Studies of Adeno-Associated Virus-Induced DNA Damage Responses

A thesis submitted to the University of London for the degree of doctor of philosophy

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Abstract

Adeno-associated virus (AAV) infection triggers a DNA damage response in the cell. This response is not induced by viral proteins but by the structure of AAV ssDNA being recognized by the cell as damaged DNA. The consequence of this is the killing of cells lacking p53 activity. We have observed that cells that lack p21 or pRb activity are also sensitive to AAV-induced cell death. We report that cells respond to AAV infection by activating two DNA damage-signaling cascades. The first activates the p84N5 protein, which in turn activates caspase-6, leading to cell death. The second cascade activates the p53-21-pRb pathway, which inhibits activation of the p84N5 protein and thus prevents cell death. The result of the antagonistic interaction between these two pathways is that cells that do not exhibit functional p53-p21-pRb signaling undergo apoptosis as a consequence of AAV infection. Cells with a functional p53-21-pRb pathway are refractory to AAV-induced cell death. These results show that p53, although a pro-apoptotic protein, together with pRb and p21 proteins, is a member of an anti-apoptotic cellular mechanism. As such, these experiments reveal features that may be exploited to specifically kill cells that lack the p53-p21-pRb pathway, such as cancer cells.

We also consider the role of DNA damage signaling in the restriction of AAV replication. We report that helper viruses may inhibit ATM, ATR and DNA-PK signaling and that this may be advantageous for AAV replication. Inhibition of ATM and DNA-PK in tissue culture cell lines led to an increase in AAV replication. We show that ATM, ATR and DNA-PK may be responsible for the hyperphosphorylation of replication protein A (RPA) in response to AAV infection. We speculate that hyperphosphorylated RPA may be a fundamental restriction factor for viral replication and delineate novel experimental tools to test such a hypothesis. The implications of these observations for oncolytic therapy are discussed.

Table of Contents

Section 1 Introduction

Chapter 1 The Parvoviruses

- **The family of parvoviruses**
- **AAV**
- **AAV replication**
- **Productive AAV infection**
- **AAV latency**

The family of parvoviruses

The virus family of *Parvoviridae* exhibit a broad host range spanning from invertebrates to humans. The family is comprised of the two virus subfamilies *Parvovirinae*, which infect vertebrate hosts and the *Densovirinae*, which infect insects. Here we will focus upon the vertebrate parvoviruses that may be further sub-classified into two groups based upon their replication lifestyles. The autonomous parvoviruses, which encompasses the genera *Parvovirus* and *Erythrovirus*, may replicate autonomously and require the host cell to pass through S phase for viral DNA synthesis. The second group is represented by the genus *Dependovirus*. These viruses require co-infection with a helper virus for functions that facilitate a productive infection. The intricacies of helper virus aided replication have been extensively studied, particularly the helper functions of Adenovirus, and are discussed in the following section of introduction.

The initial identification of Adeno-associated virus (AAV) came in 1965 when 20 μm particles were discovered in association with several human adenovirus strains and with the simian adenovirus, SV 15. Initial observations (178, 183) led to the conclusion that the 'adeno-associated particles' were related to the adenovirus genome and were in fact a structural component derived from the inside of the adenovirus particle. At this point AAV strains were to be known as ASVs; adeno-associated satellite viruses. This was based upon the definition of a satellite being a

sub-viral agent composed of nuclei acid molecules that rely upon a helper viral co-infection for productive replication and that a satellite virus is one such satellite that encodes the coat protein in which its nuclei acid is encapsidated. It was only later that these small adenovirus-associated satellite virus particles were considered as distinct viral entities whereupon the name Adeno-associated virus was adopted (12). Although at the time, acknowledging AAV as a satellite virus may have seemed appropriate, with a better understanding of the biology of AAV we can certainly see that AAV is far more independent than being deemed as a satellite virus would suggest.

At the time of its discovery AAV was thought to very closely resemble the smallest RNA viruses recognised at that time know as the picornaviruses. It was thus suggested by Mayor and Melnick (179) that in keeping with such nomenclature but recognising the DNA nature of the viral genome, that these small DNA viruses be known as a picodnavirus. At such a time it was believed that unlike helper-independent small DNA viruses whose genome had been identified as a single-stranded DNA molecule; AAV had a double-stranded genome. We can now, with hindsight, emphasise that this is not the case but rather AAV demonstrates a single-stranded genome but it meant that the whole group be known as parvoviruses and thus the family of *Parvoviridae* was born.

The *Parvoviridae* can be further subdivided into the two subfamilies of Densovirinae and Parvovirinae, (278). The Densovirinae subfamily are parvoviruses of invertebrates and their subfamily is broken down into three genera; Densovirus, Brevidensovirus and Iteravirus, (27). Members of the Densovirinae subfamily include viruses that infect an enormously wide range of insect orders from across the globe. Together they are characterised by their ability to replicate autonomously and their capacity to encapsidate either plus or minus strands of their single-stranded DNA genome. Each virus of the family is named in a logically uninspired way whereby they can be identified by either a two letter or three letter abbreviation of the host name (one letter of the genus and two letters of the species). As such the examples AvDNV fom *Agraulis vanillae* and LdiDNV from *Lymantria dispar* typify the members of this subfamily. These viruses are notably fatal to their hosts, typically causing paralysis (266) or tumour lesions of the intestines.

In contrast to the arthropod hosts of their relatives, the Parvovirinae infect only vertebrates. The Parvovirinae subfamily is yet again separated into three distinct genera; Erthythrovirus, Dependovirus and Parvovirus. Erythrovirus replication predominantly takes place in erythrocyte precursors, hence the name. The Dependoviruses are classically perceived to be reliant upon a helper virus for replication and hence the name. The Parvoviruses however are capable of efficient autonomous replication. The most widely recognised Erthythrovirus as a consequence of its widespread pathogenesis is the Human Erythrovirus genotype 1 (formally B19) whose main targets of infection are erthrocyte progenitor cells found within the host bone marrow. Depending upon the immunological and haematological state of the host human erythrovirus will cause a range of pathological symptoms from transient aplastic crisis to chronic anaemia, (35). The autonomous parvoviruses such as minute virus of mice, MVM, infect only a limited range of hosts and tissues. However, the spotlight here is particularly centred upon the Dependovirus, AAV.

AAV

In 1966 the first four serotypes of AAV were isolated from adenovirus stocks and shown to be antigenically distinct by means of antisera reactivity experiments, (122). The advent of new serological data meant that seroepidemiological studies could be considered. It was shown that in humans the most prevalent serotypes were types 2 and 3 whereas types 1 and 4 were seen to be simian viruses. Subsequent years have yielded two further serotypes, types 5 and 6 (16, 229). Although type 5 demonstrates significant variation from the others, type 6 very closely resembles AAV type 1 and may not be an additional serotype as may be the case for the proposed serotype AAV3H, seen to be a genomic variant of AAV type 3, (229). In 1999 Erles *et al.* examined the seroprevalence of IgM and IgG antibodies to AAV-2 by means of AAV-ELISA and found that in each of the three different geographical regions investigated (Germany, Brazil and Japan) approximately 50% of those tested were seropositive (88) in addition to which latent AAV is thought to be found widely disseminated throughout both human and non-human primates (99). Despite the startlingly high prevalence of AAV within the population it must be emphasised that AAV has not been associated with any negative pathology.

It has been demonstrated that AAV particles are capable of binding cells via heparin sulfate proteoglycans (HSPG) at the cell surface (284). Heparin sulfate is one of the most abundant form of glycosaminoglycan molecules at the surface of the cell and has been shown to be the initial attachment molecule for several viruses including herpes simplex virus (301). The wide distribution of HSPG at the surface of many cell types may in part be responsible for the broad host range of AAV. Indeed cell lines that are deficient in their production of HSPG do not favour AAV-binding nor infection. However, AAV cellular binding and entry also appears to require co-receptor molecules. Two such molecules have been identified, the human fibroblast growth factor receptor 1 (216) and αvβ5 integrin (265). It is thought that HSPG binding and co-receptor interactions may mediate efficiency in terms of viral attachment and entry respectively. However, for AAV there is not a singular, defined receptor-coreceptor interaction. Mutants of AAV-2 for example that cannot bind HSPG are still infectious and AAV4 and 5 have also been shown to bind sialic acids during infection, (284). Publications on AAV receptor and coreceptor binding have generated a plethora of reported interactions as demonstrated in Table 1.1.1. Following receptor binding it is thought that AAV is internalised via clathrin-mediated endocytosis (21). It is speculated here that HSPG binding may be facilitated by the presence of fibroblast growth factor receptor and that internalisation process is facilitated by the presence of αVβ5 integrin. Internalisation within the marginally acidic early endosome allows entry of the virus into the cytosol following which AAV aggregates in a perinuclear local. AAV is then capable of penetrating the nucleus and although the manner by which this occurs is somewhat unclear it is presumed to be via nuclear pores (232). The processes of viral uptake and trafficking are by no means black and white and it is perceived that viral capsids may passage through the cell by one of at least three different mechanisms (240). Rapid endocytosis of viral capsids into the cytoplasm, free diffusion of both the endosome and virus within the cytoplasm and nucleus or directed trafficking via cytoplasmic motor proteins and nuclear tubule structures constitute these three distinct processes. Once within the nucleus, regardless of the permissive state of the cell, initial second strand synthesis is required to allow for any subsequent replication or cellular integration into the host genome.

Table 1.1.1. Reported AAV receptors and co-receptors. Adapted from (284).

AAVs linear single-stranded genome is 4,675 nucleotides in length. Strands of either plus (+) or minus (-) polarity may be encapsidated within the viral particle (257) though *in vivo* experiments have demonstrated that AAV transcription only occurs from the (-) strand and that this transcription is mediated by host cell RNA polymerase II (174).

The AAV genome comprises only two open reading frames (ORF) one of which encodes the non-structural proteins (Rep proteins), the other of which encodes structural proteins (Vp proteins), (28). The Rep ORF encodes four proteins with overlapping amino acid sequences; Rep 78, Rep 68, Rep 52 and Rep 40. Similarly the Vp ORF encodes the structural proteins, Vp1, 2 and 3 whose amino acid sequences overlap also. The single strand of AAV DNA exhibits both 5' and 3' palindromic, inverted terminal repeats (ITRs) of 145 nucleotides in length (257) that are considerably self-complementary and lead to the formation of stable, terminal, T-shaped hairpin loop structures. Several functions required for the AAV life-cycle have been attributed to the viral ITRs. The Rep-dependent origin of DNA replication is contained within these regions and they are also involved in the regulation of gene expression. The ITRs are also crucial for the site-specific integration of the AAV genome into chromosome 19 during latent infection. They are also necessary for rescue from the latent state when conditions are permissive. Finally, they are required for the packaging of AAV DNA into the viral capsids (157). Despite only having two open reading frames the genome has three promotors known as p5, p19 and p40. Thus there are three transcriptionally active sections all of which overlap. There is however a shared polyadenylation site at position 95 and a splice donor site at position 41 (142, 280). All three of these transcription units make use of the same splice acceptor site at position 48, while transcripts originating from p40 are also capable of using an acceptor site at position 47. The generation of both spliced and unspliced mRNAs from these three promoters lead to the formation of the four Rep proteins and explains their overlap in sequence (184). Similarly, the three capsid proteins originate from two mRNAs and also have overlapping sequences. The AAV genome may be small but is able to efficiently produce a functionally effective and diverse range of proteins. This is schematically represented in Figure 1.1.1, which illustrates the three promoters, alternate transcripts and splice variants that yield the seven mature RNAs.

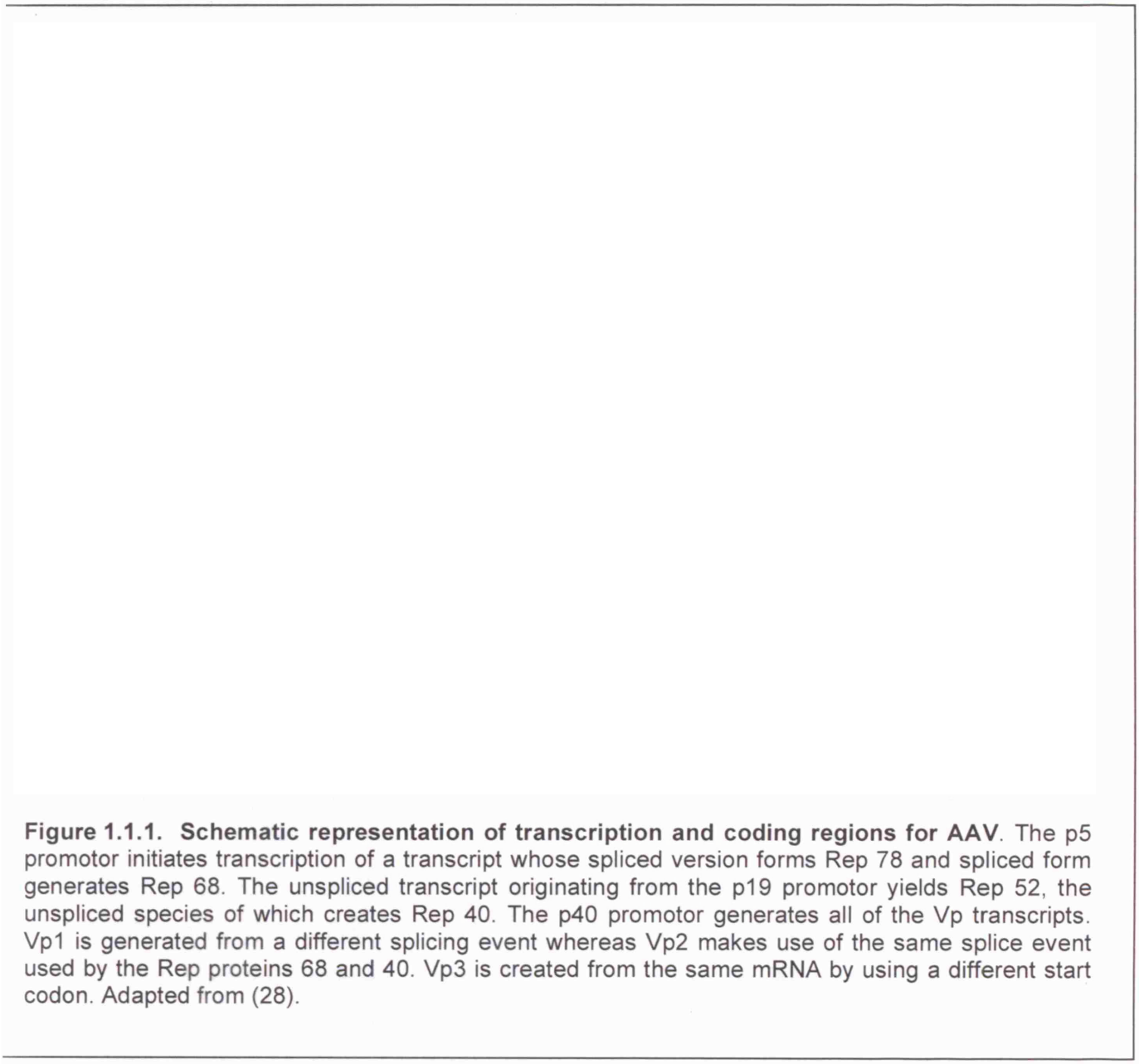


Figure 1.1.1. Schematic representation of transcription and coding regions for AAV. The p5 promotor initiates transcription of a transcript whose spliced version forms Rep 78 and spliced form generates Rep 68. The unspliced transcript originating from the p19 promotor yields Rep 52, the unspliced species of which creates Rep 40. The p40 promotor generates all of the Vp transcripts. Vp1 is generated from a different splicing event whereas Vp2 makes use of the same splice event used by the Rep proteins 68 and 40. Vp3 is created from the same mRNA by using a different start codon. Adapted from (28).

AAV transcription is orchestrated by a combination of viral Rep proteins, helper viral proteins and cellular components. How the process of transcription proceeds is dictated by the permissive status of the cell. As would be expected, transcription of viral genes is repressed during a latent infection. This is thought to involve the action of Rep 78 and Rep 68 proteins, which are capable of efficiently suppressing transcription from both the p5 and p19 promoters (137). Repression occurs by the direct binding of Rep proteins to the viral DNA, as demonstrated by Kyostio *et al.* 1995 (137). It was shown that a region in between the p5 TATA box motif and transcription initiation site represented a binding site from which Rep 68 represses transcription from the p5 promoter. Aside from direct interaction with viral DNA, Rep proteins contribute to the suppression of gene expression during latency by interfering with the cellular transcription components via protein-protein interactions. Suppression in this manner occurs during non-permissive conditions but when productive infection is permitted, the Rep proteins act as very powerful activators of viral promoters. More specifically Rep proteins may both repress and enhance AAV transcription in the presence of a helper virus (210). Furthermore, AAV Rep proteins have been shown to inhibit helper virus replication (Adenovirus for example) and repress transcription from helper virus promoters (197).

AAV DNA replication

As is the case for most DNA viruses, the parvoviruses replicate in host cell nuclei but unlike many viruses they are unable to promote entry of cells into S phase and yet display a heavy dependency on host cell factors for replication. While the autonomous parvoviruses are restricted to replicating in cells that by natural course enter S phase (274), the Dependoviruses i.e. AAV, require co-infection with helper viruses that will promote S phase entry in addition to their virally-encoded helper functions. While these other virally-encoded helper function will be discussed in the following section, this section will deal with the intricacies of the molecular mechanisms of AAV DNA replication, reiterating the importance of genomic structure and sequence.

AAV genome replication occurs by a uni-directional, single strand displacement mechanism that demonstrates no requirement for Okazaki fragments and thus no lagging strand (275). The AAV ITR structure favours the formation of a 3' self-

complementary region that acts as a primer for DNA synthesis. This is essential for converting the single-stranded viral genome into a double-stranded template for transcription and replication. While the structure of AAV ITRs is considered the most important aspect of their involvement with replication, the ITR sequence contains two motifs that represent the minimal origin for DNA replication; the Rep-binding site (RBS) and the terminal resolution site (TRS) (292). Figure 1.1.2 graphically illustrates the process of AAV genome duplication. Complementary strand synthesis originates from the 3' end of the viral genome that folds over to form the primer region of the ITR. This produces a linear duplex molecule in which the 3' hairpin loop structure is covalently closed. The viral Rep 68/78 proteins then nick the hairpin structure at the TRS. This creates a 3' primer region that will allow synthesis of the ITR region creating a complete duplex molecule. The folding of one end to form the ITR structure generates a substrate for single strand displacement replication again as such the duplex form represents a single completed progeny single-strand genome that is ready for packaging and another substrate for replication, and so the cycle continues. However, figure 1.1.2 does not depict the outcome of failed TRS nicking and terminal resolution (123). If TRS nicking does not occur to facilitate terminal resolution of the hairpin region from the linear duplex molecule, the formation of double-stranded dimers takes place as a consequence of continued synthesis generating head-to-head or tail-to-tail dimmers joined by a single ITR. This highlights the importance of Rep proteins in the process of AAV DNA replication.

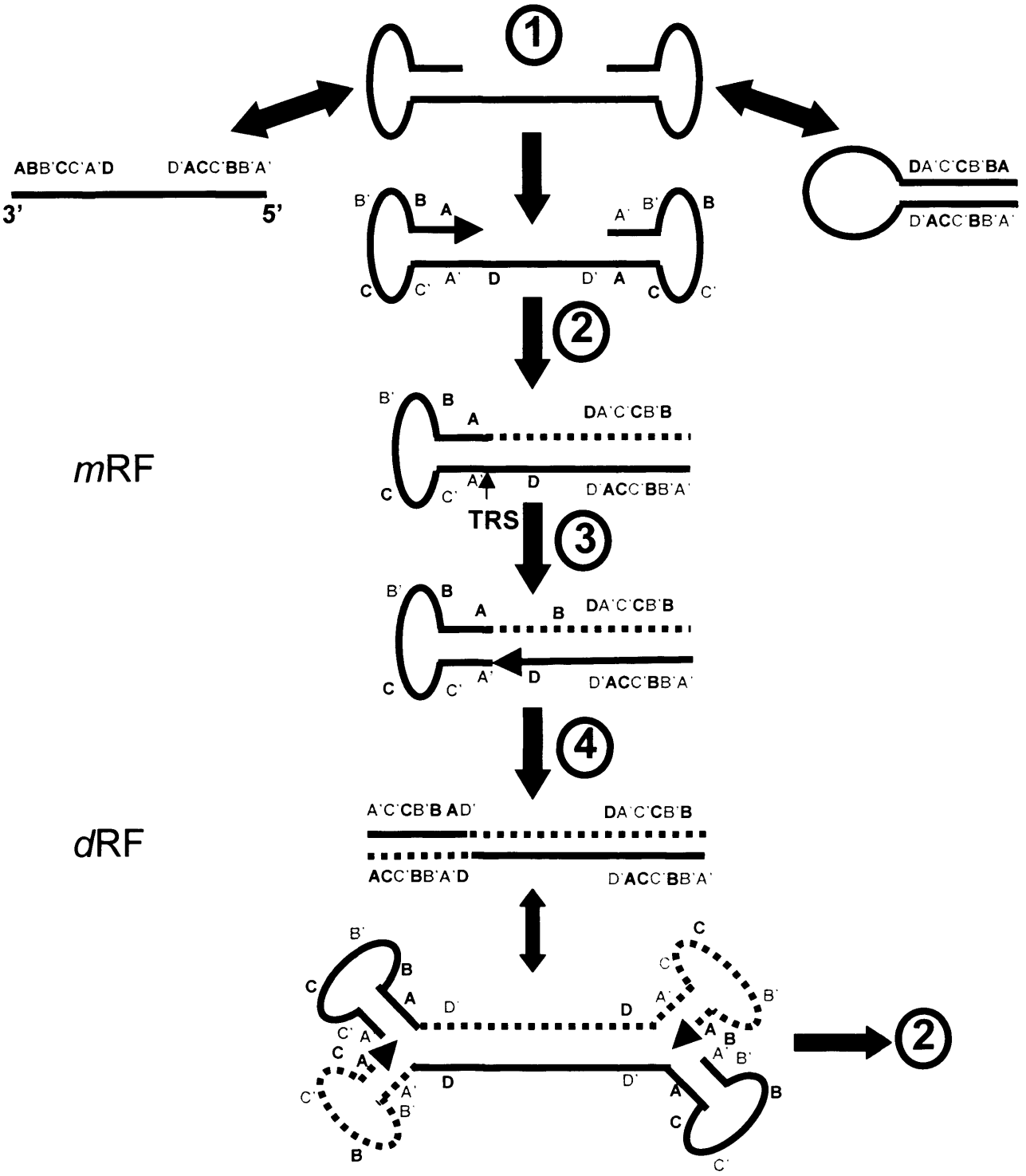


Figure 1.1.2 A step by step model of the single strand displacement mechanism by which AAV DNA is replicated. The linear single strand of DNA shown in step 1 does not occur naturally but rather the palindromic terminal repeat regions form a stable hairpin loop that functions as a primer for step 2. Step 2; elongation occurs from the priming region to form a duplex replicative intermediate. Step 3; the hairpin intermediate is nicked at the terminal resolution site (TRS). The nick site functions as a primer while the strand generated in step 2 acts as a template for synthesis to restore the 3` end of the parental strand. Strand separation may occur as in step 4 whereby the 3` terminus is free to fold over to then prime a new round of replication. Alternatively duplex dimers may be formed if the original hairpin crosslink is not resolved. The monomeric replicative forms (mRF) and duplex replicative forms (dRF) are indicated.

Productive AAV infection

AAV may receive the help that it requires for replication (and thus a productive infection) from a number of viruses including adenovirus, human papillomavirus and herpes simplex virus. The viruses capable of aiding a productive AAV infection are seemingly unrelated and there is no clear common link between the ways in which these viruses are able to facilitate AAV replication (200). The ways in which adenoviral coinfection elicits the help required for productive AAV infection have been well characterised *in vitro.* Adenoviral early functions involve the proteins E1A, E1B, E2A, E2B, E3 and E4, all of which (with the exception of E2B and E3) are involved in adenoviral helper functions towards AAV by affecting both cellular and AAV gene expression (225). It is of interest that neither adenoviral DNA polymerase nor terminal protein are involved in aiding AAV replication. In contrast herpes simplex virus-encoded enzymes are involved in AAV DNA synthesis. As a comparison, the two cases serve to demonstrate the variability in function of helper virus coinfection along side that of AAV.

Despite having described the requirement for helper virus co-infection for permissive conditions for AAV replication, productive infections have also been observed in cells that would normally be characterised as non-permissive. It has been demonstrated that in the absence of a helper virus, certain cell lines may become varyingly permissive for AAV DNA replication following the application of genotoxic stress treatments (303). A shared characteristic of the treatments that generate permissive conditions for productive infection is that they are DNA-damaging agents that are capable of both disrupting cell cycle progression and causing the over-expression of certain cellular genes. (304). Yakobson *et al.* proposed several mechanisms by which UV irradiation is able to relieve AAV's requirement for helper viral coinfection. It is suggested that AAV may encode a suppressor of its own replication, that a UV-induced cellular protein may competitively inhibit or that an alternative UV-induced protein may act as a trans-activator. However, these and other hypotheses depend upon UV-irradiation-induced change or expression of positively influencing specific proteins. No such proteins have been identified and so the mechanisms by which DNA damaging agents such as UV are capable of lifting the AAV replication block remain unclear.

Furthermore it has also been demonstrated that AAV can replicate autonomously in an *in vitro* replica of a differentiating epithelium, the keratinocyte raft system (185), and also in synchronised cell populations (304). While the precise mechanisms by which synchronisation or differentiation can provide help have not been clarified it would not be surprising if the mechanisms by which UV irradiation, synchronisation and differentiation impinge on the same mechanism to generate permissive conditions. Indeed mechanisms of synchronisation such as hydroxyurea treatment will almost certainly induce DNA damage responses that may be reminiscent of genotoxic stress-induced responses following UV treatment. In addition to this, many of the proteins that are involved in processes of differentiation and DNA damage responses may be the same and these processes may be intertwined.

AAV latency

In the absence of a permissive cell state, latent infection occurs. In such cases, AAV is capable of intergrating its genome into the cellular DNA. The AAV virion will enter the cell whereupon it is trafficked to the nucleus and uncoating takes place. Shortly following uncoating a small quantity of Rep protein is produced, the function of which is to suppress any further expression from the AAV genome as discussed. The viral DNA genome is then integrated into the host cellular genome in a site-specific manner on chromosome 19q (136) and remains there without phenotypically detectable effects until such a time that conditions are permissible, (28). In such cases, co-infection with a helper virus activates and rescues the integrated viral genome from the cellular DNA.

The pre-integration site has been cloned and sequenced and is known as AAVS1. The AAVS1 site contains the same Rep-binding motif and terminal resolution motif found in the viral ITR. Rep protein can simultaneously bind the viral ITR and the AAVS1 Rep binding site and this facilitates integration by initiating Rep-mediated nicking of the AAVS1 terminal resolution sequence (298). The model for AAV integration is somewhat speculative but following nicking it is proposed that a nick-induced primer promotes replication by the assembled cellular replication factors and Rep 68/78, which hold a circularised duplex of AAV genomes in close proximity of the AAVS1

site. The ability of Rep proteins to promote strand-switching during replication as suggested in the model of AAV DNA replication provides the basis for the proposed mechanism of the replication events that underlie AAV integration though there is little evidence for the actual mechanism of integration (158).

