therefore, it would be interesting to study whether E4ORF3 expression alters the methylation status at promoter regions of TIF1γ/SMAD-regulated genes. Similarly, the possibility that TIF1γ is SUMOylated should be investigated since E1B55K-mediated SUMOylation has been found to inhibit p53 transcriptional activity (Muller and Dobner, 2008, Pennella *et al*., 2010).

While data presented in Chapter 5 was unsuccessful in identifying the E3 ubiquitin ligase accountable for Ad-induced TIF1γ degradation, conventional CRLs were ruled out. This may not be surprising since E4ORF3 lacks the BC box motif necessary to mediate an interaction with these complexes. There is however an abundance of other proteins that may be responsible, and as mentioned in Chapter 5 the possibility that TIF1γ targets itself for proteolytic destruction via autoubiquitylation cannot be discounted. As is common for many cellular Ad substrates, TIF1γ was also relocalized into nuclear tracks during Ad5 and Ad12 infection in an E4ORF3-depndent manner. The ability of Ad3, Ad7 and Ad11 to relocalize

TIF1γ will also be interesting to determine since nuclear track formation is not a prerequisite for proteolytic degradation, and these serotypes are unable to divert p53, Mre11 or TOPBP1 away from VRCs.

Further studies are needed to determine the significance of TIF1γ inactivation by adenoviruses, especially since its conserved nature may reflect its importance to viral persistence. It is reasonable to suggest that TIF1γ abrogation prevents some unknown function of the protein that would otherwise be detrimental for the virus. TIF1γ is known to have tumour suppressive properties in CMML and PDAC, and hence could initiate programmed cell death and cell cycle arrest in infected cells (Vincent *et al*., 2009, Aucagne *et al*., 2011). Furthermore, recent data from our laboratory suggest that TIF1γ is required for M and S phase progression (Sedgwick and Turnell, unpublished observations); therefore adenoviruses could exploit TIF1γ degradation as an additional means of inducing, or sustaining, an S phase-like environment in host cells to promote viral replication. This theory could be examined through the stable over-expression of TIF1γ in Ad infected cells and by using flow cytometry to assess cell cycle distribution.

Over-expression studies could also be used to investigate the impact of TIF1γ on E4ORF3 function. E4ORF3 mutants are defective for certain aspects of Ad infection. For example, the E4ORF3-null virus *dl*341 is unable to promote viral DNA replication, late viral mRNA accumulation, and late viral gene expression in the absence of E1B55K (Shepard and Ornelles, 2004). Over-expression of TIF1γ could impede the *wt* functions of E4ORF3, or in contrast, the transcriptional nature of TIF1γ suggests that it could assist in the activation of late viral gene expression and/or cellular gene expression. Therefore, it will be interesting to establish if cells lacking TIF1γ through siRNA-mediated knockdown, or cells transfected

with the ligase-dead TIF1γC>A mutant, are able to rescue the phenotype of E4ORF3 mutants.

The ability of TIF1γ to function as a transcriptional repressor and de-repressor indicates that there are many other possible roles for TIF1γ during Ad infection. Indeed, adenoviruses are well known to transcriptionally inactivate the tumour suppressive properties of p53 and pRB either directly or indirectly. It would be interesting to utilise ChIP analysis to determine if TIF1γ can directly associate with p53 or pRB-responsive promoters. If proven, further studies could be carried out using RT-QPCR to determine the status of TIF1γ transcriptional targets in infected cells over-expressing TIF1γ, or cells infected with an E4ORF3 mutant virus. Although in the latter case it would be difficult to separate TIF1γ function from p53 relocalization and transcriptional regulation by *wt* E4ORF3 (Soria *et al*., 2010).

Given that mass spectrometric analysis identified USP9 and SMURF2, in addition to TIF1γ, as potential Ad12E1B54K-interacting proteins, it will be interesting to explore the impact Ad infection has on the TGF-β signalling pathway. This pathway arrests cells in G_1 to inhibit proliferation and promote apoptosis, and previous data has demonstrated that species C E1A and E1B55K proteins overcome TGF-β growth inhibition (Datto *et al*., 1997, Ravitz and Wenner, 1997, Tarakanova and Wold, 2003, Tarakanova and Wold, 2010). This antiviral defence mechanism is attributed to the transcriptional inhibition of SMAD-containing complexes and interference of mitochondrial activation. While the role of TIF1γ needs exploring, it is interesting that another Ad substrate, Daxx, is also known to regulate SMAD4-mediated TGF-β signalling (Chang *et al*., 2005, Schreiner *et al*., 2010). Ad-mediated degradation of Daxx is suspected to inhibit its transcriptional co-repressor properties such as its ability to enhance p53 activity (Schreiner *et al*., 2010), but prior to its

degradation it could also be involved in the regulation of TGF-β signalling pathways by early Ad proteins.