Prior to integration the AAV single strand genome must undergo second strand synthesis and these duplex forms will frequently circularise (78, 79). While integration of AAV DNA represents one long-standing mechanism by which AAV can establish persistence in host tissues. It has become clear that wild-type AAV DNA can exist for a period of latency via circular episomal forms (237) in agreement with previous observations that recombinant AAV vectors achieve latency over extended periods in a similar form (236). The circular forms also represent a substrate for potential integration and non-circularised forms may only be a very transient state.

Chapter 2 The Eukaryotic Cell Cycle

- **The eukaryotic cell cycle and its regulation**
- **DNA replication**
- **Regulation of growth and proliferation**

The eukaryotic cell cycle

The eukaryotic cell cycle revolves around the process of DNA replication. The cell cycle is segregated into four phases; G1, S, G2 and M phase. S phase represents the period of the cell cycle in which DNA synthesis takes place, hence S (for synthesis) phase. The process of cellular DNA synthesis requires that each and every chromosome is replicated once and only once per cell division. The process of chromosomal duplication begins in regions of the DNA known as replication origins. A pair of duplicated chromosomes is known as a pair of sister chromatids each of which must be segregated into each of the daughter cells during mitosis (M phase). Following this, the cell undergoes cytokinesis in which the cell physically divides and becomes two daughter cells. Prior to M phase the nuclear membrane dissolves and the duplicated chromosomes must be equally segregated into the daughter cells. This requires the formation of the mitotic spindle microtubular networks to which sister chromatids are anchored. Chromatid pairs are separated to oppiste poles by the mechanical pull of the microtubules. Ultimately the cell then divides into two, each containing a complete set of chromosomes.

The G1 and G2 phases are considered as gap phases that separate S phase and M phase and provide time for cells to acquire the appropriate cellular conditions and context required for DNA replication or division. These gap phases also represent important periods for cell cycle regulation to prevent un-timely transitions into replication or division. G1 in particular represents a key transition point in terms of passage through the cell cycle. Cells pause in G1 until conditions are favourable for DNA replication. Cells may also remain in this non-dividing state for a prolonged period of time. A prolonged non-dividing state in this phase of the cell-cycle is also known as G0.

When the cell cycle is conceptually reduced down to the four phases of G1, S, G2 and M it gives the illusion of a simple system. Indeed, the cell elegantly performs the process of extremely precise replication of its genome to produce two identical daughter cells with ease. However, this does not emphasise the pivotal importance of high fidelity with regards to DNA replication, segregation and division without which complex organisms might never survive. In order to achieve such accuracy, a complex regulatory network rigorously controls the simple cycle from G1 through to mitosis and cytokinesis.

For the purpose of understanding the control of cell-cycle progression we must consider the three major cell cycle transition points. The restriction point, is the point at which the cell is considered to enter the cell cycle and occurs in the late stages of G1. The G2/M transition represents the point at which entry into mitosis takes place and ultimately the final stages of mitosis are controlled at the metaphase-anaphase transition. There are several networks that compose biochemical switch-like operations to carefully regulate passage through the cell-cycle and regulate transition at these three key points. Central to this control system are the cyclin-dependent kinases (Cdks) and their regulatory components, the cyclins. The following sections will discuss Cdk and cyclin function, regulation and their control of cell cycle progression.

The family of Cdk proteins share the properties of being catalytic kinase proteins that are regulated by cyclin protein partners. Regulation is such that the activation of Cdks requires binding to cyclins. Further regulation takes place at the level of phosphorylation as full Cdk activation requires threonine phosphorylation proximal to the catalytic site. Upon activation, Cdks cell-cycle progression by phosphorylating cellular proteins at serine or threonine residues in a sequence-specific manner. The process of cyclin-binding to Cdk partners is able to regulate Cdk activity because the active site of the Cdk when it is bound to cyclins. Together, cyclin-Cdk interactions are fundamental in being able to govern cell-cycle progression since the oscillating changes in the activity of Cdks dictate the phosphorylation status of target proteins that affect the cell cycle.

To achieve oscillating changes in Cdk activity one might imagine that the concentrations of individual Cdk proteins may change in phase with the changes in cell-cycle status. This however is not the case. Instead changes in Cdk activity are achieved by changes in levels of the cyclins while Cdk concentrations remain constant throughout the cell cycle. Different pairs of cyclin-Cdk complexs are formed at different stages of the cell-cycle and specifically govern progression from one phase to another. In *Homo sapiens* the nomenclature for cyclin-Cdk proteins is different to that of other model systems used for considering the biology of the cell-cycle. The simple model in *H. sapiens* is that the G1 cyclins are predominantly cyclin D1,2 and 3 in complex with Cdks 4 and 6. Cyclin E and Cdk2 govern the G1 to S phase transition (190). Cyclin A and Cdks 2 and 1 regulate S phase and cyclin B in complex with Cdk1 regulates mitosis. Cdk1 is also known within the literature as cdc2.

Although, cyclin-binding is a pivotal determinant in terms of Cdk activation, full Cdk activation requires further phosphorylation and de-phosphorylation steps. Cdk-activating kinases (CAKs) phosphorylate Cdks at a threonine residue adjacent to the catalytic site and may confer full activation to cyclin-Cdk complexes. Such phosphorylation however may only take place after the binding of cyclin to Cdk. In vertebrates the CAK responsible for phosphorylation of Cdk proteins consists of a trimer formed from Cdk7 in combination with cyclin H and Mat1. Recent studies have also identified a further mammalian CAK protein, p42 (162).

Although threonine phosphorylation of Cdks by CAK is required for Cdk activation, this phosphorylation is not used to regulate Cdk activity. Of greater importance are two sites of inhibitory phosphorylation. Instead inhibitory phosphorylation of Cdks occurs at the conserved tyrosine residue number 15, and additionally in vertebrate cells, on the threonine residue at position 14. Phosphorylation of these sites inhibits Cdk function (208). The balance between two enzymes; one a phosphatase and one a kinase regulate phosphorylation of Thr 14 and Tyr 15. In vertebrates Wee1 and Myt1 control the phosphorylation of these two residues whereby Myt1 is the key inhibitory kinase, phosphorylating both Thr 14 and Tyr 15 and Wee1 phosphorylates only Tyr 15. In addition to which the Cdc25 family of phosphatases carries out opposing dephosphorylation. As such Wee1 and Myt1 are inhibitory toward Cdk activity while Cdc25 proteins are stimulatory. The phosphorylation status of the two inhibitory sites

on Cdks determined by the balance between Cdc25 and Wee1/Myt1 protein activity facilitates G1/S phase and G2/M phase transitions.

As we have already described, Cdk levels remain unchanged throughout the cell cycle. Instead it is changes in cyclin levels and phosphorylation status of Cdks that regulate Cdk activity. A further mechanism by which Cdk activity is controlled is by Cdk inhibitor proteins (CKIs). These proteins are able to inhibit Cdk activity by direct binding to Cdk or cyclin-Cdk complexes. These inhibitors are particularly important in terms of checkpoint activation in response to DNA damage or inhibitory extracellular signals.

In mammals there are two main groups of CKI proteins that are distinguished by their mode of Cdk inhibition. These two families are known as the Cip/Kip family of CKIs comprising p21 (Cip1/Waf1), p27 (Kip1) and p57 (Kip2) and the INK4 family, which includes $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$ and $p19^{INK4d}$. Members of the Cip/Kip family inhibit Cdk activity by physical interaction with Cdk and cyclin-Cdk complexes (109). Their primary function is to inhibit the G1/S and S-Cdks but they may also activate the cyclin D-Cdk4 complex as will be discussed below. Conversely, the INK4 proteins interact with Cdk4 and Cdk6 inhibiting their propensity for complex formation with cyclin D (109, 247).

The biochemical activity of the Cip/Kip proteins is rather complex. Although they are inhibitors of cyclin-Cdk2 complexes, (which are predominantly G1/S and S-Cdk complexes) the Cip/Kip proteins also aid in the activation of the G1-Cdks; Cdk 4 and 6. The Cip/Kip proteins aid activation in this scenario by enhancing binding affinity and thus promoting complex formation between cyclin D and Cdk4 or 6 (138, 246) and may help target these complexes for specific function.

CKI expression is modulated in tune with the cell cycle (247). In G1 the CKIs are highly expressed to inhibit progression through the restriction point, into S phase. Mitogenic signalling can promote cell cycle entry by promoting D type cyclin expression for example. The cyclin D-dependent kinases show substrate affinity for pRb and promote its phosphorylation and thus transcription of S phase genes (120). The predominantly inhibitory presence of the CKI proteins must be relieved for cell

cycle progression and this is achieved by targeted destruction of CKI protein in response to Cdk-mediated phosphorylation. Thus, as G1/S Cdk activity increases then so does the destruction of CKI thereby allowing S phase progression. Cell cycle progression relies upon the biological switch like activation or inhibition of Cdks, which is strictly governed by several overlapping mechanisms as described. This highlights the importance and the role of redundancy in biological systems. To guarantee that the system as a whole can survive disruption each part is controlled by multiple mechanisms and as such should one or more fail the system as a whole can survive.

One of the crucial elements of a switch-like activation is that when activation is appropriate is should occur in full without the possibility of partial activation. One of the elements of maintaining such switches is the process of proteolytic degradation of regulatory elements. This is principally carried out by proteosomal degradation following ubiquitin targeting. Ubiquitin-mediated degradation is important not only for multiple aspects of cell cycle regulation but also in terms of viral life-cycles and many viruses encode their own ubiquitin ligase enzymes as indicated in latter sections. As such we will discuss the process of ubiquitination and the enzymes involved before highlighting their importance for cell cycle progression. Ubiquitination involves the attachment of the small protein ubiquitin to target proteins in multiple copies or chains which are then recognised by the 26S proteasome that will then process the targeted protein into peptide fragments (Hershko and Ciechanover 1998). Ubiquitination is facilitated by a chain reaction that begins with the ATP-dependent attachment of ubiquitin to the ubiquitin-activating enzyme E1, followed by the transfer of the ubiquitin protein to the ubiquitin-conjugating enzyme E2. The ubiquitin-conjugating enzyme E2 in conjunction with the ubiquitin ligase E3 then co-ordinates the attachment of ubiquitin to the target protein via the generation of a peptide bonds. The E3 enzyme represents the mediator of target specificity and as such the key regulator in this process.

As inferred so far, the process of ubiquitination is crucial for several cell-cycle transitions; particularly the G1/S phase transition and the metaphase to anaphase transition. To control these transitions there are dedicated ubiquitin protein ligases that target the relevant proteins for degradation. Indeed cyclins and CKIs are subject

to periodical proteolysis during the cell cycle (196). These processes are predominantly carried out by the SCF complex and the anaphase promoting complex, APC. The multisubunit enzyme, SCF, is made up of **S**kp1, **C**ul1, **F**-box protein as well as the Rbx protein, which binds the E2 ubiquitin conjugate. The SCF unit appears to act as a platform for ubiquitination of target proteins and the F-box protein subunit determines such target specificity (126). The interchangeability of the F-box protein allows regulation of protein targeting since each F-box protein has affinity for different protein substrates. The binding of the F-box protein to its substrate is regulated on a further level since F-box proteins typically only bind to phosphorylated substrates. The cell-cycle regulation of SCF function can thus be determined by Cdk phosphorylation of F-box target proteins.

The metaphase to anaphase transition is a critical point during mitosis when sister chromatid separation occurs. Degradation of proteins that control sister chromatid cohesion and mitotic cyclins is carried out by a multisubunit enzyme analogous to the SCF complex, known as the APC. The APC is composed of up to 13 different components and unlike the SCF is regulated by several mechanisms including the involvement of activating subunits (191).

So far we have described how the driving force of cell cycle progression, Cdk activity, is regulated into oscillations of activity via (a) protein phosphorylation, (b) requirements for subunit binding, (c) CKIs and (d) targeted proteolytic degradation. However, yet another level of regulation takes place by the control of cyclin gene expression. The cell cycle dependent synthesis of cyclins is of prime importance since they contribute to the complex regulation of Cdk activity. Transcriptional regulation is at its most pivotal at the key cell-cycle transition points; Restriction point, G2/M and mitotic exit. While the regulators of gene expression and the genes that they control are moderately well-characterised in other model systems such as yeast, they are not fully understood in metazoan systems. One of the most crucial families of transcriptional regulatory gene products is the E2F group. E2F transcriptional complexes are made up of two subunits composed of one E2F family protein, of which there are six in mammals, and one of the two mammalian DP proteins. E2F complexes are activators of transcription and they stimulate the transition from G1 to S phase by increasing cyclin E and A expression as well as the expression of many

factors required for DNA replication and thus S phase progression. Functional complex formation of E2F/DP heterodimers is controlled by the RB family of pocket proteins pRB, p107 and p130 (37, 47, 55). RB proteins inhibit E2F-DP mediated gene expression by direct binding to E2F components. The mode of action of inhibition depends upon the specific RB and E2F components. While direct binding of RB proteins to some E2F proteins is sufficient to simply prevent E2F-mediated transactivation there is evidence to suggest that certain combinations of E2F and RB proteins yield repressor complexes that may actively repress the expression of certain genes (295, 296). While pRb protein mediated regulation of E2F complexes is an important way in which pRb inhibits cell cycle entry, pRb may also regulate transcription by modulation of chromatin structure (314).

The RB family proteins act as the guardians of entry into the cell cycle since they function to inhibit G1/S phase gene expression and thus prevent entry through the Restriction Point by direct binding to E2F family members. Progression into the cell-cycle can only be made by promoting the dissociation of RB-E2F protein complexes and this is mediated through phosphorylation of the RB protein component. RB phosphorylation is mediated by Cdk activity and pRB, p107 and p130 harbour multiple sites for Cdk phosphorylation and upon phosphorylation demonstrate extremely low affinity for E2F protein binding. Phosphorylation may also lead to targeted destruction since the levels of p130 decrease considerably upon phosphorylation (253). The pRB protein purportedly has two stages to its inactivation, firstly mediated by cyclin D-dependent kinases and then by cyclin A or E-dependent kinase activity (188). The activity of each cyclin regulated kinase is thought to phosphorylate a subset of available phosphorylation sites. Furthermore it is thought that the activity of each Cdk is activated by distinct signalling pathways originating from growth factor stimulus such as ras signalling (188) thus directly integrating various growth factor stimuli with cell cycle control. While the relationship between RB proteins and regulation of E2F is considered to be well understood there are several aspects that do not fit into such a philosophy of understanding. Despite the fact that RB is considered to principally regulate E2F activity, the proportion of pRb-E2F complex represents a minority of the total cellular pool of E2F complexes. As such there appears to be a gap in our knowledge as to what fully regulates E2F

activity. Perhaps E2F phosphorylation, subcellular localisation and degradation are equally as important in terms of maintaining E2F activity in context.

Furthermore recent studies demonstrate that each of the different RB family members regulate E2F in different ways and may be responsible for different aspects of RB's biological significance (84). As a potent tumour suppressor, much of pRB's role in preventing oncogenesis has been attributed its role in regulating E2F activity. While this is nevertheless crucial, it is suggested that E2F activity is not totally deregulated by a lack of pRB and furthermore interaction with E2F and DP family proteins contributes less than 20% of pRB's reported protein interactions. It is clear that the roles of both E2F and pRB are more complex than may have been first envisaged and herein lies the gaps in our understanding of vertebrate regulation of cell cycle transitions by transcriptional regulation. To emphasise this we can draw on the recent suggestion that E2F may function not only as a potential oncogene but also as a tumour suppressor. The role of E2F as oncogene can be easily envisaged since E2F activity can promote cell-cycle entry and indeed the overexpression of E2F-1 in mice can lead to increased rates of tumour acquisition (211). Conversely E2F-1 knock out mice demonstrate a wide susceptibility to a variety of tumour types and as such E2F-1 must also be considered as a tumour suppressor (305). Several theories attempt to reconcile these two observations. The ability of E2F to also act as a tumour suppressor is proposed to arise the dual modulation of E2F binding domains in functioning toward both activation and repression of gene expression depending upon the status of RB proteins as mentioned above. Additionally E2F-1 has been shown to possess pro-apoptotic function that may be partially responsible for the perceived role of E2F in tumour suppression. Perturbations of RB-mediated growth control are considered particularly responsible for activating E2F-1-mediated apoptosis via p53. Specifically, E2F-1 is thought to regulate apoptotic signalling through ATM, NBS and CHK2 activity (215, 227), demonstrating a role for E2F-1 in relaying defects in RB-mediated growth control and DNA damage signalling to induce p53 mediated apoptosis.

Many of the proteins described herein as key regulators of cell cycle checkpoints are so crucial that it is not surprising they are frequently lost in cancers and similarly

abused by viruses since their inactivation can promote the ideal environment for viral DNA synthesis and progeny production. These interactions will be discussed in more detail when we consider viral interaction with cell cycle checkpoints and DNA damage response (Chapter 4). The cell cycle is clearly very intricately maintained both internally by many check and balance mechanisms and externally in terms of its response to extracellular inputs. As such the cell is able to function competently in the context of its environment and is ideally honed to respond to neighbouring cells as part of a unified multicellular organism. This concept will be considered in more detail in section 2.3 once we have discussed the intricate process of DNA replication that occurs once a cell has finally been granted the right to move into S phase.

DNA replication

DNA replication must occur with the strictest of order to maintain genomic integrity and the process of eukaryotic chromosomal replication is a formidable task. Understanding the mechanisms that govern DNA replication is important for our understanding of DNA damage responses as well as viral replication and the cellular factors required for such processes. Therefore, while other aspects of the cell cycle are important to gather a thorough understanding of growth and proliferation, the processes that occur during S phase will be considered in considerably more detail.

Once the decision has been made for cells to replicate their DNA the control of S phase progression hinges upon origins of replication. Such origins represent the starting points for DNA replication fork progression and the pivot for the 'once and only once' dogma for origin firing and thus regulated DNA replication that ensures daughter cells emerge with the correct measure of DNA. A cell prepares its genome for replication during G1. Preparation involves the formation of pre-replicative complexes (pre-RCs) at origins of replication in a manner known as licensing and may only occur when Cdk activity is low and APC activity is high. In higher eukaryotes origins are not defined in a sequence-dependent manner and are thought to be in part a product of epigenetic modelling (319). Origin firing during S phase involves the transformation of pre-RCs into pre-initiation complexes, which involves the preliminary unwinding of DNA and loading of DNA synthesis components as a consequence of S phase Cdk activity. The presence of Cdk activity inhibits the re-

licensing of origins and this represents one method by which the cell can attempt to ensure that the replication of each region of DNA occurs only once.

The ordered formation of the pre-RC involves licensing the genomic origins and this process begins with origin recognition by the origin recognition complex, ORC. The mammalian ORC is made up of 6 subunits and binds to replication origins in an ATP-dependent reaction that is inhibited by Cdk activity and thus cannot take place outside of G1 (67). DNA-bound ORC provides the platform for recruitment, of Cdt1 and Cdc6. These proteins faciltate the loading of the DNA helicase complex; Mcm2-7 (64). As described, this process of pre-RC formation is restricted to late mitosis and early G1 as a simple consequence of inhibition by Cdk activity and by the activity of the APC. The role of the APC involves a protein known as geminin. Geminin functions as an inhibitor of pre-RC formation by its ability to bind Cdt1 and prevent its interaction with ORC. Consequently geminin levels are kept low during G1 by APC-mediated ubiquitination and thus proteolytic degradation, to allow pre-RC formation. In the absence of APC activity in the moments prior to S phase, geminin levels are able to rise and as such Cdt1 is again sequestered from facilitating pre-RC assembly again until the late stages of the following mitosis (181). The manner in which Cdk activity inhibits pre-RC formation is less well defined. One mechanism proposes that Cdk-mediated phosphorylation of Cdt1 targets it for SCF-mediated ubiquitination (160) while Cdk activity may also inhibit the DNA binding ability of Cdt1 (263) both of which are independent of geminin interactions with Cdt1. Furthermore, unbound Cdc6 may also be a target of cyclin A-CDK2-dependent proteolysis (59).

The assembly of the pre-RC in which multiple but inactive complexes of Mcm2-7 are bound represents a potential starting point for replication but requires the loading of pre-initiation complex components in response to cyclin A-CDK2 and Cdc7 activity. Cdc7 acts as a direct activator of origin firing and its activity is cell cycle regulated in a similar way to that of Cdk activity regulation by cyclin proteins. Cdc7 requires its regulatory subunit Dbf4 for activation and Dbf4 levels are strictly regulated by APC-mediated ubiquitination. Inactivation of the APC at the G1/S phase transition allows the stabilisation of Dbf4, its association with Cdc7 and the activation of Cdc7 which facilitates the activation of replication origins. The positive activity of Cdc7 and S

phase Cdks leads to the loading of the pre-initiation complex components; Mcm10, Cdc45, the Dbp11 complex and the GINS complex that recruits polymerase enzymes and promotes the unwinding of the DNA helix to initiate DNA replication. The binding of the pre-initiation complex promotes the displacement of Cdt1 and Cdc6 and the prevention of them re-binding is pivotal to prevent re-replication. Cdk activity promotes the binding of Cdc45 to the pre-initiation complex, which is required for binding of polymerase α-primase (322). The initial primase polymerase synthesises a short RNA primer using the template DNA strand. This provides a nuclei acid 3' end to which the more processive DNA polymerase enzymes can add new nucleotides. The polymerase α-primase enzyme is displaced by replication factor C (RFC) to facilitate loading proliferating cell nuclear antigen (PCNA). The more processive polymerase enzymes polymerase δ and ε can associate with PCNA and conduct rapid and continuous synthesis of the leading strand in a process that depends upon the presence of the single-stranded binding protein, RPA (312). Simultaneously the lagging strand is synthesised by discontinuous synthesis with DNA Okazaki fragments. Specialised helicase enzymes such as Dna2 helicase and nuclease remove RNA primers. DNA polymerase enzymes continue synthesis until they reach the 5' end of a previously synthesised fragment at which point DNA ligase I bridges the gap to produce a continuous stretch of DNA. Each region of DNA synthesised from a single origin is referred to as a replicon and while replicon size can vary from 30 to 450 kb, replicons within a cluster of origins tend to be of similar size. This raises the question of which origins fire during mammalian replication since we know that far more origins are prepared as pre-RCs than will actually fire (204). The factors that determine origin licensing and timing of origin firing are two important questions in terms of understanding vertebrate replication. Both of these areas appear to require consideration of chromatin structure. Indeed this may be a contributing factor to origin determination but also plays an important part in origin usage. It is recognised though not fully understood, that transcriptionally active regions of DNA replicate early in that origins fire prior to the latter origins of more transcriptionally silent DNA. The difference between these euchromatic and heterochromatic regions respectively is not fully characterised but is undoubtedly shaped by chromatin structure. DNA damage responses may also play a part in this regulation since ATR

and CHK2 (discussed further in section 3) are important in determining origin firing (244).

Chromatin structure represents the folding and packaging of the DNA of each chromosome around histone proteins to form nucleosome structures that compact the structure of the DNA to allow its ordered and yet compacted packaging into the nucleus. DNA is wound around a protein core known as the histone octomer, which is made up two copies of each histone H2A, H2B, H3 and H4. Higher order compaction of the chromatin into fibres involves interactions between histones in adjacent nucleosomes and non-histone proteins and this largely relies upon covalent modifications of histone tails and the linker histone H1 (114). Chromatin structure determined by such factors, particularly post-translational modification of histone proteins, is a crucial determinant of gene expression and regulation of DNA repair and replication. As a reflection of chromatin importance to such processes it is important to note that DNA replication during S phase requires not only the precise replication of the cellular genomic sequence but also the chromatin structure. As such histone synthesis and nucleosome assembly are highly regulated during S phase to allow accurate epigenetic inheritance (116).

The result of S phase is a cell with a nucleus that contains a duplicate set of chromosomes that are ordered into tightly associated pairs by sister-chromatid cohesion. The accurate duplication of the vertebrate cell requires that sister chromatids be separated and segregated into two separate daughter cells via mitosis. Mitosis is rigorously controlled and cells should not enter into cell division unless any aberrancy that arises during S phase has been resolved, similarly mitosis must continue to completion if at all and as such the entry into and exit from mitosis represent important checkpoints in vertebrate cells. The temporal organisation of mitosis is divided into phases; early metaphase, prophase and metaphase involving centrosome separation and spindle assembly and nuclear envelope breakdown as sister chromatids attach to the spindle and align along the central, metaphase plate. The latter stages of mitosis involve anaphase and dissolution of sister chromatid cohesion and separation of sister chromatids into opposite spindle poles and contractile ring formation to facilitate daughter cell cleavage occurs during telophase, the final stage of mitosis. The reformation of nuclear envelopes around separated

chromosme sets in daughter cells and cytokinesis mark the end of mitosis. The G2/M transition and the metaphase to anaphase transition are most important in terms of entry and exit. Progression through mitosis is controlled by cyclin A and B and the activity of the M phase Cdks is regulated by Wee1 and Cdc25. The events of early prophase are initiation by cyclin A-Cdk activity since unlike cyclin B-Cdk1, these complexes are active and within the nucleus at this stage and they may contribute to the activation of cyclin B-Cdk complexes (98). The profile of cyclin B1-Cdk activity corresponds to the entry of cyclin B1 into the nucleus in mid-prophase and is required to stimulate centrosome separation, nuclear envelope breakdown and spindle assembly. Levels of the nuclear cyclin A and associated Cdk2 activity rapidly fall during mid-prophase in concert with the complete and irreversible activation of cyclin B-Cdk1. The inhibitory phosphorylation of Cdk1 restrains cyclin B-Cdk1 complexes in an inactive state by the inhibitory phosphorylation activity of the Wee1 family proteins, Myt1 and Wee1 kinases. The activity of these kinases falls rapidly during mitosis and as such provides the opportunity for the activation of Cdk1 by the Cdc25 phosphatase enzymes of which there are three isoforms; Cdc25A, Cdc25B and Cdc25C (203). Together these three isoforms contribute to the robust on-off switch transition for Cdk1 activation. The rapid activation of Cdk1 during prophase is a consequence of the sudden increase in Cdc25A and Cdc25C activity with the concomitant decrease in Wee1 and Myt1 activity as a consequence of changes in phosphorylation status of all of these proteins. Positive feedback loops lie at the heart of Cdk1 activation, which is in part catalysed by its own activation of Cdc25 isoforms and inactivation of its kinase inhibitors. Changes in the subcellular localisation of cyclin B1-Cdk1 also govern its activation as does the localisation of Cdc25C whose nuclear translocation during prophase is governed by phosphorylation. While Cdk activity is certainly pivotal in regulating entry into mitosis, the polo like kinases (Plks) are particularly important in the regulation of mitotic exit though they also retain some function in early mitosis. They and the aurora kinase enzymes are important in controlling spindle assembly and sister chromatid segregation (Barr et al 2004). However, spindle assembly and sister-chromatid separation can only occur in the correct conditions to progress through the metaphase-anaphase transition. The metaphase to anaphase transition is driven by the activity of the APC with Cdc20 activator subunit as described earlier and as such any spindle defects will inhibit or block the activation of APC^{Cdc20} and thus mitotic exit.

Successful DNA replication and cytokinesis to produce identical daughter underlies the process of proliferation. Of course for a single cell to become two requires growth in concert with replication and division. The processes of growth and proliferation, particularly in multicellular organisms, must be controlled and such control must be dynamic to support development and complex form. Having summarised various aspects of cell-cycle control and the concept of the cell cycle in general, it is now appropriate to introduce the mechanisms that regulate growth and proliferation, at the heart of which is the cell cycle.

Control of cell proliferation and growth

Start, or the restriction point as it is known in mammals, represents the point at which a cell becomes committed to entering into the cell cycle and completing a round or replication and cytokinesis. In multicellular organisms, such proliferation is strictly governed and cells do not proliferate in the manner that we may be accustomed to seeing in a tissue culture context. As one can imagine there are differences between developmental phase and cell type that require changes to rates of proliferation. It would be unfeasible for cells of a multicellular organism to proliferate continuously. For multicellular organisms, rates of cellular proliferation depend upon both tissue specific genetic programming and signals from the extracellular environment, including other cells. The extracellular signals that stimulate cell cycle entry are known as mitogens. Mitogenic signalling influences the rate of cell division by their effects on the regulators of the G1/S transition, which embodies the restriction point. Once we have described the elements that regulate this transition past the restriction point, we can approach the mechanisms by which mitogens and developmental programming can affect these regulators.

Mitogens are small soluble proteins or peptides that stimulate intracellular signalling following binding to extracellular receptors. Mitogen binding promotes receptor dimerisation and then phosphorylation of tyrosine residues of the mitogen receptors via the kinase domain. The phosphorylated tyrosine residues recruit different SH2-domain containing proteins, prompting several signalling cascades. Ras and Myc pathways are involved in several mitogenic signalling cascades. Activation of the membrane bound GTPase Ras promotes the activation of the MAP kinase pathway.

Signalling via the MAP kinase pathway results in translocation of MAP kinase (MAPK) into the nucleus to phosphorylated proteins that facilitate transcription of immediate-early genes such Fos. Immediate-early proteins such as Fos can assemble with other factors to yield the transcription activator complex AP-1 to stimulate the transcription of genes encoding proteins such as cyclin D1, which is extremely important for promoting pRb phosphorylation and entry past the Restriction Point. Myc is another immediate-early gene product that stimulates progression through the Restriction Point by stimulating various G1 and G1/S cyclin-Cdk components (2).

An alternate pathway that is activated by mitogenic signalling is phosphoinositide-3-kinase (PI3K) signalling. PI3K signalling via the phosphorylation of membrane bound phospholipids can promote Akt protein kinase activity, which can directly control aspects of G1-Cdk activity. Since cyclin D activity is one of the principle requirements for progression through G1/S transition, it is intuitive that several mitogenic signalling cascades promote cell cycle entry by promoting cyclin D activity. Cyclin D levels and activity are typically elevated by mitogenic signalling by both increased expression and by promoted active complex assembly. The Ras MAP kinase pathway is thought to promote cyclin D activity by both of these methods via AP-1mediated transcription of cyclin D1, while Myc induces transcription of cyclin D2 and Cdk4. As discussed previously the Cip-Kip Cdk inhibitors are involved in the activation of cyclin D-Cdk4 and it is suggested that the assembly of cyclinD-Cdk4-p27 requires a MAP kinase-activated assembly factor (50). Mitogenic signalling can also stabilise cyclin D levels by inhibiting the activity of glycogen synthase kinase 3β (GSK3β), which normally functions to promote the nuclear export and proteolysis of cyclin D in the cytoplasm (71). This pathway for cyclin D stabilisation requires PI3K and Akt activity, and by promoting nuclear retention of cyclin D-Cdk complexes, it enhances their activity toward RB proteins.

The activation of cyclin D-Cdk complexes is extremely important for entry into the cell cycle. Mitogens promote cell cycle entry in part by enhancing and stabilising cyclin D protein. Although the Cip-Kip Cdk inhibitors function to activate cyclin D-Cdk complexes, the INK4 Cdk inhibitors are potent inhibitors of cyclin D-Cdk4 and 6 complex formation. INK4 family inhibitors play an important role in the other side to

mitogenic signalling via anti-mitogens, which function to inhibit cellular progression through the restriction point. One such anti-mitogen is transforming growth factor β (TGFβ). TGFβ can induce an increase in the levels of the INK4 family inhibitor $p15^{INK4b}$ promoting G1 arrest (6). As with so many aspects of cell cycle regulation and cell biology the balance between anti-mitogenic and mitogenic signals determine whether a cell will progress into cycle.

Chapter 3 DNA Damage Response in Eukaryotes

- **Sensing DNA damage**
- **Transmitting a DNA damage response**
- **Transcriptional response to DNA damage**
- **The regulation of cell fate in response to DNA damage**
- **Cell-cycle deregulation and genomic instability in cancer**
- **The molecular basis of cancer and cancer therapy**

In order to understand how DNA damage responses influence the cell cycle it is important to first thoroughly understand the unperturbed regulation of cell-cycle progression. As such this detailed discussion of DNA damage responses is positioned after the description of cell-cycle regulation. However, these two chapters are essentially inseparable and should be considered as a whole in order to hold in perspective a view of a cell cycling through time. This section gives some insight into how cell cycle progression and regulation is manipulated in response to DNA damage. Predominantly this involves understanding the concept of cell-cycle checkpoints and the manner in which cellular transcription is influenced in response to DNA damage to facilitate arrest and repair. We will also consider the regulation of cell fate in response to irreparable damage by discussing programmed cell death, namely apoptosis.

Sensing DNA damage

As discussed in the previous chapter there are several regulators that over-see the progression of the eukaryotic cell cycle through G1, S phase, G2 and mitosis. Of pivotal importance are the Cdk proteins and their regulation by association with the cyclins. We have already considered how Cdk subcellular localisation and catalytic sub-unit phosphorylation can modulate their activity and thus speed of transition while regulation of cyclin synthesis is of prime importance in this process.

When a cell senses the occurrence of DNA damage, sensor protein signalling facilitates cell-cycle arrest. Cells pause at one of the key cell-cycle transition points

i.e. G1/S or G2/M hence why these transitions are often known as checkpoints. Since cyclin-Cdk regulation represents a key mode of regulation for cell-cycle progression it is not surprising that signalling to trigger checkpoint activation usually impinges upon cyclin/Cdk regulation in some manner. In subsequent sections we will discuss the observations that AAV infection can cause cell cycle arrest via DNA damage signalling and thus it is relevant here to discuss mechanisms via which cell cycle arrest in the event of DNA damage is achieved. To understand how cell-cycle arrest is achieved, it is important to understand how damage is sensed in the first instance.

Signalling, which relays the presence of DNA damage to the system that regulates checkpoint activation, originates from sensor proteins that function in close proximity to DNA lesions themselves. There are several key sensor proteins that will be discussed here as general sensors of DNA damage. Maintaining genomic stability requires careful monitoring of DNA replication and as such there are dedicated damage sensor systems that function during S phase, these S-phase specific sensors will also be considered.

One group of proteins central to the sensor system is the phosphatidylinositol-3 kinase-like kinases (PIKKs). This group of proteins comprises the ataxia telangiectasia mutated protein (ATM), ATM and Rad3 related protein (ATR) and the catalytic sub-unit of DNA protein kinase (DNA-PKcs). These proteins appear to sense DNA-damage in a lesion-specific manner and as such will be considered individually. ATM is a ubiquitously expressed protein localised predominantly in the nucleus, which despite its homology to the PI3 kinases, is a protein kinase (228). One of the first important demonstrations of ATM's ability to phosphorylate proteins *in vivo* was the observation that ATM phosphorylated p53 at serine 15 in response to DNA damage (15). This is also one of the first premises upon which one can surmise that ATM acts as a damage sensor. Unlike many proposed sensor proteins ATM does not appear to have a dedicated interacting partner that directly binds to DNA. However, the MRN complex, composed of Mre11, Nbs1 and Rad50 proteins has been shown to be important for ATM activation (145). Since the MRN complex displays various DNA-binding capabilities including an ability to assemble linear single-stranded DNA fragments together with ATM to form signalling complexes (57), it is likely that the MRN complex provides the DNA-binding platform for the sensor activation of

ATM in response to DNA damage. However, ATM has been shown to possess some DNA binding propensity with a preference for double-strand breaks (267). In the absence of cellular stress, ATM molecules exist as dimers or multimers that are catalytically inactive. It is considered that ionising radiation for example causes some manner of changes to chromatin structure that in turn activates ATM. Activation involves the intermolecular phosphorylation of serine 1981 causing the disruption of dimer molecules, and takes place within minutes of DNA damage. Dimer dissociation releases ATM molecules whose catalytic domains are no longer concealed and are thus liberated to phosphorylate ATM's many downstream substrates (18). These downstream substrates include proteins involved in co-ordinating cell-cycle arrest, DNA repair and apoptosis.

ATR is somewhat distinct from ATM in function since it appears to be important in the regulation of responses to UV irradiation or replicative stress (Abraham 2001, Shiloh 2001) as oppose to ionising radiation-induced strand breaks. Furthermore, unlike ATM, ATR is not maintained in a catalytically inactive state by dimerisation. However, ATR does appear to have a dedicated DNA-binding partner protein known as ATR-interacting protein, ATRIP. In mammals ATRIP colocalises with ATR into nuclear foci upon DNA damage or inhibition of replication, and is phosphorylated by ATR (56). The single-stranded binding protein, replication protein A (RPA) is required for ATRIP to bind irradiation-induced sites of DNA damage and as such the recruitment and activation of ATR is thought to require regions of single-stranded DNA (320).

There are two further potential sensor complexes thought to function in sensing damage outside of S phase; the 9-1-1 complex made up of RAD9, RAD1 and HUS1 and the RAD17-RFC complex. The presence of RPA on ssDNA is thought to promote RAD17-RFC binding to gapped and primed ssDNA and subsequently facilitate the binding of the 9-1-1 complex (321).

For simplicity we frequently discuss the sensors of DNA damage and indeed their signalling targets as acting independently, with one sensor for a discrete lesion type for example. However, it is undoubtedly the case that there is cross talk between sensors, which will affect downstream signalling events. In some respects it is not in

our favour to attribute very definite roles to these proteins as it may hinder our understanding of their roles as more information becomes available but the complexity of DNA damage signalling is such that it is important to establish some order for descriptive purposes

The above discussion of sensors of DNA damage does not encompass our understanding of surveillance of replication during S phase. During S phase the cellular genome is perhaps at it's most vulnerable to damage particularly from replication fork inhibition or collapse and it is of prime importance that the cell be prepared to arrest and be primed for repair. There are several specific mechanisms that allow for replication to be completed even in the presence of damage known as damage tolerance pathways both of which require properly co-ordinated replication arrest. Sensors facilitate the initiation of two types of replication arrest following damage during S phase. The G2/M checkpoint, which prevents entry into mitosis in the presence of unreplicated or damaged DNA and the intra-S phase checkpoint, which represents the slowing of DNA repliaction by the inhibition of further origin firing (207). Several of the sensors already discussed are important in terms of generating S phase arrest. The MRN complex in particular is important for S phase arrest caused by ionising radiation but not UV irradiation-induced S phase arrest (155). ATR has also been shown to directly interact with replication forks and may, in concert with RPA, survey replication to facilitate rapid checkpoint signalling in the event of replication stress (63). It is also true that replication lesions may be converted into strand breaks for instance that may be recognised by the sensor mechanisms already discussed. There is also evidence for S phase-specific damage sensors dedicated to monitoring replication fork progression and co-ordinating arrest. Although much of this evidence comes from observations made in yeast there have been suggestions of a sensing role of interaction between both TopBP1 and RAD9 and between polymerase ε and RAD17. This is similar to the potential role of the 9-1-1 complex and RAD17-RFC already implicated in damage sensing (170, 214).

As is the case for much of the field of DNA damage research there are always discrepancies between the many model organisms from yeast to Xenopus through to humans. Despite this, a picture does emerge for eukaryotic damage sensing. This picture portrays a myriad of sensor proteins that may have their specialities but also

demonstrate considerable cross talk and interaction. It is quite spectacular to consider the many levels at which this suggests a cell may be prepared for the considerably diverse forms of damage that may be encountered. It is of importance to note that some of the most profound pathologies of disorders relating to DNA damage responses such as Ataxia telangiectasia involve loss or mutation of key sensor proteins. It is not surprising that efficient surveillance and this very first step of damage signalling is of pivotal importance and that any impairment in our ability to sense damage will be detrimental in the extreme.

Transmitting a DNA damage response

Sensing DNA damage represents the first step of a DNA damage response that will ultimately lead to arrest, repair or even death. To reach any or all of these outcomes, the cell relies on the process of signal transduction from sensors to the key regulators of cell cycle progression. This transduction can be simply described stepwise as the activation of effector protein kinases by phosphorylation originating from sensor inputs. Inputs from sensor proteins are thought to be conveyed to effectors by mediator proteins. In humans two of the most prominent effector proteins of DNA damage are the protein kinases CHK1 and CHK2, and their role in generating a transmissible damage signal will be discussed here.

CHK2 protein structure is highly conserved and consists of a key regulatory domain known as an activation loop and at least one SQ/TQ residue-rich domain, which represents the favoured residue combination for ATM/ATR-dependent phosphorylation (17). CHK2 also possesses a 'fork head associated' (FHA) domain, which is involved in phosphopeptide recognition and is thought to facilitate a conditional interaction based on phosphorylation status of an interacting protein partner (82). For example this may allow the preferential formation of a platform for signal transduction in response to DNA damage-induced phosphorylation of sensor proteins.

DNA damage or the inhibition of replication leads to the rapid phosphorylation of CHK2 (177) by ATM. This is then followed by further auto-phosphorylation facilitated by the FHA domain (144). There is considerable importance for the FHA

domain in terms of the phosphorylation of CHK2. As such it is speculated that there is a mediator protein involved, which must be recognised by the FHA domain. The 'mediator of DNA damage checkpoint' protein, MDC1, is thought to play this role though evidence also exists for the importance of 53BP1 as mediator and in fact the two bear several similarities. Both MDC1 and 53BP1, first identified as a p53 interacting protein (124), are phosphorylated by ATM. Both proteins demonstrate physical and functional interaction with phosphorylated H2AX; (phosphorylated form denoted by γH2AX) the phosphorylation of which is ATM-dependent following ionising radiation, and ATR-ATRIP-dependent following UV irradiation. Strong support of 53BP1's mediatory role comes from the fact that in response to ionising radiation, 53BP1 binds ATM and this binding is necessary for subsequent ATM-dependent phosphorylation of CHK2 (73). MDC1's role may be somewhat more complex since MDC1 also interacts with the MRN complex and strengthens ATM binding to double-strand break lesions (166). In addition to the potential role of MDC1 and 53BP1 in feedback loops to increase ATM activity in response to DNA damage, there are clear positions for these protein in mediating signal from sensor to effector i.e. ATM to CHK2.

CHK1 is the second effector for signal transmission from ATM and ATR to cell cycle regulatory substrates. Experiments suggest that CHK1 plays a pivotal role in coordinating a G2/M checkpoint and may be important in replication fork recovery and monitoring origin firing (93). CHK1 appears to be the principle recipient of ATR kinase activity, and human claspin is thought to play the part of mediating the signal from ATR (51). Following DNA damage or replication stress, claspin associates with ATR, the 9-1-1 complex and CHK1, and is required for CHK1 phosphorylation. Ultimately the effector roles of CHK1 and CHK2 may overlap but they display individual characteristics of function that are distinct from one another. For the sake of simplicity, it is more important here to highlight the hierarchy of signalling that takes place to generate a transmissible signal.

Firstly, sensor proteins recognise DNA damage. Activation of PIKK proteins occurs via mediator proteins and chromatin modifications. Ultimately signal amplification facilitates effector kinase activation. At this point we need to consider the downstream targets of these streams of signalling, which regulate cell cycle progression and DNA

repair processes. These two aspects depend upon and are very much intertwined with transcriptional regulation in determining cell fate. One of the key elements in the process of transcriptional regulation in relation to DNA damage is p53. The relationship between DNA damage sensing and activation of downstream signalling components such as p53 are highlighted in figure 1.3.1. As a key target of cell cycle checkpoint pathways, an understanding of p53 function is pivotal to our understanding of how cell signalling of DNA damage can be transduced from the point of effector activation.

Simple statistics demonstrate the importance of p53 in maintaining genomic stability. A staggering proportion of cancers have been linked to mutations of p53 with between 50 and 70% of lung, colorectal and breast cancers, exemplifying why p53 is one of the most renowned tumour suppressors discovered. First and foremost, p53 is a transcriptional activator of genes that contain p53-responsive elements but p53 can also function as a transcriptional repressor of genes that do not (134). Understanding p53's role as a transducer of a DNA damage signal requires an understanding of how p53 is regulated. The functional domains of the p53 protein and various sites of the post-translational modifications of relevance to p53 regulation are summarized in figure 1.3.2.

As a transcriptional regulator, the levels of p53 are of prime importance to its function but do not solely dictate its activity. Indeed the levels of p53 and its ability to function as a transcriptional activator are predominantly regulated by a considerable array of post-translational modifications. Phosphorylation, acetylation, methylation, sumoylation and ubiquitination all play important roles and the extensive possible modifications reflects the importance of p53 in integrating signals from many sources in order to govern a cell's response to a variety of stress signals, not merely DNA damage.

Figure 1.3.1

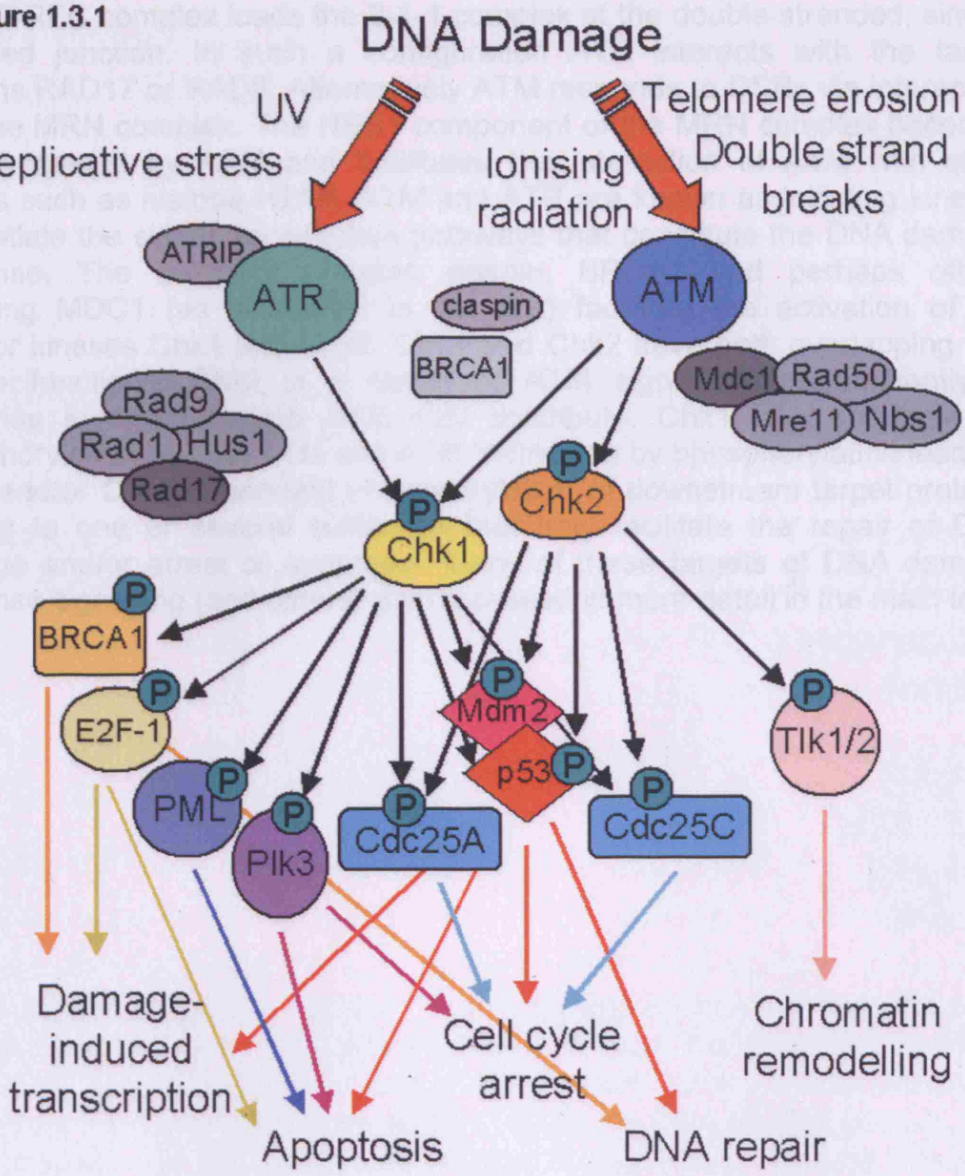


Figure 1.3.1. DNA damage leads to the cell-cycle arrest, apoptosis, DNA repair, damage induced transcription and chromatin remodelling via complex signal transduction. DNA damage may be caused by a plethora of both endogenous and exogenous means. The examples highlighted here include UV irradiation, replicative stress, ionising radiation and telomere erosion. Two of the primary DNA damage sensor protein kinases involved in the recognition of DNA damage are ATM and ATR. ATR functions in concert with the ATR interacting protein, ATRIP, which is phosphorylated by ATR and colocalises with ATR in intranuclear foci following DNA damage and replicative stress. Double strand breaks (DSBs) may be resected yielding

single stranded sections that become bound by RPA. Concomitantly the RAD17-RFC complex loads the 9-1-1 complex at the double-stranded, single-stranded junction. In such a configuration ATR interacts with the target proteins RAD17 or RAD9. Alternatively ATM responds to DSBs via interaction with the MRN complex. The NBS1 component of the MRN complex becomes phosphorylated by ATM and facilitates the interaction of ATM with other targets such as histone H2AX. ATM and ATR are known as initiating kinases that initiate the signal transduction pathways that constitute the DNA damage response. The mediator proteins claspin, BRCA1 and perhaps others including MDC1 (as discussed in the text) facilitate the activation of the effector kinases Chk1 and Chk2. Chk1 and Chk2 have both overlapping and unique functions. Chk2 is a target of ATM signalling predominantly in response to DSBs though ATR may contribute. Chk1 is a substrate for phosphorylation by both ATM and ATR. Activation by phosphorylation leads to Chk1 and/or Chk2 dependent phosphorylation of downstream target proteins leading to one or several outcomes that may facilitate the repair of DNA damage and/or arrest or apoptosis. Many of these targets of DNA damage response signalling (and others) are discussed in more detail in the main text.

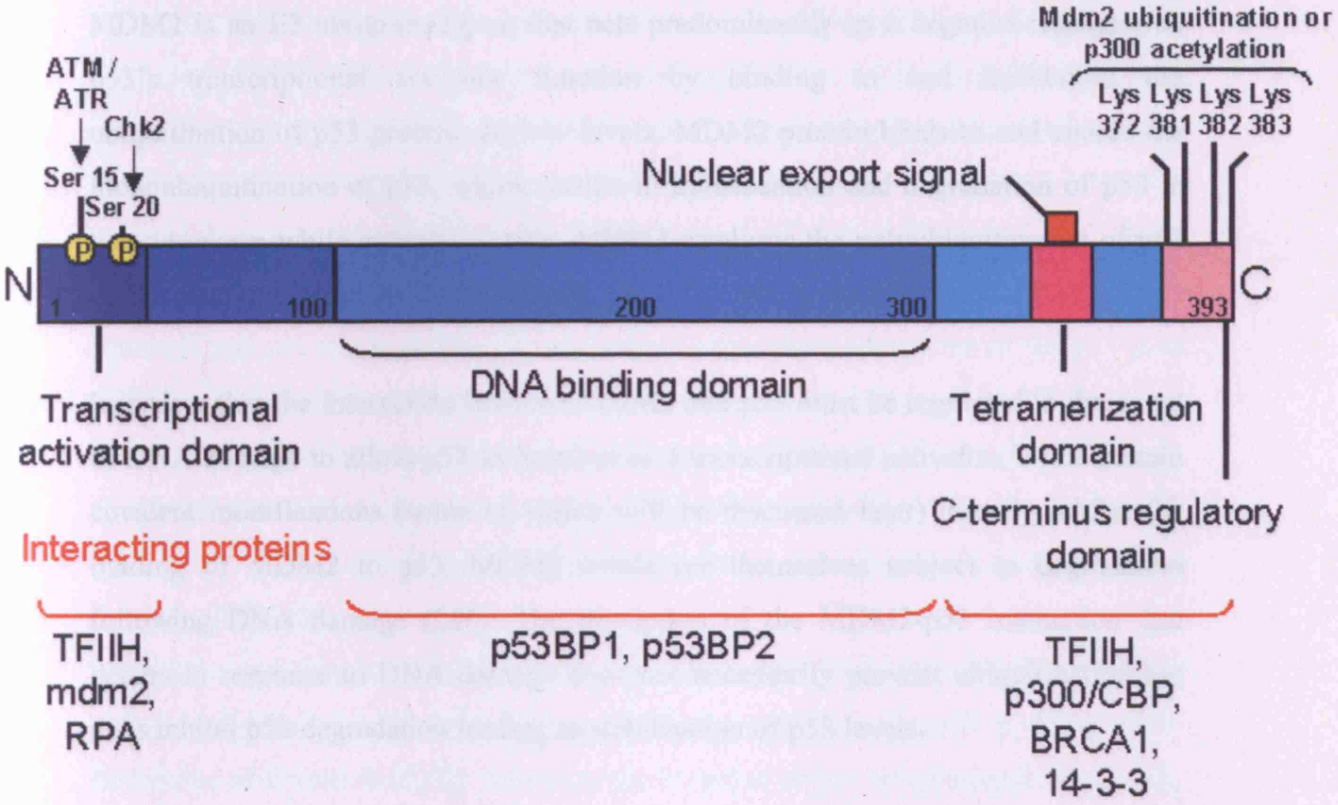


Figure 1.3.2. A summary figure of the functional domains of the p53 protein, various protein interactions and examples of post-translational modifications. The transactivation domain of p53 spans the N terminal region from residue 1 to 42. The more centrally located region of the protein, the DNA binding domain, is located within residues 102-292. The tetramerisation domain spans residues 323-356 within which the nuclear export signal is located. The regulatory domain occupies the C terminus between residues 363 and 393. The reported protein interactions with these p53 protein domains are extensive, some of which have been discussed to some degree in the main text and are indicated in the lower part of the figure. The p53 protein undergoes considerable post-translational modification via phosphorylation, acetylation, methylation, ubiquitination, NEDDylation and sumoylation. Some of these modifications occur in response to non-genotoxic stresses such as osmotic stress but p53 is predominantly modified in response to genotoxic stress such a s replicative senescence, UV and DNA breaks as a result of ionising radiation. Only a few of these modifications are indicated here in accordance with those referred to in the text. The key kinase enzymes involved in the phosphorylation of p53 are Chk1, Chk2, ATM, ATR, CAK, p300. The post-translational modification of p53 in response to such stress regulate both the levels and activity of p53 as a transactivational activator.