Finally, the possibility that TIF1γ is phosphorylated (or autophosphorylated) during Ad infection could be verified by further phosphoproteomic profiling, combined with phosphopeptide enrichment using a TiO_2 affinity purification column. If this were confirmed, it would be interesting to carry out *in vitro* kinase assays to determine the specific protein kinase responsible, and the development of phospho-specific TIF1γ antibodies or site-directed mutagenesis of the TIF1γ motif could help to study the modified protein's biological function. Furthermore, mutational analysis could also help to establish whether phosphorylation of TIF1γ during Ad infection is required to promote TIF1γ degradation.

6.3 A ROLE FOR TIF1γ IN THE DDR

While investigating the relationship between adenoviruses and TIF1γ, a secondary thread of research developed when TIF1γ was found to interact with the DDR kinase ATR. This interaction was confirmed by *in vivo* protein-binding assays, however TIF1γ protein expression remained unaffected by UVA damage, and the protein was not relocalized to sites of DNA damage during UVA microirradiation. In spite of this, there are many other avenues that need to be investigated to decipher the role, if any, TIF1γ has in the DDR.

Firstly, IR radiation could be used to study the effect a different DNA lesion has on TIF1γ protein expression and cellular localization; although it should be noted that some DDR proteins, such as Chk2, do not concentrate at sites of DNA damage and are instead distributed throughout the nucleus to enhance signal transduction (Lukas *et al*., 2003). While the ability of ATR to interact with TIF1γ was identical in mock- and Ad12-infected cells, it will be

interesting to determine by co-immunoprecipitation if this association is enhanced or reduced by DNA damage. In a similar manner, protein-binding assays could be carried out between TIF1γ and other members of the DDR, particularly the ATR-regulatory protein ATRIP and those identified in the mass spectrometric analysis of TIF1γ immunoprecipitates (Chk1, p53 and DNA ligase IV). These results may provide further clues regarding the putative significance of TIF1γ function in the DDR.

In addition to the putative phosphorylation site identified in Chapter 4, TIF1γ also has two SQ/TQ motifs located within its BRD and coiled-coil domains. These domains are associated with protein binding and transcriptional repression, and E3 ubiquitin ligase activity in the case of BRD, therefore it will be interesting to determine whether these SQ/TQ motifs are ATR or ATM targets by *in vitro* kinase assays (Reymond *et al*., 2001, Meroni and Diez-Roux, 2005, Liu *et al*., 2006, Agricola *et al*., 2011). This could lead to the development of phospho-specific TIF1γ antibodies to help determine the function of this modified protein.

It would be invaluable to use TIF1γ-specific siRNA to investigate the requirement of this protein in DNA damage-induced signalling events and relocalization of DDR proteins. Moreover, colony survival assays could be employed to measure the sensitivity of TIF1γ-knockdown cells to IR and UVA irradiation. In the event of TIF1γ having a confirmed role in the DDR *in vivo* studies could also be carried out. While knockout of TIF1γ in mice causes embryonic lethality from excessive Nodal signalling, conditional knockout mice have previously been generated using the Cre-Lox recombination system (Kim and Kaartinen, 2008, Vincent *et al*., 2009). Treatment of these mice with DNA damaging agents, or inhibitors of DNA replication, could be used to determine if they are radiosensitive or defective for checkpoint signalling, and help identify a role for TIF1γ.

The hypothesis that TIF1γ functions in the DDR is given some credibility by the fact that TGF-β is rapidly activated in response to IR in order to induce p53-dependent apoptosis (Ewan *et al*., 2002). This is supported by two recent publications; one which provides evidence that BRCA1 interacts with SMAD3 to modulate TGF-β signalling and growth inhibition in response to oxidative stress, and another which suggests that the synthetic 3-indole compound induces cell cycle arrest partly through the contribution of SMAD3 pathways (Li *et al*., 2009, Huang *et al*., 2011). Therefore, the function of TIF1γ as a negative regulator of TGF-β signalling could implicate it as a molecular switch that either allows apoptosis, or obstructs it to encourage DNA repair. Furthermore, the TIF1 family member TIF1β is already known to function in the DDR, whereby activated ATM phosphorylates TIF1β to de-repress the expression of genes involved in promoting cell cycle control and apoptosis (White *et al*., 2006, Li *et al*., 2007).

As an alternative to radiation-induced DNA breaks, it may also be worth investigating the implication of TIF1γ in DNA replication fidelity as ATR is fundamental for its maintenance (Bakkenist and Kastan, 2004, Petermann and Caldecott, 2006). Exposure of cells to chemical agents which cause faults in origin activation and fork progression, such as HU and aphidicolin (APH), could be useful in studying the involvement of TIF1γ in DNA replication. Correspondingly, the possibility that ATR interacts with TIF1γ in a non-DDR capacity cannot be disregarded, although so far reports on TIF1γ function are scarce.

While the function of TIF1γ in the DDR remains unknown, its ability to regulate transcription, associate with chromatin, function as an E3 ubiquitin ligase and putatively function as a protein kinase renders it a credible candidate DDR protein, and it will be worthwhile to investigate the above hypotheses.

CHAPTER 7

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