In terms of levels of p53 protein, the most important interactions are between p53 and MDM2 and ubiquitination since these two factors essentially maintain the normal, low cellular levels of p53 that are essential for normal cell cycle progression (307). MDM2 is an E3 ubiquitin ligase that acts predominantly as a negative regulator of p53's transcriptional activator function by binding to and facilitating the ubiquitination of p53 protein. At low levels, MDM2 protein binds to and causes the monoubiquitination of p53, which results in translocation and degradation of p53 in the cytoplasm while at higher levels, MDM2 catalyses the polyubiquitination of p53 and its degradation in the nucleus (150).

It is clear that the interaction between MDM2 and p53 must be regulated in the event of DNA damage to allow p53 to function as a transcriptional activator. While certain covalent modifications (some of which will be discussed later) directly inhibit the binding of MDM2 to p53, MDM2 levels are themselves subject to degradation following DNA damage (260). The disruption of the MDM2-p53 interaction that occurs in response to DNA damage does not necessarily prevent ubiquitination but does inhibit p53 degradation leading to stabilisation of p53 levels.

Phosphorylation is another key determinant of p53 activity and there are over 20 characterised phosphorylation sites. The MDM2 binding region in particular contains several residues that are phosphorylated in response to DNA damage, of particular interest are serines 15 and 20. Serine 15 phosphorylation has been reported to positively influence the transactivation capability of p53 (81)while other reports suggest an inhibition of MDM2-mediated cytoplasmic export of p53 and thus inhibition of degradation (315). Reports suggest that ATM and ATR are the primary kinases responsible for serine 15 phosphoryltion in response to both UV and ionising radiation, while DNA-PK activity has also been implicated (15, 148, 277). Serine 20 phosphoylation is reportedly mediated by CHK1 and CHK2 in response to UV, ionising radiation and reactive oxygen species damage (46, 248). The outcome of serine 20 phosphorylation appears to be the inhibition of the MDM2-p53 interaction. Discussion of only these two phosphorylation sites does not cover the breadth of knowledge with regards to kinases implicated in p53 phosphorylation. However, it does demonstrate how DNA damage signalling can impinge upon p53 from various levels i.e. both sensor and effector kinase activity. Various other protein modifications

can be made to p53, such as acetylation, which may depend upon previous modification by one or more of the mechanisms discussed above. Ultimately, we see a complex picture of modifications that influence p53-regulated genes via p53 activation. The transcriptional targets of p53 underlie p53's role in orchestrating both G1/S and G2/M phase cell cycle arrest in response to DNA damage. The steps involved in co-ordinating these two cell cycle checkpoints will be discussed and the factors downstream of p53 activation will be highlighted.

Although p53 activity is important for both G1/S and G2/M phase arrest it is clear that above all p53 activity is pivotal for G1/S phase arrest since abrogation of the p53 gene entirely prevents G1 arrest in response to ionising radiation. One of the most important targets of p53 transcriptional activation in generating G1/S arrest is p21. As described in earlier sections, the transition from G1 to S phase requires cyclin-Cdk complex formation and kinase activity. Of particular significance is cyclin E-CDK2 complex formation in progression from G1. As a promiscuous Cdk inhibitor, p21 disrupts a broad range of Cdk proteins and their activity but pertinent to the role of p21 in maintaining a G1/S phase arrest is the inhibition of cyclin E-CDK2 (85). Inhibition of cyclin E-CDK2 promotes G1/S phase arrest by ultimately impinging upon the activity of the retinoblastoma tumour suppressor protein pRb. The function of pRb is so crucial to cell cycle progression because it governs the activity of the E2F transcription factors, the activity of which is to promote S phase by the transcription of proteins required for replication. When pRb is phosphorylated it is no-longer able to bind E2F and thus E2F is free to conduct it's transcriptional activities. Phosphorylation of pRb is a product of both CDK4/6-cyclin D and CDK2 and cyclin E complex kinase activity and since p21's activity as CDK inhibitor inhibits such phosphorylation we see p21 promoting a hypophosphorylated state for pRb, thus inhibiting cell cycle progression into S phase (19). There are additional proposed mechanisms for prevention of entry into S phase and inhibition of concurrent S phase progression but the role of p53 and the p53 transcriptional targets remain of paramount importance.

While the role of p53 and hence the role of transcription in generating cell cycle arrest is indisputable it is also true that a transcriptional response represents a 'slow' mechanism of modulating any immediate signalling system. Indeed, in addition to the

p53-dependent process delineated above there is also a proposed system for a rapid response following DNA damage to achieve G1/S phase arrest involving both the degradation of cyclin D1 (4) and the inhibition of CDC45 binding to the origin (ORI) recognition complex (58). The destabilisation of cyclin D1 inhibits cell-cycle progression because during normal progression, p21 binds and activates the cyclin D1-CDK4 complex allowing cyclin E-CDK2 to promotes entry into S-phase. Degradation of cyclin D1 releases p21 and allows p21 to bind and inactivate cyclin E-CDK2. The inhibition of CDC45 ORI binding is the eventual result of signalling originating from DNA damage-induced activation of ATM and ATR. The resulting phosphorylation of CHK1 and CHK2 promotes the phosphorylation of CDC25A thus targeting it for ubiquitination and eventual degradation by the SCF proteasome. Without the phosphatase activity of CDC25 the inhibitory phosphate groups are not removed from CDK2 and without cyclin E-CDK2 activity CDC45 will be unable to bind to the ORI and thus origin firing is inhibited.

As suggested earlier the G2/M checkpoint response following DNA damage relies less heavily upon p53, though a G2/M arrest is a prominent cell-cycle checkpoint. As is the case in terms of G1/S phase arrest there are essentially two arms to the regulation of the G2/M checkpoint involving the transcriptional activator potential of p53 on one hand and the regulation of CDC25 phosphatase on the other. There are at least two key targets of p53 transcription that are involved in maintaining G2/M arrest. The increased transcription of p21 functions in part to inactivate CDK1 via its broad range Cdk inhibitor activity. CDK1 inactivation also requires the p53-dependent transcription of 14-3-3σ protein. In this case 14-3-3σ promotes the nuclear export of CDK1 and cyclin B into the cytoplasm thus preventing the activation of CDK1 required for the onset of mitosis (117). Inactivation and sequestration of CDK1 and cyclin B is pivotal to maintenance of G2/M arrest and thus prevention of mitotic catastrophe, the process of fatal entry into mitosis in the presence of DNA damage following the failure of cell cycle checkpoints (44). The transcription of 14-3-3σ also promotes a positive feedback loop since it blocks the MDM2-dependent ubiquitination and nuclear export of p53 thus promoting p53 stabilisation and in turn augmenting its own transcription (306). The additional facet to G2/M arrest is the prevention of CDC25 phosphatase-mediated removal of the inhibitory phosphate

group from CDK1. There are three isoforms of CDC25 and there is some controversy as to the isoform of functional importance in this aspect of checkpoint signalling. Despite controversy the important model is that the phosphorylation of either CDC25A or CDC25C by CHK1 inhibits the binding of CDC25 phosphatase to CDK1 or promotes destabilisation of CDC25 (169, 209) via cytoplasmic sequestration by 14-3-3 binding (note the alternate isoform, not the 14-3-3σ isoform).

While the G1/S and G2/M transitions represent the most clearly characterised checkpoints, there is also evidence for both an S phase checkpoint and an M phase checkpoint. S phase arrest most likely involves the inhibition of origin firing. Further to this is the role of the *structural maintenance of chromosomes* protein, SMC1, as a substrate for phosphorylation by both ATM-MRN and BRCA1-dependent pathways in response to ionising radiation, UV radiation and hydroxyurea treatment (131, 132). The precise mode of function of SMC1 in generating S phase arrest remains unknown. As part of the cohesion complex, which functions to maintain sister chromatid cohesion following fork progression, it is possible that phosphorylation somehow affects its function in cohesion and perhaps in turn fork progression. Similarly the Fanconi anemia product protein, FANCD2 is phosphorylated in an ATM-dependent reaction that is also required for S phase arrest though the mechanism is not clear (273). Further to these pathways of S phase arrest is the anticipated role of replication protein A (RPA) in slowing S phase in response to DNA damage. RPA not only plays some roles in detecting DNA damage but may also be the recipient of checkpoint signalling during S phase. RPA is essential for DNA replication and plays a part in repair and homologous recombination. The function of RPA is modulated by phosphorylation (30). In particular hyperphosphorylation of the 34kDa subunit of RPA mediated by PIKK protein kinase activity is thought to inhibit RPA's function in replication and promote function in repair processes. This may be a consequence of hyperphosphoryltion excluding RPA from replication origins (282).

The existence of an M phase checkpoint remains highly speculative and much research into this potential checkpoint comes from the study of yeast. In mammalian cells DNA damage during mitosis does not necessarily cause arrest though may cause a delay in exit from mitosis (254). A potential checkpoint involved in mitotic integrity

is the mitotic spindle checkpoint which signals diminished centromere function following considerable DNA damage (186).

After an extensive discussion of cell-cycle arrest in response to DNA damage it is important to consider the potential benefits of such arrest. It is clear that repair mechanisms are likely to be important if resolving DNA damage is a possibility and it emerges that checkpoint signalling may influence the transcription of repair proteins, their intracellular localisation and such factors may also be the target of effector kinase activity. These concepts will be considered in more detail in the following section in which we will describe some of the outcomes of sensing, and then transmitting a DNA damage signal as the transcription response to DNA damage.

Transcriptional response to DNA damage

It is not surprising that the vertebrate transcriptional response to DNA damage is enormously complex. It involves not merely the transcriptional regulation of genes involved in DNA repair processes but also growth factors or growth factor receptors and proteins more commonly associated with tissue injury and inflammation (119), all of which are extremely interesting when we consider the response of the organism as a whole to the presence of DNA damaged cells for example the role of the immune system. In fact the pathways that are activated by DNA damage are extremely diverse and differs considerably from that of prokaryotes such as bacteria that can summon a defined repair response, the SOS response, following DNA damage. This likely corresponds to the obvious difference in organism complexity since the vertebrate response is not only undoubtedly cell type specific to some degree but must also integrate extracellular cues and manage the outcome of a balance between promotion of growth and survival with signals of arrest and apoptosis. Compounding this is the point that DNA damage is not the only form of cellular stress that proteins such as p53 respond to. Furthermore even in the case of DNA damage, a response is not only lesion-specific but also modulated in concert with dose.

We have already emphasised the importance of the transcriptional activation properties of p53 in relation to the generation of cell cycle arrest. p53 is also

important in terms of translational regulation of proteins and processes involved in repair mechanisms. In many respects p53 co-ordinates cell-cycle progression in concert with repair and cell death, acting very much as the integrating factor of the complex system depicted above. It is important to reiterate the point that while enhancement of transcription is a very significant property of p53, the repression of transcription has also been mentioned as an additional factor in p53-mediated translational modulation (316, 317). However, our aim here is to summarise some of the understood p53-dependent as well as independent transcriptional responses to damage signalling, many of which also involve repair processes.

When we consider repair mechanisms it is important to remember that repair processes are largely lesion-specific and of course lesion type is often a reflection of DNA damaging agent. In light of this, p53 plays a role in co-ordinating repair of various lesion types in response to diverse DNA-damaging insults. First we might consider the role of p53 in double-strand break repair such as that resulting from exposure to ionising radiation. The process of double-strand break repair involves both non-homologous end-joining (NHEJ) and homologous recombination (HR). One component to the involvement of p53 in double-strand break repair is the p53 target gene, p53R2, a ribonucleotide reductase. The role of p53R2 in response to DNA damage is thought to be that of supplying the dNTPs that are urgently required for repair. It may not only function in the maintenance of the dNTP pool but also directly in the protein repair complexes (272). There is also implication for p53's interaction with components of the NHEJ and HR repair pathways (100), though these direct protein interactions may not dictate the functional importance of p53 in terms of repair.

One of the important repair mechanism employed by cells to repair lesions such as those introduced by UV radiation is that of nucleotide excision repair (NER). Evidence has come to the fore that implicates p53 in the regulation of NER. In one respect p53 appears to directly influence the transcription of proteins involved in nucleotide excision repair such as XPC and DDB2, whose roles in global genomic repair have not been thoroughly elucidate (3). The contribution of p53 to NER may also be by the modulation of chromatin accessibility. The p53 responsive gene product GADD45a may in part facilitate this by binding damaged DNA (40).

GADD45a and b may also contribute to G2/M arrest by regulation of the CDK1-cyclin B1 complexes (313). On the other hand p53 may also have a direct role in DNA repair processes. p53 has been reported to be able to recognise and bind to regions of damaged DNA in a mode distinct from its sequence-specific transactivational DNA binding activity (146, 221, 299). p53 has also been shown to posses exonuclease activity that may have role in DNA repair (194).

The regulation of cell fate in response to DNA damage

Thus far we have discussed the sensing of DNA damage, the modulation of cell cycle progression, changes to the cellular transcriptional profile and DNA repair. Although mutation is the driving force of evolution, gross damage to the cellular genome cannot be tolerated. If DNA damage cannot be resolved, that is, repair mechanisms are insufficient and/or damage is too great, the likely outcome for a metazoan cell is cell death. Cell death may manifest itself from one of several processes, but the essential mechanistic form of cell death is that of programmed cell death, apoptosis. Cells may enter into the cell cycle in the presence of DNA damage, by the process of adaptation but this will frequently end in what has been termed as unregulated cell death, or mitotic catastrophe (42). Adaptation in the absence of death is almost certainly the prequel to cancer. Although this is generally perceived as a passive mechanism, there is data from studies on Drosophilia to suggest that it may indeed be an actively regulated system to prevent genomic instability (269), perhaps depending upon CHK2 activity. In vertebrates it is generally perceived that this form of cell death is unusual since apoptotic mechanisms will generally prevail.

However, if DNA damage can be resolved, checkpoint signalling should cease and allow the cell cycle to resume. Perhaps when DNA damage no-longer persists there is no-longer an activating signal and thus cells are able to overcome cell-cycle checkpoint arrest. Perhaps understanding the process of adaptation will teach us something about the more favourable process of recovery following successful DNA repair and indeed the mechanisms behind both may well overlap. One mechanism that is proposed to be significant to recovery from checkpoint arrest following double-strand breaks involves DNA-PKcs (106). The manner in which this takes place has

not been clarified but the dephosphorylation of DNA-PK by protein phosphatase 5 may well be one such mechanism (293).

The role for p53 in response to DNA damage may extend to the process of recovery since several pathways that negatively regulate p53 function appear to be involved in the course of recovery; this is not surprising since we have already discussed how p53 functions so strongly in activating checkpoints. Proposed factors implicated in recovery include c-Jun and MYC protein. The mechanism of c-Jun-mediated cell cycle re-entry is thought to involve repression of p53-mediated transactivation and thus inhibition of p21 induction (242). MYC protein, more commonly recognised as the product of an oncogene, may also function to inhibit the upregulation of p21 in a similar mechanism to that of c-Jun via inhibition of p53 through the protein Miz1 (118).

However, repair is not always possible and for the safety of the whole organism the cells of multicellular organisms have the ability to commit themselves to a cell death programme, apoptosis. In many respects apoptosis represents the ultimate fail-safe mechanism. We have considered the many pathways that run in parallel to sense damage, propagate a signal and give a cell the best opportunity to repair any abnormalities in the light of DNA damage. Many of these pathways are at least semi-redundant since the cell appears ready for at least one or more pathway to fail and ultimately the cell is prepared with a complex array of pathways to ensure that emerging aberrant cells cannot perpetuate.

Apoptosis involves the co-ordinated disintegration of the cell by protease and nuclease enzymes. An apoptotic cell will typically shrink and the cell membrane will bleb out. Chromosome condensation is followed by internucleosomal endonuclease activity to yield DNA fragmentation (176). Apoptosis takes course by one of two well-characterised pathways, the extrinsic and the intrinsic pathways. Activation of the extrinsic pathway involves an extracellular stimulus whereas the intrinsic pathway is stimulated from within the cell; as such many of the components of either pathway are fairly distinct though they may ultimately converge upon similar effectors. Of particular importance are the caspase enzymes, which play a pivotal role in apoptotic cascades. The family of caspase enzymes are cysteine proteases that facilitate the

proteolytic cleavage of cellular proteins upon activation of an apoptotic programme (226). The caspases are broadly grouped into two classes, the initiator and the effector caspases. In humans the initiator caspases include caspase-2, -8, -9, and –10, while the effector caspases are –3, -6 and –7. The initiator caspases are auto-activated triggering downstream caspase cascades.

The intrinsic pathway responds to intracellular signals and instigates cell death in a mode that involves the cytoplasmic release of proteins from the intermembrane, mitochondrial space. Cell death stimuli is transduced to the mitochondria via the BH3-only domain proteins BID and BIM while the pro-apoptotic BCL-2 family members such as BAX, BAK and BOK influence the release of mitochondrial factors probably by influencing mitochondrial membrane integrity. There are also anti-apoptotic members of the BCL-2 family and these two may interact such that a pro-apoptotic member can inhibit the anti-apoptotic activities of the other. Many pro-apoptotic proteins are products of genes that contain p53 responsive elements including BAX, PUMA, PIGs (p53-induced genes), NOXA, FAS, APAF-1 and PERP, many of which impose their pro-apoptotic ability by localising to the mitochondria and inducing membrane potential changes that lead to the cascade of events that begins with cytochrome c release (285). In particular the PIGs are redox-regulating enzymes that control membrane integrity by the formation of reactive oxygen species that damage mitochondrial membranes (213). The expression of several anti-apoptotic proteins has been shown to be repressed by p53 including Bcl-2, MAP4 and survivin (195). The ability of p53 to function at the level of DNA-binding and transcription is not the only device at hand for p53's pro-apoptotic function. On one level p53 appears to be directly imported into mitochondria and functionally interact with both BAK and BAX to induce membrane potential destabilisation (52, 149).

Various pro-apoptotic proteins are released from mitochondria including SMAC (second mitochondria derived activator of caspases) and DIABLO (direct inhibitor of apoptosis binding protein with low pI), which function to prevent the activity of the inhibitors of apoptotic proteins (IAPs). The release of cytochrome c is of fundamental importance however since it promotes the activation of APAF-1 by allowing binding of ATP and the formation of the apoptosome, which results in the activation of

initiator caspase-9. The activation of pro-caspase-9 by the apoptosome leads to the activation of the effector caspase 3 and subsequently a collection of downstream components that function to dissemble chromatin and encourage DNA fragmentation.

One of the better-studied modes of extrinsic activation involves the tumour necrosis factor (TNF) receptor family member, FAS. The binding of FAS ligand to the FAS death receptor results in the multimerisation of the intracellular portion of the receptor known as the death domain. Multimerisation allows binding of the FAS-associated death domain (FADD) adaptor, which facilitates the recruitment of the initiator caspase, caspase 8. This large complex is known as the death inducing signalling complex (DISC) and its formation leads to the autocatalytic cleavage of caspase 8 and thus its activation. Caspase-8 activation leads to activation of the effector caspase-3 and a cascade of caspase activation ensues. It is possible that the extrinsic pathway can activate the intrinsic pathway via caspase-8 mediated cleavage of BID to facilitate release of mitochondrial proteins. A brief summary of the extrinsic and intrinsic pathways of apoptosis is described in figure 1.3.3.

It is important to emphasise that both the intrinsic and extrinsic pathways can be of significance to DNA damage-induced cell death even though one might imagine that DNA damage would be purely sensed by intrinsic mechanisms. For example, DNA damage increases the expression of FAS receptor and ligand (130), and UV-induced cell death may be mediated by death receptor pathways in some instances (223). However it is intuitive that in the majority of cases, it is considered that apoptosis in response to DNA damage involves the mitochondrial pathway and that p53 is the co-ordinator of DNA damage-induced cell death.

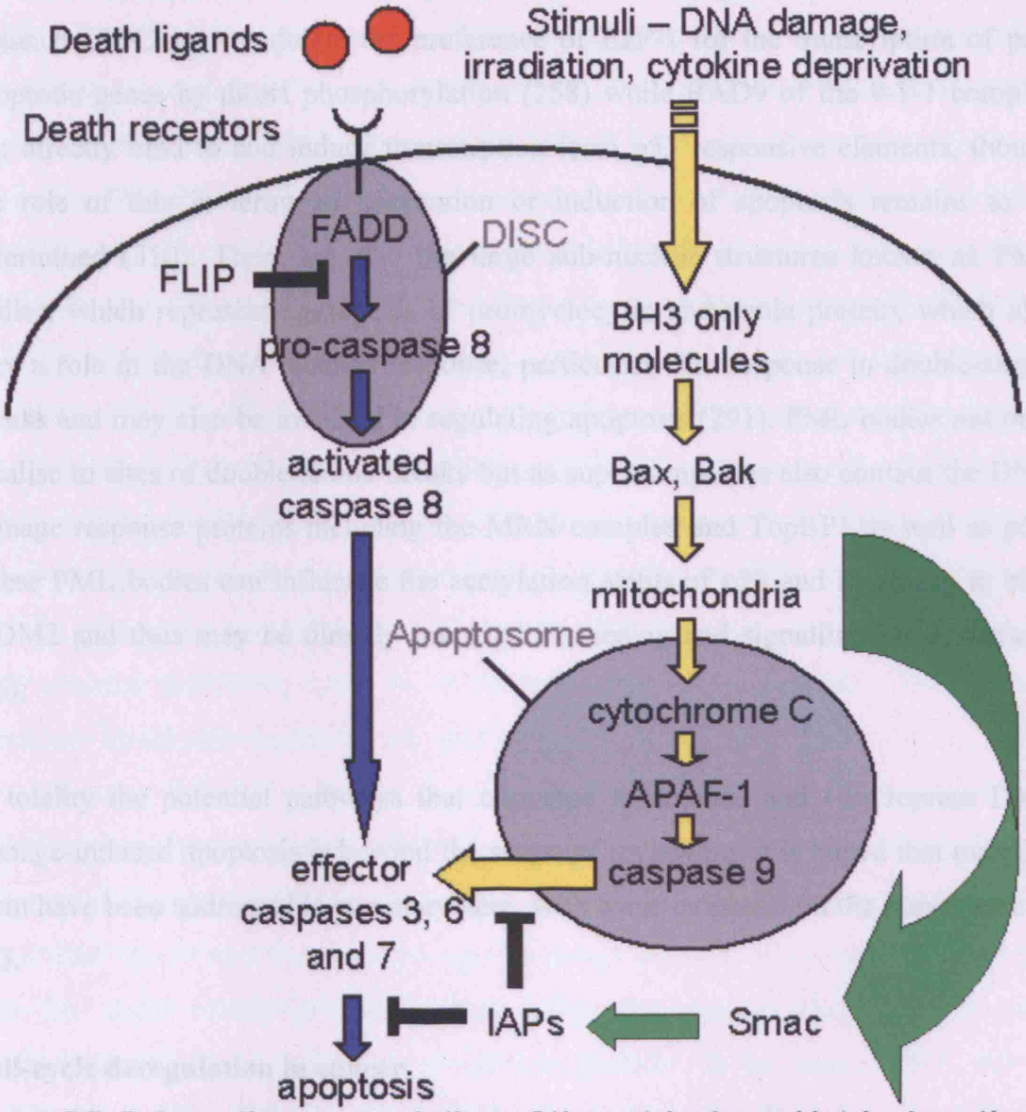


Figure 1.3.3. Schematic representation of the extrinsic and intrinsic pathways to apoptosis. The left side of the diagram shows the extrinsic apoptotic pathway. The extrinsic apoptotic pathway is initiated when death receptors at the cell surface encounter specific 'death ligands'. Ligand binding induces the formation of the death inducing signalling complex (DISC), which in turn cleaves and activates the initiator caspases, which in turn activate a second group of caspases known as effector caspases, which conduct the apoptotic process via the degradation of key intracellular substrates. The right hand side of the diagram represents the intrinsic pathway of apoptosis though it is important to note that the distinction between intrinsic and extrinsic is purely descriptive and there is cross-talk between both pathways. The intrinsic pathway of apoptosis centres around the mitochondria and more importantly the process of mitochondrial outer membrane permeabilisation.

Several of the kinase proteins involved in checkpoint responses also play a role in apoptosis. Their regulation of transcription factors (in addition to p53), may mediate the observed requirement for these proteins in DNA damage-induced cell death. For instance CHK2 can modulate the preference of E2F-1 for the transcription of pro-apoptotic genes by direct phosphorylation (258) while RAD9 of the 9-1-1 complex can directly bind to and induce transcription from p53 responsive elements, though the role of this in terms of prevention or induction of apoptosis remains to be determined (310). There are also the large sub-nuclear structures known as PML bodies, which represent aggregates of promyelocytic leukaemia protein, which also play a role in the DNA damage response, particularly the response to double-strand breaks and may also be involved in regulating apoptosis (291). PML bodies not only localise to sites of double-strand breaks but as supercomplexes also contain the DNA damage response proteins including the MRN complex and TopBP1 as well as p53. These PML bodies can influence the acetylation status of p53 and its ability to bind MDM2 and thus may be directly involved in sensing and signalling DNA damage (66).

In totality the potential pathways that converge to activate and / or repress DNA damage-induced apoptosis is beyond the scope of review but it is hoped that many of them have been addressed in summary here, with some emphasis on the importance of p53.

Cell-cycle deregulation in cancer

In 2000 Hanahan and Weinberg (111) provided the field with a comprehensive evaluation of the changes that must be required for a cell to become a cancer cell. Several key features of cancer cells were put into the spotlight as 'the hallmarks of cancer'. These properties include the ability of cancer cells to evade apoptosis, insensitivity to anti-growth signals, a limitless replicative potential and self-sufficiency in growth signals. We know that the manner in which a cancer cell achieves such properties is via a process of accumulation of both genetic and epigenetic changes to genes that regulate such processes. As a consequence of their pivotal importance to normal cell existence, many of the genes that are frequently mutated in human cancers are known as oncogenes or tumour suppressors and are

almost always involved in the processes described as hallmarks of cancer, such as replication, growth and proliferation, cell cycle checkpoints and apoptosis.

There are various mechanisms by which oncogenes may be activated from their normal 'proto-oncogenic'. Gene amplification, classic mutation and chromosomal rearragments all play roles in some of medicine's most well characterised forms of cancer specifically associated with oncogenes such as Burkitt's lymphoma in which the *MYC* gene undergoes chromosomal translocation. MYC whose function is as a regulatory factor during mitogenic signalling is rearranged to be under the control of an immunoglobulin gene regulatory element that results in excessive and abnormal expression of MYC. Many of the other commonly mutated oncogenes are involved in mitogenic signalling such as RAS, EGFR and PDGR. Conversely the disruption of tumour suppressors typically involves their inactivation and the mutation of tumour suppressor genes usually requires inactivation mutation of both alleles of the gene. The list of tumour suppressor genes is inseparable from the cancer pathology that their absence promotes, such as ATM and ataxia talengiectasia, BRCA1 and hereditary breast and ovarian cancer, pRB and retinoblastoma.

Of course not all cancers are associated with disruption to chromosomal structure or sequence. Of particular pertinence are the many cancers associated with viruses such as cervical cancer and the causative agent, human papillomavirus (HPV). As is the case for many virally-initiated cancer states, the process frequently involves mechanistic inactivation of tumour suppressor proteins. In the case of HPV, this at least involves the viral-induced inactivation of p53 and pRb via interaction and degradation by the viral proteins E6 and E7 (74).

When we consider the process of cell cycle and checkpoint deregulation in terms of cancer it is important to bear in mind the idea that for a cell that is a member of a multicellular organism, proliferation is not the default. These cells do not undergo continuous proliferation and only proliferate when given the correct series of stimuli from extracellular mitogens, growth and survival factors. We know that one of the classic 'hallmarks' of cancer is indeed a loss of dependency upon these signals for proliferation. As such it follows that cancer cells frequently acquire oncogenic mutations in aspects of mitogenic signalling such as MYC, as exemplified above, or

even cell-surface mitogen receptors. Ultimately mitogenic signalling is integrated into cell cycle control and cancer cells frequently carry mutation that affects cell cycle entry and thus G1/S phase gene expression, which is usually regulated by the E2F family of transcription factors. All cells, regardless of their tissue origin, must activate cyclin-CDK complexes to progress into cycle via phosphorylation of pRB and ultimately activation of E2F. Frequently, cancer cells display mutations that are associated with pRB's regulatory role in the G1/S phase transition and affect pRb's ability to restrain E2F's transcriptional capabilities (245). Potential mutations affect the cyclin-CDK complexes that phosphorylate pRb either by overproduction of cyclin or CDK subunits or by mutation that renders CDK molecules refractive to CDK inhibitors or tumours actually lose a gene required for CDK inhibition such as $p16^{INK4a}$. While less frequent than mutation of the CDK4/cyclinD1- $p16^{INK4a}$ –pRb pathway, which is deficient in high frequency in human cancers (172) pRB may itself be lost or compromised (201, 212).

Multicellular organisms are typically of a particular size and shape because the tissues that constitute the organism are carefully regulated in their size by restriction to growth and proliferation while tumours represent cell populations that have lost their normal growth restrictions. Growth factors stimulate growth in part by PI3 kinase activation, which stimulates PIP3 synthesis and in turn leads to Akt activation. Akt activation stimulates the protein kinase TOR, which promotes protein synthesis and growth in part by increasing the production of ribosomes (175). To achieve a state that forgoes growth inhibition, cancer cells frequently harbour mutations of the PI3 kinase-Akt signalling pathway (230).

While growth factors and mitogenic signalling herald the potential to actively encourage growth and proliferation, we know that survival factors function to suppress apoptosis and thus grant cells survival in the appropriate context. Cancer cells frequently do not require such pro-survival signalling if they have mutations of apoptotic apparatus such as over-production of anti-apoptotic mediators such as Bcl-2 family or loss of the FADD adaptor Apaf1.

Unlike cancer cells, normal cells would not respond to constitutively active mitogenic signalling but rather this would induce cell-death or permenant withdrawal from the

cell-cycle, i.e. senescence via upregulation of $p16^{INK4a}$ or ARF (156). This response is known as the hyperproliferation response and is of course frequently suppressed in human cancers either by the over-production of survival signals such as Bcl-2 or inhibition of pro-apoptotic devices. Since ARF signalling in response to hyperproliferation promotes cell death via p53, we highlight another reason why loss of functional p53 is beneficial to cancer. The loss of p53 is of profound importance to human cancer and almost warrants an acknowledgement as a defining 'hallmark'. As a crucial hinge between life and death in response to diverse stress stimuli, not merely DNA damage, it is clear how the disruption of p53 function alone can promote genomic instability.

The concept of genomic instability essentially represents that way in which we perceive the process that drives tumorigenesis. It designates a state of increased genetic mutation, loss or rearrangement and potentially arises from an initial phase of spontaneous mutation. Several forms of instability may contribute to genome wide instability arising from defects or mistakes in various processes. The DNA damage responses are of particular importance to this concept since mutations in aspects of DNA damage responses and checkpoint signalling frequently predispose genomic instability such as loss of p53 or ATM. One form of instability is known as microsatellite instability and manifests itself as an increased number of point mutations in microsatellite regions of DNA i.e. regions of repetition in DNA sequence. This form of instability arises from defects that affect the mismatch repair machinery. Defects in mismatch repair leave the genome vulnerable to the accumulation of point mutation. Components of this pathway and the analogous repair process of base excision repair are not usually associated with defects that promote genomic instability. However the hereditary disease, xeroderma pigmentosum, is characterised by a high predisposition to cancer as a consequence of defective nucleotide excision repair and thus inadequate repair of UV-induced DNA lesions. Despite the involvement of these processes in generating genomic instability, one of the most significant paths is via chromosomal instability arising from defects in chromosome number or structure i.e. when regions from one chromosome may be exchanged or attached to another chromosome. Chromosomal instability is thought to arise from DNA damage response defects, telomere erosion as well as defects in chromosomal segregation and cell division. Of course not all rearrangements that

arise from breakage-fusion events will be viable and in many cases such translocation and recombinations will be lethal; it is in the few instances when cells survive with gross reassortments that chromosomal instability becomes a significant cancer risk.

Defects in the cellular response to double-strand breaks such as MRN and BRCA1 deficiencies can lead to chromosomal instability via failures in the process of homologous recombination. Additionally, telomere defects that allow the emergence of chromosome ends unprotected by the telomere cap, will facilitate the fusion of broken ends between chromosomes, or following replication, between sister chromatid ends (11). This will frequently result in the production of dicentric chromosomes that will undoubtedly be broken by opposing spindle pole bodies during division, yielding further broken ends. This represents the model of breakage-fusion-bridge cycles that can promote the loss or amplification of large portions of chromosome. While the shortening of telomeres during cell division is important in preventing unrestrained proliferation, eroded telomeres can promote the form of chromosomal instability described above when p53 defects abrogate the mode by which eroded telomeres would normally signal the onset of apoptosis or senescence (94).

It is not unusual for cancer cells to harbour unusual (aneuploid) numbers of chromosomes and frequently this arises as a consequence of the accidental generation of a tetraploid state (four copies of each chromosome) through abnormal cell division for example. Subsequent chromosomal segregation may result in uneven and irregular chromosomal distribution, expression from which may result in abnormal protein expression profiles. Furthermore cancer cells may not harbour the correct numbers of centrosomes for segregation and division and this is frequently associated with alterations to the spindle checkpoint system such as Mad2 and Bub1 (135).

Frequently, the described chromosomal instabilities are accentuated by defects in p53 and our understanding of how DNA damage repair mechanisms and checkpoint signalling overlap and potentiate gross instability is part of how understanding tumorigenesis may progress in the future. Understanding how combinations of defects and deficiencies manifest themselves in human cancer supports our ability to treat

tumours. Indeed, many of our weapons against cancer abuse the very factors that promote cancer manifestation itself.

The molecular basis of cancer and cancer therapy

Thus far we have discussed how normal cells control their cell cycle, growth and proliferation and how DNA damage responses are intimately linked with these control mechanisms. We have summarised how DNA damage can be sensed and communicated to effectors of cell-cycle arrest, repair and cell death and described the important mechanisms that drive tumorigenesis. Finally it is important to consider how our molecular understanding of cancer evolution can aid our efforts to minimise cancer mortality and target tumours.

The ideal world for cancer therapy would be the complete eradication of all tumour cells leaving normal cells unscathed. Our current methods for cancer treatment are comparatively barbaric when we consider such an ideal. While surgical removal of primary tumours can, in many cases be successful, surgery is frequently not an option and in such cases radiotherapy and chemotherapy are the alternatives.

In traditional radiation-based therapy, the molecular basis of cancer is exploited by modulation of both dose and frequency to utilize the potential differences between tumour cells and normal cell radiosensitivity. The basis for this lies in understanding how genotype might predict resistance to both radio and chemo therapeutic agents as cancer cells are frequently more sensitive to DNA damaging agents such as radiation and drugs that inhibit DNA synthesis or directly damage DNA (108). For example a loss of p53 results in genetic instability as a result of loss of cell cycle checkpoints. However loss of p53 can also promote increased survival and radiation-resistance since the pro-apoptotic activities of p53 are also abrogated. As such the activation of alternative apoptotic pathways that are independent of frequently abrogated tumour suppresors such as p53 is an important therapeutic research focus (108).

The manipulation of checkpoint responses is a further strategy for consideration to widen the susceptibility window for treatment. Broad range inhibitors of checkpoint kinases such as caffeine and CHK1 inhibitors are currently of interest in the

sensitisation of p53-deficient cells to cell death (318). Another strategy is combination therapy devised to trigger DNA damage checkpoints in normal cells to protect them from the secondary agent thus making the secondary drug more selective. For example the tubulin modifier Taxol that disrupts spindle function is lethal to cells in mitosis. Low dose DNA-damaging agents will cause arrest in normal cells that still retain normal checkpoint function while cancer cells that are frequently checkpoint deficient will continue to cycle thus rendering them more susceptible to subsequent Taxol treatment (31).

Chapter 4 Viral Interactions with the DNA Damage Response

- **DNA viruses and the DNA damage response**
- **Retroviruses and the DNA damage response**
- **Tumour viruses and cell cycle checkpoints**
- **Oncolytic viruses**

- **Thesis Aims**

DNA viruses and the DNA damage response

Aside from the various interactions that viral proteins have with cellular factors and the potential genomic instability that they may cause (as discussed in more detail in section 4.4), there is a fundamental level upon which viruses may interact with the DNA damage response. This is because of the nature of viral DNAs and their replication intermediates, which appear as abnormal structures that may potentially induce a DNA damage response. As such many viruses have evolved ways counteract such responses, which might otherwise prevent or inhibit productive viral replication. Furthermore, some viruses may well benefit from such responses.

The DNA damage responses do not constitute our traditional perceptions of anti-viral responses such as the interferon response. Indeed more recently this traditional understanding of anti-viral interferon responses was observed to feed into DNA damage responses as one of its antiviral mechanisms (270). The DNA damage surveillance mechanisms are so well honed through evolution that it seems almost natural that this system, so adept at recognising aberrant DNA structures, would be employed as an anti-viral defence. Furthermore since DNA damage responses function with great efficacy as effectors toward DNA substrates, it is not unlikely that the various systems that we recognise as contributing toward cellular defence to viral pathogens integrate on many levels and also revolve around DNA damage networks.

The past decade has seen a considerable increase in our appreciation of viral interactions with DNA damage responses and the literature is now teeming with reported interactions. In many cases these interactions are not always associated with a known function (249) but several other such interactions highlight the importance of inactivating the DNA damage response to permit a successful viral life-cycle. Alternatively some viruses do appear to benefit from a DNA damage response and these too will be discussed below. There are very few instances in which viral RNA interactions with DNA damage responses have been reported. Viral RNA interactions are more thoroughly considered at the level of retroviruses and as such integration and these interactions will be discussed in the following section (4.2). This section will attempt to address some of the reported interactions of DNA viruses with the DNA damage responses and will specifically deal with experiments that more completely demonstrate a well-understood story.

The Adenovirus lifecycle represents one of the best-characterised examples of how viruses may elicit a DNA damage response that can be detrimental to viral reproduction. Adenovirus harbours a double-stranded DNA genome with flushed ends. These DNA ends are recognised by the cellular repair machinery, which attempts to join these ends in a repair-like manner. This is detrimental to the virus since it results in circular concatemers of newly replicated genomes that cannot be packaged into viral capsids. As such Adenovirus has evolved mechanisms to evade the DNA damage repair machinery. The ability of wild-type Adenovirus to evade the cellular repair machinery usually masks the actual DNA damage response. As such, much of the work that has alluded to the cellular response to Adenovirus comes from the use of mutant viruses that are unable to produce the viral proteins that are required to derail the cellular response.

When the E4 protein-encoding region of Adenovirus is deleted and mutant viruses produced and used to infect cells, the activation of a response reminiscent of the cellular response to double strand breaks is observed. In particular, the formation of viral replication centres coincides and colocalises with the accumulation of activated ATM and the MRN complex (261). The MRN complex functions as a platform for ATM activation in response to cellular DNA damage. The observation that MRN complex colocalises with these replication foci suggests that the MRN complex

recognises some structures present as viral replication intermediates as damaged DNA. As the replicative cycle of these mutant viruses continue it was observed that replicated viral genomes are joined together to form concatemers that cannot be packaged and thus in many respects this represents an unsuccessful productive infection since progeny viruses are not produced despite replication taking place. The process of joining of these viral genomes appears to involve cellular repair mechanisms, particularly the non-homologous end-joining (NHEJ) pathway for DNA repair and particularly, DNA-PKcs and ligase IV activity, both of which function as important components of the NHEJ response to double strand breaks (33, 261).

The mechanism by which this takes place involves the co-ordinated effort of two proteins; the E4orf6 and E4orf3 proteins. These two viral proteins disrupt MRN complex activity. E4orf3 expression prevents the accumulation of Mre11 to replication centres and promotes the redistribution of MRN complex components into aggresome-like structures (9). While redistribution of MRN prevents concatemer formation, the direct degradation of Mre11 by the complex of E1B-55K and E4orf6 attenuates DNA damage signal transduction thus ablating ATM activation, while simultaneously removing the catalyst for viral genome joining (41, 261).

The manipulation of DNA damage signalling and DNA repair mechanisms by the Adenoviral oncoproteins E4orf6 and E4orf3 represents an important part of the adenoviral life cycle. These aspects of interaction with the cellular DNA damage machinery are not the limits of adenoviral manipulation of such responses and further aspects of oncoproteins function are considered below in the context of tumour viral interactions with cell cycle checkpoints and control.

Viral interactions with PML bodies are also considered to be significant with regard to viral replication since several viruses have been seen to influence these intranuclear structures also known as ND10s and PODs. PML bodies are composed of PML protein and many other diverse proteins involved in transcriptional regulation, apoptosis and cell cycle control. PML bodies are thought to play a role in DNA damage responses including apoptosis and chromatin modification as well as gene expression and p53 function. PML bodies are considered to function as part of the

cellular anti-viral defence and as such it makes evolutionary sense that several viruses appear to have the ability to manipulate PML body structure and function (199, 222).

Herpes Simplex Virus 1 (HSV1) and Adenoviral interactions with PML bodies have been considerably investigated while Epstein-Barr Virus (EBV), SV40 and AAV also have reported interactions (89, 96) though for AAV this lies in the context of helper viral co-infections. HSV1 and PML body manipulation has been extensively investigated and will be used as an example here to explore the concept of viral-mediated changes to PML body structure and function. Addition features of the HSV lifecycle in terms of viral-induced changes to DNA damage and repair protein localisation will also be considered.

PML nuclear bodies are not considered to be particularly mobile structures within the nucleus but they are thought to be relatively dynamic in nature in that it is imagined that these bodies represent pools of various proteins that can be readily manoeuvred to required sites for function such as regions of DNA damage as proposed in previous section 3.2. In the case of HSV for example it is suggested that proteins associated with PML bodies become localised adjacent to incoming viral genomes (91). For several DNA viruses one also observes the recruitment of DNA damage response and repair proteins to viral replication centres. In some cases as described previously (for Adenovirus) this may not be beneficial. In a similar way to Adenovirus, HSV1 has a liner double-stranded genome. However, in the case of HSV1 the recruitment of the MRN complex and activation of subsequent DNA damage responses is considered to be advantageous for viral replication since when Mre11 is removed, viral replication is less efficient (153). Conversely, HSV1 must disrupt PML bodies for optimum viral replication. For HSV-1 the principal protein involved in PML body remodelling is the E3 ubiquitin ligase, ICP0. ICP0 activity promotes the proteasome-dependant degradation of PML (90). If PML-remodelling is inhibited by disruption of ICP0 expression, viral gene expression is repressed and lytic infection suppressed.

In addition to the degradation of PML protein, HSV ICP0 protein also degrades DNA-PKcs, which is thought to be beneficial to HSV replication for several reasons relating to the role of DNA-PKcs, which includes processing and repair of double strand breaks. On one hand it is thought that ICP0 maintains the HSV genome in a

configuration that is optimal for viral replication by inhibiting circle formation and it is thought that ICP0 may in part achieve this by the degradation of DNA-PKcs (205). The latency aspect of HSV infection is an important facet of herpesvirus pathology and relies upon the circularisation of the viral DNA to promote its dormancy. As described, it is considered that HSV may also benefit from some aspects of DNA damage signalling and in terms encouraging viral latency, it is thought that HSV may benefit from some aspects of the cellular DNA repair machinery. Hence ICP0's degradation of DNA-PKcs is beneficial for the productive, replicative element of the HSV lifecycle when DNA circularisation is not an optimal configuration (125).

The exemplification of adenovirus and herpesvirus interactions with DNA damage responses highlights several similarities in terms of the recruitment of DNA damage response proteins to replication centres and disruption of PML nuclear bodies. However these two viruses are generally perceived to reside at opposite ends of a scale with regards to benefits gained from DNA damage signalling, with Adenovirus seemingly inhibited by such responses and Herpes virus supposedly benefiting (154). DNA damage signalling is extremely complex and many pathways interact perhaps via a single protein and so while abrogation of one element may hinder viral replication it is not clear what pathways such abrogation would have blocked. A balance of benefit versus detriment is entirely artificial and perhaps DNA damage signalling as a process is too complex and intricate to make such a distinction, as are viral infringements upon such signalling. However, the lessons that can be taken away from viral interactions with cellular DNA damage processes and cell cycle mechanics may be enormously beneficial to our understanding of cellular processes.

Unlike HSV and Adenovirus, the parvovirus AAV is a non-autonomous virus. Viral transcription is minimal in the absence of a helper virus and yet the potential interactions of AAV with the cellular DNA damage response is enormously significant. Unlike the majority of DNA viruses reported to have interactions with DNA damage signalling, AAV is a single-stranded DNA virus. In the absence of permissive conditions the AAV genome can be integrated in a site-specific manner into the human genome. As such the AAV genome can exist in different forms; as a single-stranded DNA with double-stranded ends, as an independent double-stranded molecule, as a, integrated DNA or as a circular double stranded form. These different

configurations may act as substrates for different elements of DNA damage signalling. Indeed in the absence of any viral transcription, AAV infection results in the activation of various DNA damage signals. The infection of various human cell lines with AAV reveals the generation of a G2/M arrest (220). This G2/M phase arrest is dependent upon the activation of an ATR-dependent response that results in the activation of Chk1. Ultimately, this culminates in the inhibition of the cyclinB/cdc2 complex by the degradation of CDC25C. The observed cell-cycle arrest occurs in parallel to the formation of intranuclear foci composed of various DNA damage related proteins that colocalise with AAV DNA. It is proposed that RPA, Rad51 and DNA polymerase delta are involved in the initial recognition of the single-stranded AAV DNA molecule (129). The hypothesis follows that if polymerase delta cannot progress along the DNA, a signalling complex reminiscent of a stalled replication fork is recruited consisting of ATR and the 9-1-1 complex, which may facilitate the ATR-dependent recruitment of BLM protein. The adaptor proteins TopBP1 and Brca1 promote the ATR kinase-mediated activation of Chk1 and subsequent G2 arrest.

More recently it has also been shown that using recombinant AAV that viral genome processing into circular double-stranded species requires both ATM and DNA-PKcs (53). Recombinant AAV is frequently used as an experimental model since considerable interest is placed in the development of recombinant AAV vectors for use in gene therapy. These vectors resemble wild-type AAV in terms of the single-stranded genome and terminal repeat structure but of course the viral genes are replaced with genes of interest and relevant promoter elements. While some genomes will integrate into cellular chromosomes it has been recently observed that both wild-type and recombinant AAV can persist in a circular, episomal form (236, 237). It has previously been suggested that DNA-PKcs is involved in promoting the formation of episomal forms, without which i.e. in the context of SCID mice, linear molecules persist that are not usually observed in normal tissues (80, 255), and that DNA-PK may inhibit the integration of AAV DNA (256).

The ability of AAV genomes to persist in tissues is one of the most important factors for consideration in terms of gene therapy development. The implication that host cell repair factors are involved in the process of circularisation and concatemerisation that is seemingly required for episomal persistence has lead to the in-depth consideration

of the cellular pathways that govern such processing. The AAV terminal repeat structures represent the substrate for genome processing. The double-strand break repair pathway is the predominant recombination mechanism involved in AAV genome processing. Using transgenic mice and mutant tissue culture cell line systems, several proteins were implicated in being significant for AAV genome recombination though the significance of each protein differed somewhat between systems. Collectively it was shown that ATM, DNA-PKcs, Mre11, Nbs1, BLM and WRN proteins were important while the activity of Nbs1 was only significant in tissue culture cells and DNA-PKcs only in transgenic animals. These differences were attributed to the proliferative nature of cell lines. The previously characterised roles of these proteins in various aspects of non-homologous end-joining (NHEJ) and homologous recombination (HR) led the authors to propose that NHEJ mechanisms were of prime importance for AAV genome processing in quiescent cells and HR mechanisms in dividing cells. In particular the authors speculate a role for the single-strand annealing mechanism of HR, a homology dependent mechanism of double strand break repair that requires only small regions of homology, which are aligned and intervening sequences are removed (25).

The described interactions of AAV DNA with DNA damage response and repair pathways focus entirely upon the cellular responses to AAV DNA as a substrate and do not infer a role for any AAV gene products. There are however some reports of the AAV Rep gene products in modulating cell cycle progression. Of particular interest is the observation that the Rep78 protein elicits a complete S phase arrest (233). The study of AAV's potential oncosuppressive properties has brought to the fore AAV's anti-proliferative activity. In particular there is a strong implication that Rep gene expression suppresses helper viral oncoprotein-mediated cellular transformation as well as DNA replication and it is likely that these observations purvey some cell cycle modulatory role for the Rep gene products. As such, the activities of AAV proteins in regulating cell-cycle checkpoints will be discussed here, along side our introduction to viral DNA interactions with DNA damage responses.

Rep 68 and Rep 78 both induce cell cycle arrest though at different phases of cell cycle progression. Rep 78 can cause complete S phase arrest while Rep 68 promotes arrest at both G1 and G2 phases of the cell cycle. The apparent difference in activity

resides in the zinc finger domain of Rep 78, the role of which may be to promote the accumulation of hypophosphorylated pRb. The mechanism behind the ability of Rep 78 to evoke an unusual S phase arrest involves two modes of action. The Rep 78 protein is able to directly induce DNA damage by nicking of the cellular DNA leading to an ATM-CHK2 dependent DNA damage response. The endonuclease activity of Rep 78 is not sufficient to generate complete S phase arrest, and it is observed that the zinc finger domain is also required. The zinc finger domain promotes the interaction of Rep 78 with the cellular protein phosphatase CDC25A resulting in stabilisation of the protein but preventing it from activating CDK1 and CDK2 thus suppressing its cellular activity (29). Whilst these two activities are inseparable in terms of their requirement for generating S phase arrest, it remains unclear how these activities may be reconciled to result in such arrest. It is proposed that the ability of Rep 78 to promote the accumulation of active pRb (233) is relevant to the ability of Rep 78 to sustain S phase arrest. It is speculated that the combined endonuclease activity and CDC25A inhibition is sufficient to cause the complete activation of cellular pools of pRb (29). The authors propose that Rep 78 can activate pRb to such a degree that this alone is capable of preventing S phase progress. This is supported by the observation that pRb a complete S phase arrest can represent 'chronic' pRb-mediated arrest (8). This is likely mediated by a lack of expression of replication factors, leading to a senescence-like programme that is molecularly distinct from a pRb-mediated pause in S phase.

The ability of AAV Rep proteins to induce cell cycle arrest in and around S phase is undoubtedly a contributing factor to the potential anti-proliferative effect of AAV infection. In addition to these interactions it seems that AAV Rep proteins can also directly inhibit the ability of tumour virus proteins to cripple the cell cycle checkpoint system. It has been shown that AAV Rep proteins can bind and stabilise p53, preventing ubiquitin-mediated proteolytic degradation (24). Rep 78 is perceived to conduct this activity by direct binding to p53 and is considered to be the principle Rep protein responsible for preventing p53 degradation by tumour viruses such as Adenovirus. Furthermore, Rep 78 has also been shown to protect the pRB family of pocket proteins (pRb, p107, p130) from inactivation by the Adenoviral E1A protein (22). It appears that this is achieved by the binding of Rep proteins to E1A thus preventing its association with the pRB proteins and rescuing them from targeted

disruption. It is also postulated that AAV Rep proteins may induce expression of the pRB family proteins and thus contribute to their elevated levels via transactivation of transcription in addition to direct protection in the presence of Adenovirus. Rep 78 may also actively suppress E2F-1-mediated transcription providing an additional mechanism to suppress cell cycle progression and DNA replication (23). This tri-partite rescue of cell-cycle checkpoint and DNA damage control proteins represents an important facet to how AAV Rep proteins mediate anti-proliferative and indeed anti-oncogenic properties.

Retroviruses and the DNA damage response

Contrary to DNA viruses, retroviruses harbour an RNA genome and there have been few reports of RNA viral interactions with DNA damage responses. This may be due to the counter-intuitive nature of searching for such interactions since RNA substrates are not considered to be as important in terms of activation of cellular DNA damage responses. However, as we probe deeper into the complex world of DNA damage signalling it is becoming clear that RNA processing and transcriptional control signalling may play a significant role and be integrated into the DNA damage response. As such perhaps forthcoming research will uncover a deeper role for DNA damage signalling responses for RNA viruses.

Retroviruses however have a complex element to their life cycle whereby reverse transcription of their viral RNA ultimately leads to the formation of a duplex DNA molecule that is then integrated into the host cellular genome. As such we can see that retroviruses present not only an RNA substrate but also a DNA substrate to the cell, which furthermore requires recombinogenic mechanisms to integrate into host DNA. In this guise there have been several discoveries that highlight the importance of cellular DNA damage repair mechanisms for retroviral life cycles.

Retroviral integration is extremely important for viral persistence but given the efficiency of surveillance of cellular DNA integrity it is not surprising that in the process of retroviral integration, cellular DNA damage proteins may become involved. There are several phases to the process of retroviral integration, which may prompt a cellular DNA damage response. The process of retroviral integration

involves a version of a virally-encoded integrase enzyme, which facilitates some end processing of the viral DNA complex, which itself represents a substrate for DNA damage signalling. This is followed by the concerted nicking of cellular DNA with ligation into the genome. This process causes localised chromatin structure disruption and the momentary introduction of a double-strand break that if not sealed at either end of the viral insertion via post-integration repair, may emerge as more complex lesion.

The process of post-integration repair is extremely important in terms of avoiding the generation of further DNA breaks that might trigger a potent DNA damage response, which could lead to perceived irreparable damage and cell death, which is not beneficial for retroviral persistence. It is observed that the process of post-integration repair is mediated by a host of cellular repair proteins, not virally encoded factors. The process and enzymes involved in non-homologous end-joining are considered to be required for post-integration repair and as such successful integration. Cells that are deficient in the non-homologous end-joining repair pathway appear to be more susceptible to cell death following retroviral infection and that retroviral transduction is reduced by up to 90% in non-homologous end-joining deficient cells (60, 62). It is speculated that cell death is a consequence of unsuccessful integration leading to DNA strand breaks. Biochemical analysis using specific PIKK protein inhibitors points to a significant role for DNA-PK and perhaps ATM in the process of successful retroviral integration though the genuine relevance of DNA-PK in the process of retroviral integration is strongly contested (10, 13).

Despite the controversy regarding the role of DNA-PK in terms of retroviral integration it seems that there is some agreement for the importance of the other PIKK proteins ATM and ATR in terms of signalling DNA damage during retroviral integration and it is clear that the process of retroviral integration does generate a DNA damage response. One of the primary indicators of DNA damage signalling and indeed changes to chromatin structure is the phosphorylation of histone H2AX. The generation of γ-H2AX is indicative of an active DNA damage response and has been shown to occur in response to retroviral integration (62). While phosphorylated histone H2AX is not required for retroviral integration and post-integration repair, its phosphorylation suggests the activation of the upstream kinases for which H2AX is a

substrate, such as ATM, ATR and DNA-PK. It has been shown that retroviral integration activates an ATR-dependent DNA damage response (61) and an ATM-mediated DNA damage response (141). Such responses are considered beneficial to retroviral integration and may aid the process of repair since caffeine treatment (a broadly effective inhibitor of PIKK activity) inhibits retroviral transduction efficiency. Indeed, ATM inhibitors, caffeine and caffeine-related substances are currently being considered for their potential in terms of inhibition of retroviral transduction and particularly in terms of HIV therapy (141, 202).

It is clear that the process of integration represents the key point during retroviral life cycles at which they are at the mercy of the host cell since cellular DNA repair response factors are so crucial to successful integration. Other factors pin-pointed in terms of having significant roles in this process are proteins known to be of significance to homologous recombination and trans-lesion synthesis, Rad52 and Rad18. The HIV-1 integrase enzyme is seen to associate with Rad18 and it is thought that the trans-lesion synthesis repair pathway may be involved in the process of retroviral integration (193).

It is surprising that so many DNA damage response pathways might be stimulated by retroviral integration and in totality be of benefit to retroviral transduction. The presence of numerous free cDNA retroviral genomes in the nucleus and the lack of detrimental DNA damage signalling suggests that retroviruses have evolved to predominantly benefit from responses that for many viruses inhibit productive life-cycles. However, it seems the case that the homologous recombination repair component Rad52 does act in detriment to retroviral integration (140). In a Rad52 null background, an increase in retroviral integration is observed. It is not considered that homologous recombination pathways are inhibitory in general but rather Rad52 presents a hurdle for retroviral integration by binding of the afore mentioned cDNA genomes. The proposed mechanism of inhibition involves the competition between Rad52 binding, which does not promote integration; with the dedicated integration complexes required for successful integration events.

The wealth of host DNA repair factors implicated as having retroviral interactions, and the clarification of their roles in viral life cycles has provided much hope for the

development of retroviral therapeutics. When we consider the purpose of striving to understand viral interactions in general with DNA damage responses it might be said that elucidating novel targets for the generation of viral therapeutics is one of the main goals of such fields. In the light of such a purpose one might say that the identification of retroviral DNA damage response and repair interactions represents one of the more significant success stories of this field with several clinical trials revolving around such observations. This may be seen as somewhat ironic when we consider that the field emerged with the expectation that DNA viruses would dominate the field.

Tumour viruses and cell-cycle checkpoints

We have already considered at length what constitutes normal cell-cycle progression and the key co-ordinating factors that ensure regulated growth and proliferation in the correct context for multicellular organisms. However, for viruses the cellular milieu is of pivotal importance to viral replication. Though this may not be the same for every virus there are some similarities in the general requirement of a cellular context that is relevant for replication and the viral life-cycle. In the process of generating such an environment some viruses may kick-start a process that may lead to tumorigenesis. These viruses are consequently known as tumour viruses because of their unequivocal association with cancers, some of which are of considerable relevance to human disease.

As described, viruses require cells to be prepared to replicate so that a virus can use some of the vital components of replication that it cannot provide itself. To do so a virus must force a cell into cycle and to do so a virus must manipulate both cell cycle and checkpoint control proteins. To achieve this a virus must encode its own tools, the relevant proteins. We have already considered the events that are required to overcome cell-cycle checkpoints and promote proliferation in the guise of tumorigenesis. Viruses of course reach the same objective by the targeting of the same proteins. The viral proteins that facilitate such activity are often referred to as viral oncoproteins since the occasional outcome of their activity is cancer.

While the list of viral oncoprotein targets is fairly considerable and includes both cyclin, CDKs as well as CDK inhibitors such as p21 and p16, there are two proteins

whose viral inactivation almost certainly promotes genomic instability and/or tumorigenesis; p53 and pRb. Since tumour viral interactions with these two proteins are so very important the two will be considered separately in conjunction with the relevant viral interactions.

HPV and Simian virus-40 (SV40), inhibit p53 by direct interaction. On the part of the SV40 this takes place via the viral large T antigen (SV40 T-ag), the mechanism of which appears to depend in part on the ability of SV40 T-ag to bind p53 and promote sequestration that prevents its function (239). The HPV E6 viral oncoprotein appears to hold similar activity to that of the SV40 T-ag in terms of inhibition of p53 activity by complex formation with E6, in addition to which HPV E6 facilitates the degradation of p53 via ubiquitin-targeted proteosomal degradation (234).

The adenovirus type 5 proteins E1B-55k and E4orf6 are also considered to inhibit p53 transactivation via direct binding (309) and targeted degradation (217, 218). The human T-cell leukaemia associated virus HTLV-1 can also functionally inactivate p53 via the viral protein Tax though the mechanism by which this occurs is uncertain. In each of these cases the end result of viral oncoprotein interactions with p53 is the inhibition of p53's tumour suppressor activities. In addition the Kaposi's sarcoma herpes virus (KSHV) encoded latancy associated nuclear antigen (LANA) also interacts with p53, suppressing its transactivational activity and disrupting its ability to induce apoptosis (97). Further to the role of the KSHV LANA protein in disrupting p53 properties it also binds to pRB proteins and promotes E2F transcriptional activity (219). As we have extensively discussed a lack of functional p53 activity is a fundamental determinant of genomic instability and thus cancer. These viruses have a profound influence on genomic stability by their negative regulation of p53 via disruption of cell cycle checkpoints and apoptosis.

Viral oncoproteins also target the tumour suppressor protein pRb. As a fundamental brake on cell-cycle entry it is intuitive that viruses might be required to manipulate pRB activity. As we have already described, pRb is one of several proteins all of which bare considerable structural homology (p107 and p130 being the further family members). Most importantly, they all share the 'pocket' domains that are responsible for their important functional interactions. Of particular significance being

transcriptional factor binding and inactivation to prevent entry into S phase (294). The polyoma viruses encode the large and small T antigens, which constitute versatile oncoproteins that have been extensively studied. SV40, BK virus (BKV) and JC virus (JCV) are the most comprehensively considered in terms of their interactions with pRb (38, 189). The HPV E7 protein has also been widely investigated for its part in pRB inactivation and carcinogenesis. The adenovirus E1A protein has considerable history with the RB family of proteins since the RB group of pocket proteins were identified as a consequence of their binding to adenovirus E1A (112).

The adenovirus E1A protein targets pRB via the B-Box domain of the protein via the LxCxE motif of the E1A protein (250) in a way analogous to that of HPV E7 proteins' pRB interaction. It is thought that interaction of these viral proteins with pRb prevents the binding of other interacting partners that also contain the LxCxE sequence motif, such as D-type cyclins (192). The E2F group of transcription factors do not contain the LxCxE binding motif but it is thought that viral oncoprotein binding via the LxCxE domain displaces E2F-pRB binding through competition for binding within the pocket region. Ultimately, displacement of E2F from pRB leads to the activation of E2F target gene transcription and drives cells into S-phase.

The high risk HPVs are so named for their oncogenic potential and are clinically associated with the majority of cervical cancers. The E7 protein of high risk HPV is capable of binding RB family proteins and also causing the release of E2F. Furthermore these E7 proteins target pRb for ubiquitin-mediated degradation, and ultimately proteolytic degradation is primarily responsible for E7's oncogenicity (103). Several mechanisms including E7s ability to bind directly to the proteasome are considered relevant for E7's ability to destabilise RB family proteins (26).

The three Polyomaviruses mentioned above, SV40, JCV and BKV are the only Polyomaviruses though to have some role is certain human cancers. While JCV and BKV are rather common throughout the population, the number of individuals serotypically positive for SV40 infection is considered low. As already mentioned, the Polyomaviruses encode oncoproteins known as T antigens that are essential for replication in the early stages of infection. The large T antigens of SV40, JCV and BKV bare considerable sequence homology and all possess the LxCxE domain that

provides a binding motif for interaction with pRb and the related pocket proteins p130 and p107.

Tumour viral interactions with both p53 and RB family proteins are predominantly responsible for much of their tumorigenicity and despite being rather different viruses, the many viruses described here have evolved exceptionally similar ways to counteract p53 and RB protein function to activate the cell cycle. However for many of these viruses, p53 and RB interactions are not the limits of their manipulation of cell-cycle regulatory mechanisms. Though some of these interactions are not as significant with regard to their contribution toward carcinogenesis they are nevertheless probably contributing factors to the overall progress of cellular transformation. These alternate mechanisms for cell-cycle deregulation are primarily based upon the disruption of cyclin-CDK complexes and function. Since the catalogue of tumour viral interactions of this nature is extensive, the fundamental strategies employed will be considered and examples of viruses and/or the relevant oncoproteins will be considered.

The principles of cyclin-CDK manipulation by tumour viruses involves three main approaches all of which function toward the same goal, functional activation of cyclin-CDKs. The principles of tumour viral cyclin-CDK activation include (a) the inhibition of both classes of CDK inhibitor, (b) prevention of the increase in CKI expression, (c) stabilisation of cyclin and CDK proteins and (d) the expression of 'viral' cyclins that are homologous to their cellular counterparts. For the Cip1/Waf1 class of CDK inhibitors the HPV oncoproteins E6 and E7 act proficiently to inhibit p21's activity. On one hand, E6 appears to reduce the transcription of p21 mRNA probably via the previously described p53 disruption, while E7 counteracts CDK inhibition by direct competition for p21 binding sites for cyclin-CDK complexes (127, 128). E7 can also cause the cellular redistribution of p21 and thus can inhibit its binding to cyclin-CDK complexes. The INK4 class of CDK inhibitors are also susceptible to viral oncoprotein disruption and the HTLV-1 Tax protein is a prominent example. Tax appears to be capable of directly inhibiting the expression of $p15^{INK4B}$, $p18^{INK4C}$, $p19^{INK4d}$ (101) It is also able to bind $p16^{INK4a}$ and prevent cyclin/CDK inhibition (268). With regard to stabilisation of cyclin and CDK protein levels, Tax protein stimulates cyclin D expression and by direct protein-protein interaction encourages cyclin D-CDK complex formation (110). However, one

innately simple and yet exceptionally effective mechanism is the viral expression of 'viral' cyclins (187). Essentially this involves viral proteins that bear considerable sequence similarity to cellular cyclins. The viral cyclin proteins function in a similar way to their cellular homologues and essentially this represents a simple and self-sufficient mechanism for enhancing cyclin expression and activity. Approximately eight viruses have been shown to encode viral cyclin homologues many of which are herpes viruses, KSHV as an example (187).

The viruses described here and their encoded oncoproteins disrupt the cell cycle and inactivate cell-cycle checkpoints to achieve a host cell environment in which they can thrive. It is probable that one consequence of this is cancer, a state that is probably rather beneficial to many viral lifecycles since it means not only continual, inappropriate proliferation but also a resistance to cell-death. Since, viruses are obligate parasites that require a host cell for replication and indeed a proliferating cell for essential replicative factors, these are undoubtedly evolutionarily relevant functions.

Oncolytic viruses

The generation of oncolytic viruses perhaps represents one way in which our developed understanding of what constitutes cancer cells and understanding the defects that define them meets our understanding of viral replication. Considering oncolytic viruses is very much the flipside to understanding tumour virus biology. Moving from the poise of how viruses might instigate a cancerous state we will now discuss how viruses might be engineered to selectively kill cells with tumorigenic potential.

Viruses represent an exceptionally efficient nucleic acid delivery system. Once introduced to a susceptible host, a virus will propagate itself within host cells to produce progeny that will be transmitted from cell to cell. Ultimately viral replication may cause considerable tissue damage as a virus and its progeny spread through a susceptible cell population. As such one can envisage harnessing such efficient powers of delivery and destruction to target cell populations that may be detrimental to a host such as cancer cells. We have considered the mutations commonly

associated with cancer cells and we know that cancers are essentially clonogenic. As such if it were possible to engineer a virus to exploit specific flaws in a cancer cell population to yield selective replication in cancer cells whilst leaving normal cells unscathed, we would have a highly refined and efficient biotherapeutic agent. If such therapy were feasible it may be that one might imagine that in the future a virus could be specifically tailored in a patient-specific manner to target specific tumour type with in the therapeutic time frame. These concepts are essentially the philosophy behind developing oncolytic viruses.

When we consider 'tailoring' viruses, the fundamental points are that the virus should selectively infect and / or replicate in cancer cells as oppose to normal cells. There are several ways in which viruses can and have been manipulated to yield such characteristics by engineering key aspects of the virus life cycle to increase specificity in this regard.

One of the key mechanisms for engineering specificity for tumour cells is to manipulate the virus so that one or several of the key viral genes are only expressed in tumour cells and as such completion of the viral life cycle may only occur in such cells. This method of engineering has been employed to produce oncolytic adenovirus, herpesvirus and parvovirus derivatives (5, 7, 171). Oncolytic viruses manipulated in this way are regarded as transcriptionally regulated and this typically involves the regulation of viral early gene products via tumour-specific enhancer and promoter elements (133).

A second method of viral manipulation is to exploit the cellular interferon response by preventing viral subversion of interferon defences. Viruses typically evoke a type 1 interferon response, which has the propensity to signal neighbouring cells to halt protein synthesis and as such inhibit virus replication. Many viruses encode proteins that can prevent an interferon response from being generated by cells in response to viral infection and cancer cells typically have defective interferon responses. As such if viruses are manipulated to no longer possess interferon suppression properties then normal cells will inhibit their replication by an active interferon response while cancer cells will remain susceptible and support viral replication (259). On a similar notion viruses can be altered in their ability to prevent induction of apoptosis. Cells typically

respond to viral infection by attempting to induce an apoptotic response and viruses have evolved ways to prevent or delay this. We know that cancer cells frequently lose their ability to regulate apoptosis and as such if viruses are manipulated such that they can no longer prevent apoptosis they should only propagate in susceptible cancer cell populations.

Viruses naturally display tissue and cell tropisms determined by the surface antigens that dictate their receptor specificity. Since the virus itself encodes viral capsid proteins, these proteins can be modified such that a virus displays the desired receptor specificity. Recombinant viruses can be generated that can only infect via cell surface receptors that are exclusively found on tumour cells for instance (198). Viruses can be made to express proteins on their surface that will be exclusively recognised by tumour antigens such as single-chain antibodies or polypeptide-binding ligands such as growth factors. As such these viruses are described as being re-directed since they are specifically targeted to cells expressing the relevant associated ligand.

Ultimately these factors can be manipulated to produce a virus that is considered to be capable of harbouring specificity to kill cancer cells. However, several other factors determine how efficiently any such virus can act as an oncolytic therapy *in vivo*. As a realistic therapeutic agent, oncolytic viruses must not only selectively propagate in cancer cells leaving normal cells healthy, but to do so they must be able to evade innate immune responses that will hinder the efficacy with which a virus can gain access to the tumour site, and adaptive immune responses that develop after subsequent rounds of treatment. Furthermore, viral propagation within a tumour must be able to sufficiently eliminate cancer cells before an immune response attempts to eliminate viral load. Several viruses naturally possess multiple serotypes and as such the neutralising antibodies that may be generated against one serotype as a consequence of an immune response may not be successful neutralising agents against the other serotypes. It may be possible to evade immune responses for sufficiently long to offer a suitable therapeutic window by switching serotypes for each round of therapy (14). Many immune evasion attributes are beyond the scope of manipulation of viral genes and proteins and require consideration of therapeutic regime and particularly combination therapy to increase efficacy of either therapeutic agent or artificial modification of these bioreagents. The process of coating viruses in a

polymer substance may be one such method of artificial manipulation to evade immune responses. It is thought that by coating viruses in a polymer that disguises antigens that would otherwise be recognised by the immune responses, one can prolong the period of time in which the virus can avoid sequestration by host immunity. It is not surprising that while polymer coating disguises viral ligands from meeting the immune system, it also restricts them from meeting there prospective partner receptor on the target cancer cell. As such it has been necessary to insert the antigens required for target cell binding into the polymer coat. Not surprisingly this leads to a vicious circle of attempting to find the balance between immune invasion and sufficient targeting and infection (105).

Alternatively traditional immunosuppressant drugs can be used to achieve direct suppression of host immune responses. When administered at the appropriate time certain drugs can be extremely effective in eliminating both B and T cell-mediated host antiviral responses. Additionally one can reduce the levels of antibodies that may facilitate an immune response by antibody depletion prior to the introduction of virus. Several mechanisms can be employed to this end and it may be less detrimental to the patient than immunosuppression. Alternative methods to avoid immune responses that are innately associated with viral infection appear to defeat the object of using viruses as a specific delivery tool. Mechanisms employed involve the direct delivery of viral genomes to target cells or by the delivery of viruses within cells to target regions. Both of these mechanism may avoid initial immune responses but with the case of direct genome delivery risks off-target destruction of healthy tissue. These mechanisms are of course short term in that they only disguise the virus for the initial step before virus meets target tissue at which point viral replication may evoke immune responses (39).

Further to the issue of what constitutes a good oncolytic therapy one must consider the ability of the virus not only to specifically infect and replicate within tumour cells but to be able to access the entirety of a tumour mass. It is important that a virus be able to efficiently move from blood vessel to tumour mass and as such many oncolytic viruses have been engineered to express not only ligands for tumour cell receptors but also for endothelial receptors to promote more efficient transfer from

blood vessel to tumour whilst maintaining specificity for specific transfer and propagation within the tumour mass.

However, what are the clinical implications of immunosuppressants at a point when cancers have already established? Can the long-term safety of patients be guaranteed with regard to cancer recurrence or progression in the face of an immunocompromised system in addition to the susceptibility of patients to potential off-target viral effects? These questions can only be answered by extensive clinical trials to determine the realistic potential of oncolytic viral therapy. These are some of the many questions yet to be answered regarding the suitability of viruses as cancer therapy. The nature of viruses as evolutionarily competent entities makes it difficult to predict safety in short term studies since one the most significant of fears is that viral evolution and recombination will lead to the propagation of potentially uncontrollable biological agents. If oncolytic therapy can progress as a reality it is pivotal that the many questions regarding their biosafety be addressed. While several viruses are currently in phase I and II clinical trials the acceleration of focus into oncolytic therapy has been surpassed by a plethora of agents whose biosafety can be more easily assessed. The use of viruses in gene therapy and the unexpected losses of lives in such clinical trials have perhaps stunted enthusiasm in this area (161, 180).

Thesis Aims

The introduction covers a considerable breadth of various fields since the aims described below straddle several sectors of biological research. The core observations from which these aims stem are described below and it is hoped that the topics of introduction provide some insight into the concepts discussed in later chapters.

In 2001 Raj *et al.* highlighted the ability of AAV viral DNA to cause a DNA damage response. This response was shown to result in the killing of cells lacking p53 activity. It was established that in the presence of functional p53, cells may undergo a transient G2 arrest characterised by stabilisation of p53 and subsequent increase in p21 and the targeted degradation of CDC25C while a lack of functional p53 resulted in a cells susceptibility to AAV-induced cell death. The mechanism by which an AAV induced DNA damage response may result in targeted cell death has not thus far been established. One of the primary aims of this work was to establish the mechanism by which selective cell death in the absence of p53 might be achieved. This aim is addressed in the first section of results.

Furthermore, subsequent publications have examined the ability of AAV DNA to induce G2 arrest (129). Such data has further highlighted the observation that AAV DNA may be recognised by an infected cell as damaged DNA. Stemming from these observations and those of others that we have introduced and have shown the detrimental effect of DNA damage response with regard to viral replication we also wanted to consider the role of DNA damage responses in restricting AAV replication. This aim is addressed in the second section of results.

The potential clinical implications for our furthered understanding of AAV DNA-induced DNA damage responses in terms of cell death and for AAV replication are later discussed.

Section 2 Materials and Methods

Chapter 1 Materials

Suppliers of reagents

Except where specified, including below, reagents were obtained from Sigma-Aldrich Company Ltd. (UK), BDH Laboratory Supplies (UK) or Fisher Scientific (UK).

Suppliers of commonly used reagents are listed below:
Agarose, ammonium persulfate (APS), ethidium bromide, sodium dodecyl sulfate (SDS) and N,N,N,N'-tetra-methyl-ethylenediamine (TEMED) were obtained from Bio-Rad (UK). Ultra Pure ProtoGel Acrylamide was obtained from National Diagnostics (UK). Complete Protease Inhibitor Cocktail Tablets were obtained from Roche (UK). Marvel was obtained from Premier International Foods (UK). Fetal calf serum (FCS) was obtained from Perbio Science Ltd. (UK). Dulbecco's modified Eagle's medium (DMEM) was obtained from Invitrogen (UK). All primers were produced by Sigma-Genosys (UK).

Media and buffers

Table 2.1.1 Buffers and reagents

2 x Laemmli's buffer	4 % (w/v) SDS, 20 % (v/v) glycerol, 0.1 M DTT, 0.12 M Tris, pH 6.8, containing a trace of bromophenol blue
10 x loading buffer	50 % (v/v) glycerol, 0.5 % (w/v) bromophenol blue, 0.4 % (w/v) xylene cyanol
LB broth	1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 86 mM NaCl
LB agar	LB broth + 2 % (w/v) Bacto agar
PBS	0.17 M NaCl, 3.3 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4
TAE	0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0

trypsin-versene	0.14 M NaCl, 2.7 mM KCl, 8.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.27 mM EDTA, 0.13 % (w/v) trypsin, 0.001 % (w/v) phenol red, pH 7.6-7.8
pen/strep stock (cell culture antibiotics)	0.6 % (v/v) penicillin, 1 % (v/v) streptomycin
SDS electrophoresis buffer	25 mM Tris base, 192 mM glycine, 0.1 % (w/v) SDS
transfer buffer	30 mM Tris base, 240 mM glycine, 20 % (v/v) methanol

Antibodies

Primary and secondary antibodies were used for western blotting and / or immunofluorescence in accordance with supplier instructions.

Table 2.1.2 Primary antibody supplier information

Antibody	Supplier	Catalogue number
Actin	Santa Cruz	SC-1616(1-9)
ATM	Abcam	Ab78-100
ATM serine 1981p	Calbiochem	DR1002
ATR	Santa Cruz	SC-1887
Caspase-3	Cell Signalling	9662
Caspase-6	Cell Signalling	9762
DNA-PKcs	BD pharmingen	556456
FLAG	Sigma-Aldrich	F3165
p21	Santz Cruz	SC-397-G
p53	Novocastra	NCL-p53-DOI
p53 serine 15p	Cell Signalling	9284
p84N5	Abcam	ab487
pRb	BD pharmingen	554136
RPA-34	Calbiochem	NA18
RPA serine 33p	Bethyl Labs	BL744#A300-246A

TopBP1	BD transduction	611875
Tubulin	Santz Cruz	SC-5546

Secondary HRP conjugated antibodies for western blotting were purchased from GE Healthcare, UK. Secondary Alexa conjugated antibodies for immunofluorescence were purchased from Molecular Probes, UK. Antibodies were used in accordance with supplier instructions.

Chapter 2 Cell culture techniques and manipulation

Cell lines

The isogenic HCT116 cell lines (a kind gift from B. Vogelstein, The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD), Saos-2 and U2OS cells were all cultured under the same culture conditions as follows. The HCT116 cells are immortalised human colon tumour cells from which lines lacking either p53 (HCT116 p53-/-) or p21 (HCT116 p21-/-) have been generated (Kholi et al. 2004). Both Saos-2 and U2OS cell lines are human osteosarcoma lines. The U2OS p53DD cell line was generated by K Raj (NIMR, London, UK). The cells express the p53DD protein, a C terminal miniprotein that acts in a similar manner to full-length mutant p53. It lacks protein residues 12-301 and causes a reduction in sequence specific DNA binding of wild-type p53 (243). ΦNXA cells are a retroviral packaging cell line, kindly supplied by (a kind gift from G. Nolan, Department of Microbiology and Immunology, School of Medicine, Stanford University, Stanford, CA). 293T human embryonic kidney cells immortalized by SV40 large T antigen.

Maintenance of cells

Cells were maintained in 10 cm tissue culture dishes (Nunc) in 10 ml of DMEM (Dulbecco's modified Eagle's Medium) supplemented with 10 % (v/v) fetal calf serum (FCS) and 1 % (v/v) antibiotics stock (pen/strep). In this study, the concentrations of FCS and pen/strep were kept constant at 10 % and 1 % respectively, unless otherwise stated, and DMEM supplemented with these reagents is termed 'complete DMEM'. Cells were incubated at 37 °C in a 5 % CO_2 environment. When the cells reached confluency, they were passaged by seeding 10 % of them into a new dish (approximately twice a week). To detach the adherent cells from the flask, they were washed with phosphate buffered saline (PBS), incubated for 5 min at 37 °C with trypsin-versene until they detached from the bottom of the flask and then suspended in more complete DMEM. When cells were required to be seeded at a particular density, the cells in suspension were counted on a haemocytometer, using a phase contrast light microscope (Nikon Eclipse TS100, Surrey, UK) to estimate the cell concentration.

Long-term storage of cells

Cells were cultured until confluent and then a suspension of ~1x10^6 cells was prepared as above (using trypsin-versene). They were then pelleted (2500 g, 5 min) and then resuspended in 1 ml of freezing medium (90 % (v/v) FCS, 10 % (v/v) dimethyl sulphoxide (DMSO)) and transferred to a cryogenic vial. This was stored at -80 °C overnight, wrapped in 12-15 layers of tissue paper to slow down the freezing of the cells, before being transferred to liquid nitrogen. When required, the cells were rapidly defrosted at 37 °C and added to 10 ml of complete DMEM. The cells were pelleted as above, resuspended in 10 ml of complete DMEM and transferred to a 10 cm dish

Virus production and infection of cells

Wild-type AAV-2 was made using the pDM single plasmid transfection system (Plasmid Factory). 293T cells were transfected with the pDM plasmid and cultured for 72 hours. Cells were then harvested along with the supernatent by cell scraper and pelleted at 1750 RPM for 5 minutes in a centrifuge. The cell pellet was resuspended in 10 mL PBS and subjected to three freeze thaw cycles in a snap-freeze bath of ethanol and dry ice. Cell debris was pelleted once more by centrifugation at 1750 RPM for 5 minutes. The supernatant was then collected and CsCl salt added to give the refractive index of 1.372 as measured using a refractometer. The adjusted solution was transferred to ultraclear tubes and balanced using liquid paraffin. These caesium chloride gradients were spun at 50,000 RPM for 30 hours at 24°C. A visible band in the centre of the gradient corresponding to AAV with a refractive index of 1.372 was collected using a fine gauge needle and syringe.

For infection cells were seeded the day prior to infection at 1 x 10^6 cells per 10cm tissue culture dish. Cells were infected with AAV at a multiplicity of infection (MOI) ranging between 2,000 and 5,000. For UV inactivation of virus the required volume of virus was added to the well of a 4 well tissue culture plate (Nunc) and PBS added to a total volume of 10 μL. The lid from the 4 well plate was removed within a UV crosslinker and the plate was then irradiated with 2400 J/m^2. UV-irradiated virus was then resuspended in tissue culture medium and used to infect cells in 10 cm tissue culture dishes. Virus was resuspend in 3 ml medium per 10 cm dish and overlayed

onto cells. After 5 hours, tissue culture medium was added to a total volume of 10 ml per plate.

Infection with Ad5, HSV-1 or Vv was as above whereby virus was resuspended in 3 ml complete DMEM and overlayed onto cells. After 5 hours, complete tissue culture medium was added to a total volume of 10 ml per plate.

Plasmids and transfection

Plasmids expressing dominant negative caspase-6 and dominant negative caspase-3 were kindly provided by D.E. Bredesen (Buck Institute, Novato CA). Plasmids expressing ATMwt and ATMkd were kindly provided by M. Kastan (St. Jude Children's Research Hospital, Memphis TN). All transfection were performed using Effectene (Qiagen) in accordance with the manufacturers supplied protocol. Cells were seeded the day prior to transfection at 1 x 10^6 cells per 10cm tissue culture dish or 0.2 x 10^6 cells per well of a 6 well plate. Cells were transfected with 2.5 μg of plasmid DNA per 10cm dish or 0.5 μg of plasmid DNA per well of a 6 well plate. 24 hours post transfection the culture media from transfected cells was aspirated and refreshed with 10 ml of fresh complete medium. 48 hours post transfection cells were selected for antibiotic resistance. Culture media was supplemented with the appropriate antibiotic to the following concentrations; 4.5 μg/mL puromycin, 5 μg/mL blasticidin or 500 μg/mL neomycin. For siDNA-PK or siCONTROL transfection, 1 μM siRNA were used to transfect 0.2 x 10^6 cells per well of a 6 well plate.

Vectors and retrovirus production and cell transduction

For shRNA expression silencing of pRb the sequences;
5'-GATCCCCGCCACTTGAAATGTTAGTCTTCAAGAGAGACTAACATTTCAA GTGGCTTTTTGGAAA-3'and
5'-AGCTTTTCCAAAAAGCCACTTGAAATGTTAGTCTCTCTTGAAGACTA ACATTTCAAGTGGCGGG-3' were annealed and cloned into the EcoRI site of the pSuper expression vector (kind gift from Thijn Brummelkamp, Whitehead Institute, Cambridge, MA). The shRNA insert and promoters were excised from pSuper via

XhoI and EcoRI restriction sites and ligated into the pRetroSuper retroviral expression vector to yield the pRSshRb construct. Once in the context of the retroviral expression vector it is possible to generate retroviruses in which the desired vector is packaged using a packaging cell line as delineated below.

The packaging cell line known as ϕNXA cells (a kind gift from G. Nolan, Department of Microbiology and Immunology, School of Medicine, Stanford University, Stanford, CA) were transfected with the pRSshRb vector or a vector containing an irrelevant (scrambled) sequence. Virus particles were harvested 48 hours post transfection by harvesting the supernatant from transfected cell monolayers. Supernatents were filtered using a sterile 0.2 μm filter and syringe. The filtrate was then supplemented with 10 μg/ml polybrene and used to infect 10-cm tissue culture dishes of cells. 24 hours following infection cells were selected for antibiotic resistance with 1.5 μg/ml puromycin.

To reconstitute p21 or pRb protein in Saos-2 cells, the pBabe Puro retroviral vector harboring these genes was used to generate retroviruses as above and used to transduce Saos-2 cells, which were then subjected to puromycin selection as described above.

The p84DDD sequence was excised from the PCR3 and inserted via EcoRI restriction sites into the pBabeBlast retroviral vector. ΦNXA cells were transfected with this construct as delineated above to generate retroviruses. Cells expressing the dominant-negative construct or the empty vector control were selected using blasticidin (7.5 μg/ml).

Protein extraction from tissue culture cells

Cells were washed in PBS and harvested using a plastic cell scraper. Cells were pelleted and then the pellet resuspended in Reporter lysis buffer (Promega) with a cocktail of protease inhibitors (Calbiochem) and incubated on ice for 30 minutes. Lysates were centrifuged at 16,000 g for 5 minutes and the supernatent collected. The protein concentration of the sample extracts was determined via Bradford assay in accordance with manufacturers instructions (BioRad).

Total DNA extraction from tissue culture cells

Cells were harvested by trypsinisation and pelleted. Cell pellets were washed once with PBS. Total DNA extraction was performed using the QIAamp DNA mini kit as per the manufacturer's protocol with the following alterations. Lysis reaction occurred at 56°C for 1 hour as opposed to 10 minutes. DNA was precipitated using 230 µl 100% ethanol as opposed to 200 µl. DNA was eluted from columns using 60 µl ddH20 as opposed to 200 µl following 5 minutes incubation at room temperature.

Hirt extraction from tissue culture cells

Cells were trypsinised from tissue culture plates and neutralized with tissue culture medium. Cells were counted and the cell concentration was adjusted to 2 x 106 cells per ml. 0.5 ml of this suspension was dispensed into an eppendorf tube. 0.5 ml of 2 x lysis solution (2% SDS, 2 mM EDTA pH8.0) was added to the eppendorf immediately. The eppendorf was then gently inverted 3 times and left to stand at room temperature for 15 minutes. 250 µl 5 M NaCl was then added and the tube inverted 3 times. The tube was then left to stand at 4°C overnight. Tubes were then spun at 13,000 RPM in a refrigerated benchtop centrifuge at 4°C for 15 minutes. The supernatant containing episomal DNA, viral DNA and mitochondrial DNA can then be collected. The supernatent was then subject to phenol/chloroform extraction and ethanol precipitation by the addition of $1/10^{th}$ volume of 3M Sodium Acetate pH5.2 and two volumes 100% ethanol. The tube was vortexed and then left to stand at -20 °C overnight. The tubes were then spun at 13,000 RPM in a refrigerated benchtop centrifuge at 4°C for 10 minutes. Pellets were then resuspended in ddH_2O.

Fixing and blocking of tissue culture cells for immunofluorescence

Cells were grown as per the experimental conditions in 10 cm dishes on 13 mm glass coverslips. Glass coverslips were harvested using forceps by removing individual coverslips from dishes and placing them into individual wells of a 4 well dish (Nunc). Cells on coverslips were washed in PBS and fixed in 5% paraformaldehyde for 20 minutes with gentle shaking. Cells were then rinsed twice in PBS and then stored in

IFF (1% FCS, 2%BSA in PBS) at 4°C for up to two weeks. Coverslips were then processed for immunofluorescence or FISH (see chapter 5 below)

Fluorescence activated cell-sorting (FACS) analysis of DNA content

Cells were harvested by trypsinisation and then pelleted by centrifugation at 660g for five minutes. Cells were fixed by resuspending the cell pellet in ice cold 70% ethanol and then incubated at room temperature for 5 minutes. Cells were then re-pelleted and resuspended in one volume RNase A (0.1mg/ml in PBS) and incubated for 10 minutes at 37°C. One volume of 40 μg/mL propidium iodide solution in PBS was then added. DNA content was then quantified using FACScan and CellQuest software (Beckton Dickinson). A total of 10,000 cells were counted for each analysis.

Methylene blue staining of tissue culture monolayers

Cell monolayers were washed twice with PBS and then a solution of 1% methylene blue in 50% methanol / PBS was overlayed onto them. Dishes were then incubated at room temperature for 30 minutes. Staining solution was then decanted from the dishes and monolayers were washed extensively with PBS. Dishes were then air dryed in the dark.

Drug inhibition of PIKK proteins in tissue culture cells

Caffeine (Calbiochem) was resuspended directly in complete DMEM to the desired concentration. Wortmannin and DNA-PK inhibitor II (Calbiochem) were resuspended in DMSO in a 1000 times stock solution. U2OS cells were plated at 1 x 10^6 cells per plate the day prior to drug application. Manipulations were conducted in the dark where possible to avoid light inactivation of wortmannin or DNA-PK inhibitor. Drugs were diluted in 10 ml complete DMEM and overlayed onto cells. Cells were incubated with drug for 24 hours prior to infection. For infection 3 ml culture medium was removed from cells and used to resuspend virus. The remaining 7 ml culture medium was removed and stored at 37°C in the dark. The 3 ml containing virus was overlayed onto cells. 5 hours post infection the 7 ml removed from each plate was replaced. Cells were cultured for a further 48 hours and then half of each plate was harvested for total protein extraction and the other half for DNA extraction.

Transformation of *E.coli* with DNA

The chemical competent *E.coli* cells, XL1-Blue supercompetent cells or BL21 Star™ (DE3) cells, were used in transformations. One μl of plasmid miniprep or maxiprep DNA (0.1-50 ng of DNA) was added to 50 μl of cells in a pre-chilled 15 ml Falcon 2059 polypropylene tube. The tube was incubated on ice for 30 min then heat-pulsed in a 42 °C water bath for 45 sec. After cooling the tube on ice for 2 min, 1 ml of SOC medium (Invitrogen, UK) was added, then the tube incubated at 37 °C for 1 h with shaking at 220 rpm. The culture (~250 μl) was spread on an LB agar plate (containing 100 μg/ml ampicillin) using aseptic technique and the plate incubated overnight at 37 °C. Cultures of any resultant *E. coli* clones were prepared the following day by transferring bacteria from the plate to LB broth using a sterile pipette tip and aseptic technique.

Glycerol stocks

E.coli clones were stored at –80 °C as a 'glycerol stock'. A glycerol stock was prepared by transferring 0.8 ml of a fresh mid-log culture (in LB broth) to a cryogenic vial and adding 0.2 ml of glycerol. This was mixed and immediately stored at –80 °C.

Purifying plasmid DNA-miniprep;

Two ml of plasmid-containing *E.*coli cultures (in LB broth with 50 μg/ml kanamycin or 100 μg/ml ampicillin) were grown overnight at 37 °C with shaking at 220 rpm. Plasmid minipreps were prepared using the QiaPrep Plasmid Miniprep Kit (Qiagen, UK) using the manufacturer's protocol. DNA was eluted using 50 μl of deionised H_2O.

Purifying plasmid DNA-maxiprep;

A 5 ml plasmid-containing *E. coli* culture was grown during the day (for ~ 6 h) in LB broth containing 50 μg/ml kanamycin or 100 μg/ml ampicillin (at 37 °C with shaking at 220 rpm). Then, 150-250 ml of LB broth (containing antibiotic) was inoculated with the 5 ml culture, and was incubated overnight at 37 °C with shaking at 220 rpm. The HiSpeed Maxi Kit (Qiagen, UK) was used to produce plasmid DNA maxipreps according to the manufacturer's protocol. DNA was eluted using 1 ml of deionised H_2O.

Quantitation of DNA

DNA concentrations were assayed using a 1201 UV-visible spectrophotometer (Shimadzu, UK). DNA solutions were diluted 1:100-1:500 and their absorbance at 260 nm measured in quartz cuvettes. DNA concentrations were calculated assuming an A_{260} of 1 is equivalent to 50 μg/ml of double-stranded DNA.

TAE agarose gel electrophoresis

To check for the presence or quantity of DNA (for example, mutagenesis PCR products, miniprep DNA and RT-PCR products), the DNA was separated on a 1 % agarose gel, buffered with TAE, containing 1 μg/ml ethidium bromide. Samples were mixed with 10 x loading buffer, loaded into the gel, and electrophoresis was carried out at a constant voltage of 70 V, limiting to 200 mA. DNA bands were visualized by trans-UV illumination and compared to DNA markers (Invitrogen, UK) of known molecular mass. Digital images were captured with a Kodak Image Station 440 and Digital Science 1D software (Perkin Elmer Life Sciences Ltd., UK).

Southern blotting

DNA was separated by TAE agarose gel electrophoresis as described above. DNA was then transferred to nylon membrane (Millipore) over night via capillary action using Solution A for transfer (1.5 M NaCl, 0.4 NaOH). For slot-blot analysis, DNA was diluted in 100 μl Solution A and applied to a MINIFOLD-II slot blot system (Schleicher and Schuell). The DNA was transferred to nylon membrane by suction. In both cases the DNA was cross-linked to membrane via 1200 J/m2 UV. Membranes were blocked in pre-hybrisation solution for 6 hours. Radiolabelled DNA probes were prepared via random priming from the relevant plasmid DNA (as described below). Radiolabelled probes were resuspended in hybridisation solution and incubated with membrane over night at 42 °C in a rotator oven. Membranes were washed at 48 °C in a rotator oven using 8 volumes of pre-warmed wash solution (0.1 X SSC, 0.1% SDS). Membranes were then wrapped in saran wrap and exposed to Kodak MXB X-ray film (X-ograph imaging systems, UK). Films were developed using a Fujifilm FPM-3800A processor (UK). Alternatively, membranes were wrapped in saran wrap and exposed to phosphorimager screen for phosphorimager analysis and ImageQuant quantification.

Pre-hybridisation solution composition, 10 ml:

4.5 ml Formamide

2 ml 20 X SSC

1 ml 1M Sodium Phosphate pH6.5

1 ml 100 X Denhardt solution

1 ml 20 % SDS

0.5 ml Herring sperm DNA (5mg/ml)

Hybridisation solution composition, 10 ml:

4.5 ml Formamide

2 ml 20 X SSC

1 ml 1M Sodium Phosphate pH6.5

0.1 ml 100 X Denhardt solution

0.2 ml 20 % SDS

0.2 ml Herring sperm DNA (5mg/ml)

2 ml Dextran sulphate (50%)

Generation of p^{32} radiolabelled DNA probes

50 ng of DNA in 10 µl were denatured at 100°C for 8 minutes and immediately chilled in ice for 8 minutes and then spun briefly. 3 µl dATP, dTTP, dGTP (0.5 mM each) were added followed by 2 µl Hexanucleotide mix (Roche), 2 µl α-32P-dCTP and 1 µl Klenow enzyme (Promega). After incubation at 37°C for 30 minutes the reaction was stopped by denaturation at 100 °C for 7 minutes and chilled for 5 minutes.

Generation of fluorescence *in situ* hybridisation (FISH) probes

For FISH of Ad5, HSV-1 and Vaccinia virus DNA a set of 4 DIG labelled probes each were prepared using the PCR DIG Probe synthesis kit (Roche Cat. No. 11636090910) as per the supplier instructions. The following primers were designed for probe synthesis. The template DNA for probe synthesis was Hirt extracted DNA from Ad5, HSV-1 or Vaccinia virus infected U2OS cells as described above.

Human Adenovirus Type 5

Primers designed from the sequence from ACCESSION AC_000008 VERSION AC_000008.1 GI:56160529 from Pubmed nucleotide.

Set 1 – A = nucleotides 920 to 939, B = nucleotides 1080 to 1099, Fragment size 179bp

A 5' – GATCTTACCTGCCACGAGGC – 3'

B 5' – GCCACAGGTCCTCATATAGC – 3'

Set 2 – C = nucleotides 11,000 to 11,019, D = nucleotides 11,180 to 11,199, Fragment size 199bp

C 5' – TTGCAAATTCCTCCGGAAAC – 3'

D 5' - TTCGTAATCACCATCTGCTG - 3'

Set 3 – E = nucleotides 21,543 to 21,562, F = nucleotides 21,711 to 21,729, Fragment size 186bp

E 5' – TAGACATGACTTTTGAGGTG – 3'

F 5' - CGGCAGCTGTTGTTGATGTT - 3'

Set 4 – G = nucleotides 29,995 to 30,014, H = nucleotides 30,196 to 30,215, Fragment size 220bp

G 5' – CATATCTCAGACACCATCCC – 3'

H 5' – AGGCTTCGGAAAGATCGCTT – 3'

Human HSV-1 Strain 17

Primers designed from the Human herpesvirus strain 17 complete short unique region with partial terminal and inverted repeats DNA, complete cds. ACCESSION L00036 L00037 M12354 M12355 M12506 M30738. VERSION L00036.1 GI:291490.

Set 1 – A = nucleotides 3072 to 3091, B = nucleotides 3254 to 3273, Fragment size 201bp

A 5' – AATAAACAACGCTACTGCAA – 3'

B 5' – GAGCTGCGGTTCGTGCGCGA– 3'

Set 2 – C = nucleotides 11,107 to 11,126, D = nucleotides 11,339 to 11,358, Fragment size 251bp

C 5' – AACTCCTCCCATCCATTCTT – 3'

D 5' – GCCATCGCCGAAGAGTCGGA – 3'

Set 3 – E = nucleotides 18,382 to 18,401, F = nucleotides 18,557 to 18,576, Fragment size 194bp

E 5' – CCAGACACTTGCGGTCTTCT – 3'

F 5' – TTACGCTGGAAACCCCAGAA– 3'

Set 4 – G = nucleotides 25,655 to 25,674, H = nucleotides 25,821 to 25,840, Fragment size 185bp

G 5' – TGTGTAGCAGGAGCGGTGTG – 3'

H 5' – GGTGAAACCAACAGAGCACG – 3'

Vaccinia Virus strain 'Western Reserve'

Primers generated from the sequence, Vaccinia virus, complete genome ACCESSION NC_006998, VERSION NC_006998.1 GI:66275797. This is strain 'western reserve' analogous to the wild-type CR-19 virus used in our experiments.

Set 1 – A = nucleotides 107,281 to 107,300, B = nucleotides 107,461to 107,480, Fragment size 200bp

A 5' – GCGTGTGATTTACTCATTTA – 3'

B 5' – CGGAATTCATTAATAAGTTC – 3'

Set 2 – C = nucleotides 117,814 to 117,833, D = nucleotides 117,994 to 118,013, Fragment size 199bp

C 5' – TAATATATGGGCGACTTCTC – 3'

D 5' – CCAAATTAAGATCTGGTACT – 3'

Set 3 – E = nucleotides 16,743 to 16,762, F = nucleotides 16,940 to 16,959, Fragment size 216bp

E 5' – GATAGATGGTCTCATTTCAT – 3'

F 5' – AGTGTACTCGTTACTAAATA – 3'

Set 4 – G = nucleotides 133,399 to 133,418, H = nucleotides 133,573 to 133,592, Fragment size 193bp

G 5' – TAATTGCTTCGGCCAACATG – 3'

H 5' – TTGAATAGGCGTTAGACCTA – 3'

For FISH of AAV DNA, biotinylated AAV specific probes were generated by random priming using the Biotin Decalabel DNA labelling kit (Fermentas) as per the supplier instructions.

Probes were used for FISH of viral DNA in tissue culture cells. The protocol for FISH using these probes is delineated in chapter 5.

Generation of retroviral expression vector harbouring the RPA32Δ33 mutant

The original sequence for the RPA32Δ33 mutant was within a bacterial expression vector as described in the following reference (30). The sequence was removed by PCR to generate BamHI and EcoRI restriction sites at 5' and 3' ends respectively. The following primers were used;

Forward

5' CGC**GGATCC**ATGGCTAGGCAAGCCGAAAA

Reverse

5' CCG**GAATTC**TTATTCTGCATCTGTGGATT

for PCR using the Thermoprime Plus DNA polymerase kit (Thermo scientific) as per the suppliers instructions. The PCR product was cleaned using the QIAquick PCR purification kit (QIAgen) as per suppliers instructions. The PCR product was cut using the BamHI and EcoRI restriction enzymes and ligated into the pBabePuro BamHI and EcoRI cloning sites.

Chapter 4 Protein methods

SDS-PAGE

SDS-PAGE was used to separate proteins according to molecular mass. It was performed using Mini Protean II apparatus (Bio-Rad, UK). 1.5 mm spacers were used and proteins were resolved on 8%, 10% or 12% gels where appropriate. The components for preparing resolving and stacking gels were as delineated in Harlow and Lane 1988. The resolving gel solution was prepared, ensuring that the APS and TEMED were added just before use. This solution was poured into the caster to 3 cm below the top, overlaid with water-saturated butanol and allowed to polymerise for 20 min. Once the resolving gel was set, the butanol was rinsed away with ddH_2O and the stacking gel solution prepared (the APS and TEMED were added just before use). The stacking gel was poured on top of the resolving gel and a gel comb inserted into the stacking gel solution. After 20 min the comb was removed and the gel positioned in the electrophoresis tank. The tank was filled with SDS electrophoresis buffer and the sample wells rinsed with this buffer.

Protein samples were then prepared for loading by mixing with an equal volume of 2 x Laemmli's buffer and denaturing by incubating at 95 °C for 2 min. Samples and protein standards (dual colour marker, BioRad, UK) were loaded into the wells of the gel. The gel was then run at a constant voltage of 150 V until the desired separation of the protein-standards was achieved.

Western blotting

Western blotting was performed to detect proteins that have been electrophoretically separated on SDS gels as above. The separated proteins were then transferred from the gel to a PVDF membrane, Immobilon™ (Millipore, UK). This was achieved using a wet transfer system, Mini Transfer Blot (Bio-Rad, UK; filled with transfer buffer), at a constant voltage of 150 V for 90 min.

The membrane was incubated in blocking solution for 1 h at R/T with gentle shaking. For most Western blots, the membrane was blocked in PBS containing 5 % (w/v) Marvel (Premier Brands UK, Ltd., UK) and 0.1 % (v/v) Tween20. The membrane was incubated with primary antibody (see Table 2.1.1 for a list of antibodies) for 2 h at

R/T with gentle shaking, in PBS, 5 % (w/v) Marvel and 0.1 % (v/v) Tween20. The membrane was then washed in PBS with 0.1 % (v/v) Tween20 three times at 15 min intervals. The membrane was then incubated for 1 h at R/T with gentle shaking in PBS with 5 % (w/v) Marvel and 0.1 % (v/v) Tween20, containing HRP-conjugated IgG from the relevant host (GE Healthcare, UK) diluted to 1:2000. Following this, the membranes were washed five times as described above. Binding of HRP-conjugated antibody was visualized by Enhanced Chemiluminescence (ECL; GE Healthcare, UK), with blots being exposed to Kodak MXB X-ray film (X-ograph imaging systems, UK). Films were developed using a Fujifilm FPM-3800A processor (UK).

Chapter 5 Staining and imaging techniques

Immunofluorescence

Cells were fixed to glass coverslips as described in chapter 1 and stored in IFF at 4°C. For immunofluorescence cells were permeabilised with 0.5% Triton-X (Fluka) in PBS for 10 minutes at room temperature with gentle shaking. Coverslips were rinsed three times in PBS and then blocked for 30 minutes in IFF at room temperature with gentle shaking. Coverslips were incubated with primary antibody diluted to 30 µl in IFF in a humidity chamber for one hour. Coverslips were then washed three times in PBS. Coverslips were then incubated with the appropriate secondary antibody conjugated to an Alex Fluor (Molecular Probes). Secondary antibodies were diluted in IFF and cells were incubated in a humidity chamber in the dark for one hour with shaking. Coverslips were then washed 3 times with PBS in the dark. Coverslips were then incubated with a solution of 1 µg/ml DAPI nuclei acid stain (Molecular Probes) in PBS for 5 minutes in the dark with gentle shaking. Coverslips were then rinsed three times with PBS and once with water and mounted onto SuperFrost Plus glass slides (Menzel-Glaser) using Citifluor (Agar Scientific).

Fluorescence *in situ* hybridisation (FISH)

Cells were fixed to glass coverslips as described in chapter 1 and stored in IFF at 4°C. For FISH cells were permeabilised with 0.5% Triton-X (Fluka) in PBS for 10 minutes at room temperature with gentle shaking. Coverslips were rinsed three times in PBS and then blocked for 30 minutes at 42°C in pre-hybridisation buffer.

FISH pre-hybridisation buffer composition, 10 ml:

5 ml Formamide

1 ml Denhardts solution 100x

0.5 ml Herring sperm DNA (5mg/ml)

2 ml 20 X SSC

1.5 ml ddH_20

FISH hybridisation buffer composition, 50 ml:

25 ml Formamide

500 µl Denhardts solution 100X

5 ml Dextran sulphate 50%

2 ml Herring sperm DNA (5mg/ml)

4 ml 20 X SSC

13.5 ml ddH_20

Probes were resuspended in hybridisation buffer. A combination of all four probes specific for either Ad5, HSV1 or Vv DNA were diluted 1:25 in a total of 50 µl per coverslip. For combined FISH of AAV DNA 2 µl of the random priming reaction was added. The 50 µl hybridisation mix was pipetted onto SuperFrost Plus glass slides (Menzel-Glaser) and the coverlip overlayed onto the mix avoiding any bubbles. The slide was then placed on a heat block (pre heated to 100°C) for 5 minutes. The slides were then placed on ice for 10 minutes. Slides were then placed into a dark humidity chamber and incubated at 42°C overnight. The following day coverslips were washed with two 10-minute washes with formamide wash buffer (50% formamide, 2XSSC, 0.05% (v/v) Tween-20) at 42°C with shaking. Coverslips were then washed with two 10-minute washes with 2XSSC at 42°C with shaking.

DIG labelled probes were fluorescently labelled using the Fluorescent Antibody Enhancer Set for DIG Detection (Roche Cat No. 1768506) as per supplier protocol. Alexa Fluor 594 conjugated streptavidin was used to fluorescently label anti-AAV biotinylated probes as per supplier protocol. All reactions were carried out in the dark where possible. Coverslips were finally rinsed three times with PBS and once with water and mounted onto SuperFrost Plus glass slides (Menzel-Glaser) using Citifluor (Agar Scientific). For combined FISH with immunofluorescence the above protocol for immunofluorescence was carried out prior to mounting after FISH labeling.

Confocal imaging

Cells were imaged using a Leica DMRXE upright microscope (Leica Microsystems Ltd, Milton Keynes, UK) and a 100x objective lens. Specimens were scanned using the following excitation (ex) and emission wavelengths (em): DAPI ex 359 nm, em 395-520 nm; Alexa-488 ex 488, em 505-685; Alexa 594 ex 568, em 595-710 nm. Fluorophores were excited sequentially to reduce cross-channel bleed through and to allow maximum emission detection. Specimens were scanned with 20 sections of the Z axis with 6 accumulations per frame. Images are presented as an overlay projection of all frames resulting in a composite image.

Section 3 Results

Chapter 1 Cells lacking the p53-p21-pRb pathway are susceptible to AAV-induced cell death via p84N5

- **AAV induces a DNA Damage Response**
- **The AAV-induced DNA damage response leads to cell death in the absence of p53 and p21**
- **Elevated levels of p21 cannot rescue Saos-2 cells from AAV-induced cell death**
- **An AAV-induced DNA damage response activates pRb**
- **Expression of pRb alone cannot rescue cells from AAV-induced cell death**
- **Reconstitution of the p53-p21-pRb pathway in Saos-2 cells is not experimentally viable**
- **Abrogation of pRb renders U2OS cells susceptible to death**
- **AAV infection leads to caspase activation**
- **Caspase-6 is the principle caspase involved in mediating cell death in response to AAV**
- **AAV activates p84N5**
- **Inactivation of p84N5 relieves susceptibility to AAV-mediated cell death**

AAV induces a DNA Damage Response

When cells undergo DNA damage by exogenous means such as UV irradiation for example, proteins involved in detecting, signalling and repairing DNA are recruited to sites of damage and form what are known as irradiation-induced, or DNA damage-induced foci. The single-stranded DNA binding protein RPA is one of the first proteins to form foci at sites of DNA damage. Furthermore, in response to DNA damage, RPA becomes hyperphosphorylated. Since AAV infection has been reported to induce a DNA damage response, we wanted to know if an AAV-induced DNA damage response would also cause the appearance of DNA-damage foci. The data described in this chapter involve the use of UV-inactivated AAV. Both wild-type and UV-irradiated AAV have been shown to induce the same responses though to a lesser degree when not UV irradiated (129). In this section UV-AAV was used to prevent

any viral transcription and thus eliminate the possibility that virally transcribed proteins were responsible for subsequent observations. To visualise AAV-induced DNA damage foci, cells were grown on coverslips and infected with AAV. Coverslips were harvested and *in situ* hybridisation techniques were employed to visualise AAV DNA in infected cells. In addition to *in situ* hybridisation for AAV DNA, the same coverslips were examined via immunofluorescence using antibodies directed to phosphorylated RPA. Figure 3.1.1A shows that when U2OS cells were infected with AAV, AAV DNA was readily detectable within the nucleus of the cells via *in situ* hybridisation. The DNA appeared to be found in discrete, punctuate foci within the nucleus. When serine 33 phosphorylated RPA was visualised in the same cells, punctuate foci, reminiscent of DNA damage-induced foci were visible, and were precisely colocalised with foci of AAV DNA. These foci are a visual representation of the DNA damage response signalling that takes place as a consequence of incoming AAV DNA.

Figure 3.1.1A

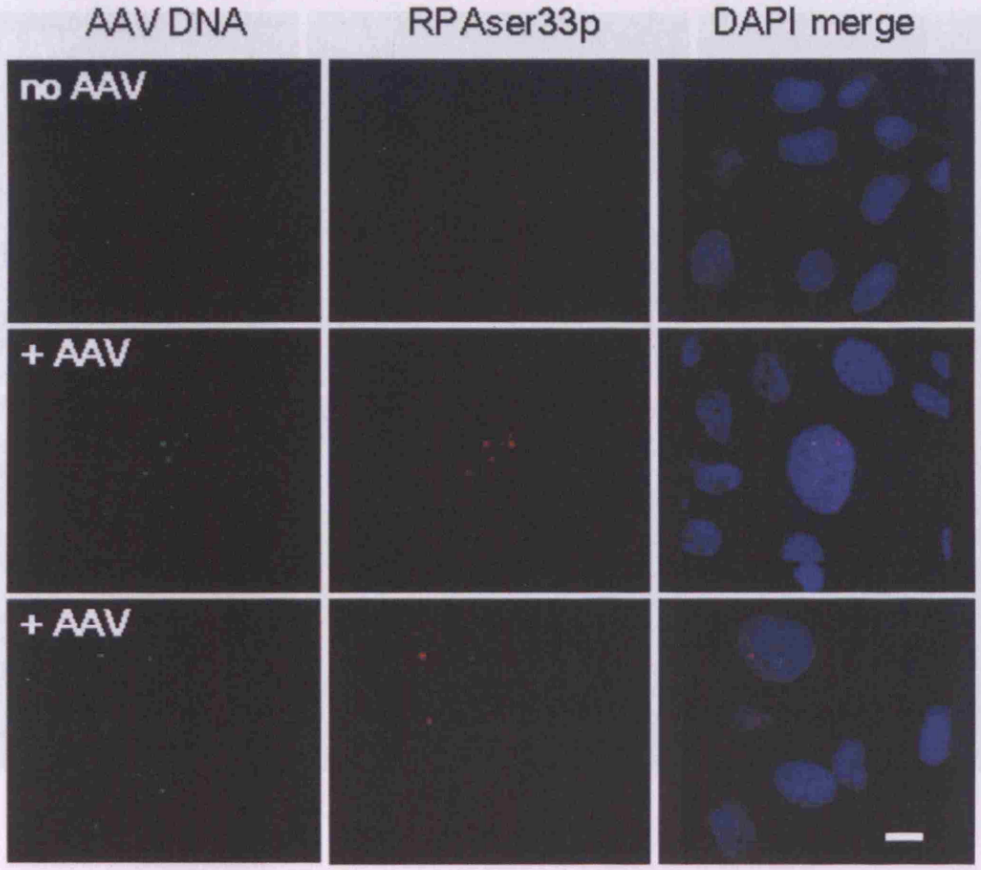


Figure 3.1.1A. Hyperphosphorylated RPA colocalises with AAV DNA in AAV-infected cells. U2OS cultured on glass coverslips and infected with AAV. 24 hours post infection cells were fixed and processed for *in situ* hybridisation with a Dig-labelled anti-AAV probe that was visualised using a FITC labelled anti-DIG staining kit. Hyperphosphorylated RPA was stained using an anti-RPA antibody specific for the serine 33 phosphorylated form of the protein and an Alexa 594 conjugated secondary antibody. RPA is shown in red and AAV DNA in green. DAPI stain highlights DNA in blue. The scale bar represents 10 μm.

Figure 3.1.1B

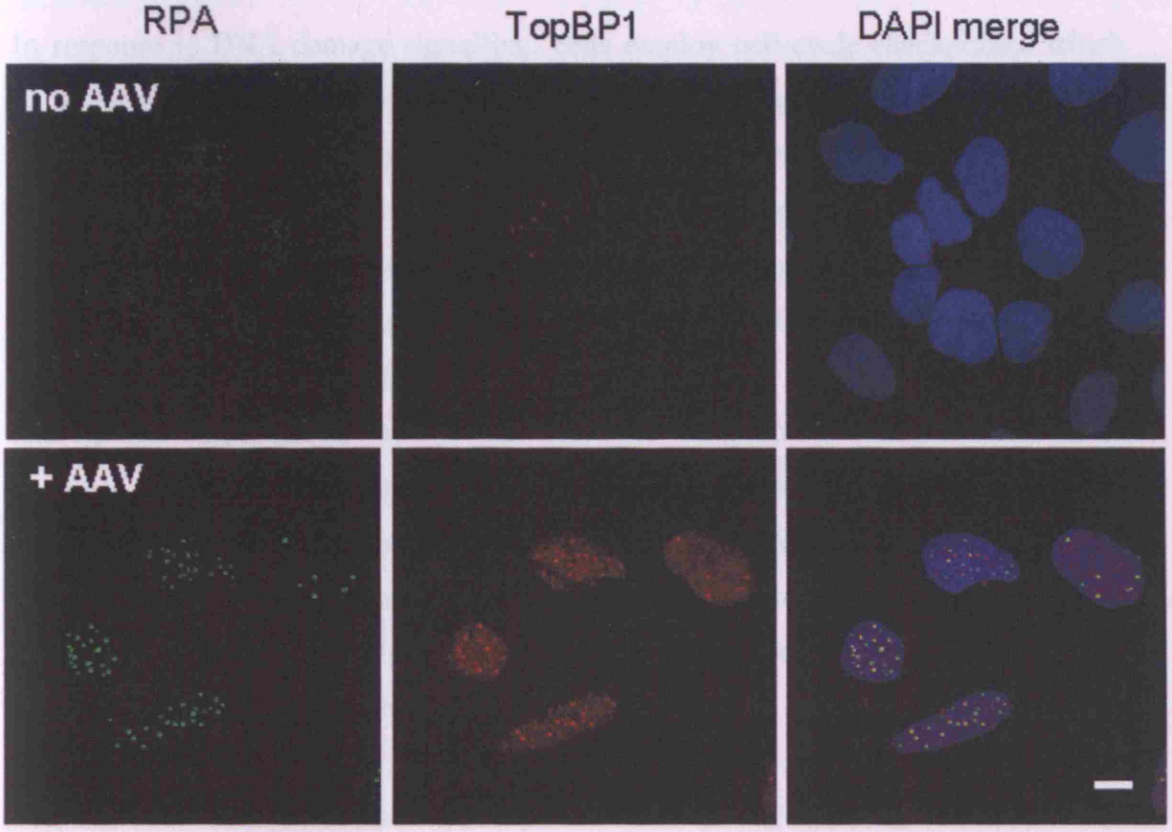


Figure 3.1.1B. RPA and TopBP1 colocalise into nuclear foci following AAV infection. U2OS cultured on glass coverslips and infected with AAV. 24 hours post infection cells were fixed and stained for immunofluoresence using anti-RPA antibodies and anti-TopBP1 antibodies. RPA is shown in red and TopBP1 in green. DAPI stain highlights DNA in blue. The scale bar represents 10 μm.

We also wanted to consider other DNA damage related proteins that might be recruited to these foci. As such we did co-immunostaining of RPA and TopBP1, which acts a recruitment factor for ATR. We observed that following AAV infection, both RPA and TopBP1 colocalised to punctate intranuclear foci, which we know also contain AAV DNA (Figure 3.1.1B).

In response to DNA damage signalling, cells employ cell cycle checkpoints, which pause the normally cycling cell to allow for repair and to prevent aberrant replication. There are two key transition points during the cell cycle at which cells may arrest; the G1/S phase checkpoint and the G2/M transition. In the event of significant damage to cellular DNA, cells may undergo cell death by apoptosis or mitotic catastrophe. The fine balance between arrest and cell death in response to DNA damage is controlled by various protein signalling cascades. One such protein is the p53 protein, whose activity has long been deemed 'the guardian of the genome' (139). A plethora of evidence highlights the means by which p53 preserves genome integrity, fundamentally by inducing pro-apoptotic cascades in the event of DNA damage. The subsequent experiments documented here stem from the observations made by Raj *et al.* 2001 in which the AAV-induced DNA damage response was seen to culminate in death of cells lacking p53 activity (220).

The AAV-induced DNA damage response leads to cell death in the absence of p53 and p21

In cells with functional p53, AAV infection results in a DNA damage response that culminates in G2 arrest. Arrest is accompanied by the activation of p53 and a subsequent rise in the levels of p21 protein, a transcriptional target of p53 activity. The p21 protein is a broad range CDK inhibitor and as such inhibits cell-cycle progression. As such cells may not progress through the G2/M transition and instead arrest with 4n DNA content. AAV-induced G2 arrest of cells with functional p53 is shown in figure 3.1.2. Cells were infected with AAV and harvested at various time points post infection for fluorescence-activated cell sorting (FACS) analysis of their DNA content. U2OS cells with functional p53 arrest with 4n DNA content whereas AAV-infected Saos-2 cells, which lack functional p53 accumulate with sub-G1 DNA content, which is indicative of cell death. Similarly when HCT116 cells with p53

were infected with AAV, they underwent G2 arrest. However, when the isogenic partner cell line HCT116 p21-/-, which expresses functional p53 but lack p21 were infected with AAV, significant cell death was observed as shown by the increase in cells with less that 2n DNA content. These observations previously reported by Raj *et al.* 2001 demonstrate that in the absence of p53 or p21, cells are susceptible to AAV-mediated cell death. In the presence of functional p53 and p21 however, cells respond to AAV infection with G2 phase cell cycle arrest (220). We show here that this is concomitant with the activation of p53 as demonstrated by the phosphorylation of p53 on serine 15 and the increased expression of p21 protein, a p53 transcriptional target gene (Figure 3.1.3). We sought to establish whether artificially enhancing the levels of p21 protein in Saos-2 cells (which lack p53) would be sufficient to protect them from AAV-induced cell death.

Figure 3.1.2

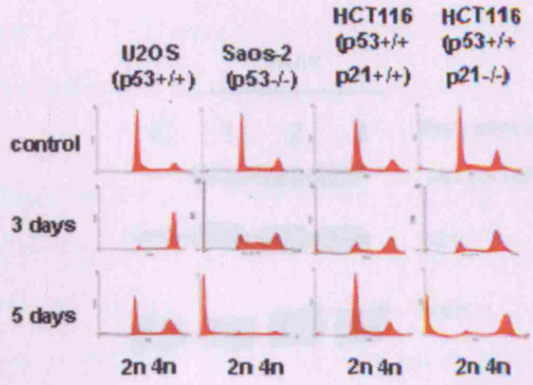


Figure 3.1.2. AAV induces G2 arrest or cell death in cells lacking p53 or p21. Analyses of uninfected cells (control) and cells infected with AAV at various times after infection. Cellular DNA was stained with propidium iodide and analysed by FACS.

Figure 3.1.3

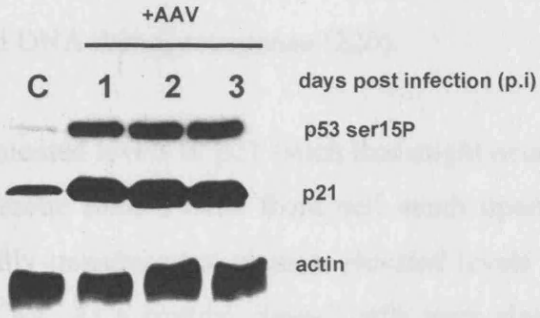


Figure 3.1.3. AAV infection of U2OS cells leads to the phosphorylation of p53 on serine 15 and increased expression of p21. *(B)* Western blot analysis of activated p53 (as shown via phosphorylation of serine 15) and p21 from lysates of uninfected U2OS cells and those at various days post-infection (p.i) with AAV.

Elevated levels of p21 cannot rescue Saos-2 cells from AAV-induced cell death

p53's ability to enhance transcription of various proteins involved in cell cycle arrest and apoptosis in response to DNA damage is well documented. For example the levels of 14-3-3σ are augmented following an AAV-induced DNA damage response. However, the presence or absence of 14-3-3σ does not dictate the survival of a cell following an AAV-induced DNA damage response (220).

To determine whether augmented levels of p21 (such that might occur in the presence of activated p53) might rescue Saos-2 cells from cell death upon AAV infection, Saos-2 cells were retrovirally-transduced to express elevated levels of p21 protein as demonstrated in figure 3.1.4A. As a control, Saos-2 cells were also transduced with the empty retroviral vector. These transduced cells were infected with AAV and harvested for FACS analysis five days post-infection (Figure 3.1.4B). Dishes of cells were also processed via methylene blue staining of adherent monolayer cells to further demonstrate cell death (Figure 3.1.4C). FACS analysis demonstrates that while uninfected cells preserve a normal profile, AAV-infected cells (both untransduced, control transduced and +p21 transduced cells) exhibit a profound increase in cells with less than 2n DNA content, indicative of cell death.

The clonogenic assay method of methylene blue staining of surviving cells represents a robust way of demonstrating long-term cell survival (34). Such analysis becomes more directly relevant in subsequent experiments in which a comparison of relative survival is required. In this case there is a very definite distinction between survival and death as demonstrated by FACS and this is reinforced by methylene blue analysis.

Although initial experiments demonstrate the importance of p21 in protecting cells from death, it is clear that elevated levels of p21 alone are not sufficient to prevent AAV DNA-induced cell death. Since the role of p21 is unmistakable, we considered factors downstream of p21 in terms of signalling cascades. One of the most documented signalling cascades originating from p53 activation is that involving the increased expression of p21 and as a consequence of p21's CDK inhibition, the maintenance of pRb in a dephosphorylated state that inhibits cell cycle progression.

As such pRb is a recipient of increased p21 activity and so we considered the possible role of pRb in preventing AAV-induced cell death. Of particular relevance here is the fact that pRb is not expressed in Saos-2 cells.

An AAV-induced DNA damage response activates pRb

We have thus far demonstrated that AAV infection activates p53 and leads to the subsequent up-regulation of p21 expression. We examined AAV-infected U2OS cell lysates to determine whether pRb status was affected by the presence of AAV DNA. Western blot analysis using antibodies directed to pRb demonstrated that upon AAV infection, pRb activation takes place as shown by its increased dephosphorylation over the course of 72 hours following infection, as demonstrated by species of pRb with increased electrophorectic mobility (Figure 3.1.5A and B). The very distinct activation of pRb in U2OS cells, which demonstrate resistance to AAV-induced cell death, reiterates the potential involvement of pRb in preventing cell death in response to AAV-induced DNA damage signalling.

Figure 3.1.4

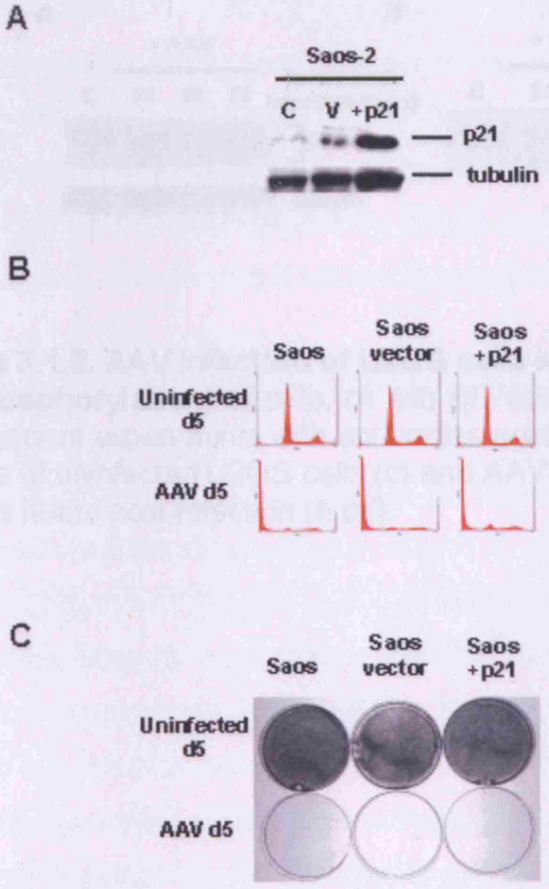


Figure 3.1.4. Saos-2 cells with augmented levels of p21 are susceptible to AAV-induced cell death. *(A)* Western blot analyses of lysates of Saos-2 cells that were untransfected (c) Saos-2 that were either transduced with empty vector (v) or vector that carried a p21 gene (+p21) *(B)* FACS analyses of DNA content and *(C)* methylene blue staining of uninfected cells and AAV-infected untransduced and transduced cells.

Figure 3.1.5

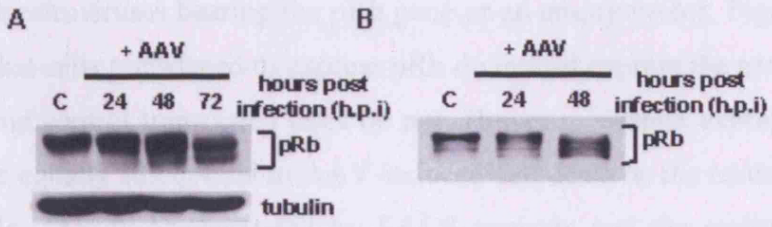


Figure 3.1.5. AAV infection of U2OS cells leads to the activation (dephosphorylation) of pRb. *(A and B)* Western blot analysis of two independent experiments with antibodies against the pRb protein of lysates of uninfected U2OS cells (c) and AAV-infected U2OS cells at various hours post-infection (h.p.i).

Expression of pRb alone cannot rescue cells from AAV-induced cell death

To examine the hypothesis that pRb plays a fundamental role here in preventing cell death, we used Saos-2 cells, which do not express pRb and are susceptible to AAV-mediated cell death. We reasoned that if pRb was crucial, then the re-introduction of pRb protein might confer protection to otherwise susceptible cells. Saos-2 cells were transduced with retroviruses bearing the pRb gene or an empty vector. Figure 3.1.6A clearly shows that cells transduced to express pRb do indeed express the protein while untransduced and control transduced cells do not. However, despite expressing pRb, these cells were equally susceptible to AAV-induced cell death as the control and un-transduced cells. This is demonstrated by FACS analysis and the methylene blue staining, clonogenic assay (Figure 3.1.6B and C respectively).

Reconstitution of the p53-p21-pRb pathway in Saos-2 cells is not experimentally viable

Experimental evidence thus far demonstrates the importance of p53, p21 and pRb in that absence of either of these proteins leads to a cell's susceptibility to death in response to AAV-induced DNA damage signalling. However, cells cannot be rescued from such susceptibility by expression of either one of these proteins alone. As such we wanted to reconstitute the full pathway in Saos-2 cells to analyse the possibility that the complete signalling pathway be required for cell survival in the event of such damage signalling. Saos-2 cells were transfected with a combination of expression vectors bearing the genes for p53, p21 or pRb protein. Figure 3.1.7 demonstrates that it is possible to achieve the expression of p53, p21 and pRb with a population of cells as shown by western blot analysis of transfected Saos-2 cells prior to selection. However, these unselected populations, which are heterogenous by nature, are unsuitable for further experiments in which we would be asking the question; will these cells survive following AAV infection? If cell death were observed one could not be certain that it was not simply a consequence of unsuccessful reconstitution of the pathway. Unfortunately, upon antibiotic selection to obtain a homogenous population of Saos-2 cells expressing p53, p21 and pRb, it was found that the emerging cells no longer express these proteins in detectable quantities. It is likely that reconstitution of this pathway in Saos-2 cells does not allow successful proliferation, and subsequently the cells selected for no longer express the proteins.

Figure 3.1.6

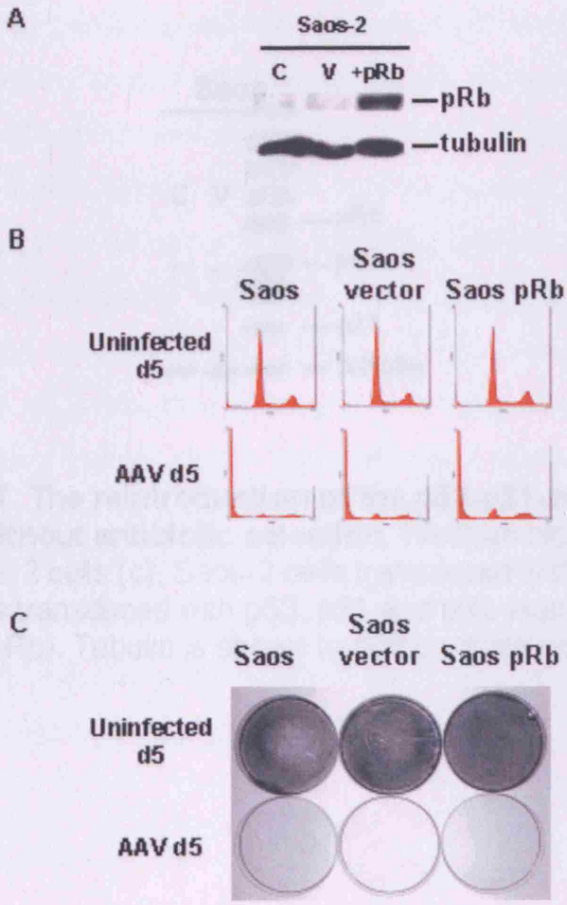


Figure 3.1.6. Reconstitution of pRb cannot rescue Saos-2 cells from AAV-induced cell death. *(A)* Western blot analysis, using antibodies against pRb, of extracts from cells that were either transduced with an empty vector (v) or a vector bearing the pRb gene (+pRb) and untransduced cells (c). *(B)* FACS analyses of DNA content and (C) methylene blue staining of uninfected cells and AAV-infected untransduced and transduced cells.

Figure 3.1.7

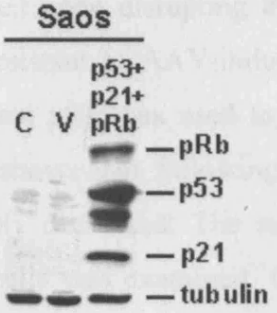


Figure 3.1.7. The reintroduction of the p53-p21-pRb pathway is only possible without antibiotic selection. Western blotting of lysates of control Saos-2 cells (c), Saos-2 cells transduced with an empty vector (v) or Saos-2 cells transduced with p53, p21 and pRb expression vectors (p53+p21+pRb). Tubulin is shown to demonstrate equal loading.

Abrogation of pRb renders U2OS cells susceptible to death

Despite Saos-2 cells being the ideal system in which to test the hypothesis that the complete signalling pathway from p53 ultimately to pRb be required to afford protection from AAV-induced cell death, a more experimentally viable approach was required. As such we focused upon disrupting the p53-p21-pRb pathway in U2OS cells which are otherwise resistant to AAV-induced apoptosis. Expression of short hairpin RNA (shRNA) against pRb was used to decrease levels of pRb protein in U2OS cells. Figure 3.1.8A shows that following shRNA transduction, the level of pRb protein was considerably decreased. The susceptibility to AAV-mediated cell death of these and control cells was examined. Cells were infected with AAV and then analysed by methylene blue staining of surviving, adherent cells 10 days post-infection. The stained plates clearly show that upon abrogation of pRb expression, U2OS cells were no longer resistant to AAV-induced cell death (Figure 3.1.8B). In combination with our previous observations, emerges the following conclusion; that the functional integrity of the complete p53-p21-pRb signalling pathway is required to prevent cell death in the event of AAV-induced DNA damage signalling. A table to summarise these data and those reported by Raj *et al.* 2001 (220) is shown in Table 3.1.1.

Figure 3.1.8

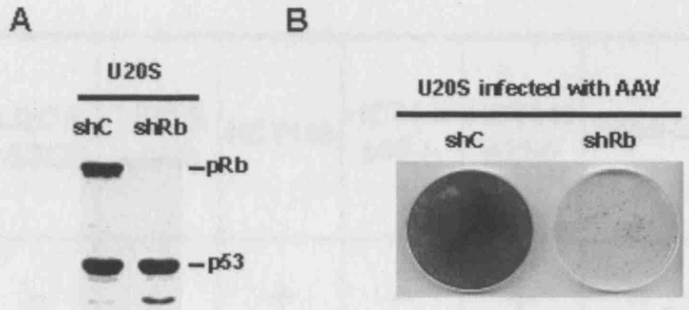


Figure 3.1.8. Expression silencing of pRb in U2OS cells renders U2OS susceptible to AAV-induced cell death. *(A)* Western blot analysis of lysates from cells that were transduced with retroviruses encoding either scrambled shRNA (shC) or pRb-specific shRNA (shRb). Antibodies against pRb and p53 were used. NB Upon extensive exposure of the blot, residual pRb protein band is observed in the shRb lane, indicating non-complete abolition of the pRb protein by the shRNA *(B)* Methylene blue staining of AAV infected cells transduced with scrambled shRNA (shC) or pRb-specific shRNA (shRb).

Table 3.1.1

	U2OS	U2OS p53DD	U2OS shRb	HCT116	HCT116 p53-/-	HCT116 p21-/-	Saos-2	Saos-2 + p21	Saos-2 + pRb
p53	+	-	+	+	-	+	-	-	-
↓ p21	+	+	+	+	+	-	+	++	+
↓ pRb	+	+	-	+	+	+	-	-	+
+AAV									

Table 3.1.1. Perturbations of the p53-p21-pRb pathway render cells susceptible to cell death upon AAV infection. The table summarises the result of AAV infection in all cell types used or generated and infected with AAV as described in the text together with those reported by Raj *et al.* 2001. The table shows the status in each line of the p53-p21-pRb pathway and the result of AAV infection in the lower row. A skull and crossbones indicates cells death upon infection. A continuous circle indicates cell survival.

We now centred our attention on understanding how signalling via this pathway might prevent death. As such it was necessary to try and establish the pathway or pathways that promote cell death to understand how p53 signalling to pRb might protect.

AAV infection leads to caspase activation

Caspase proteins represent core mediators of cell death. There are at least 11 known caspases in humans and the signature of caspase protein activation and involvement in cell death can yield a lot of information with regard to the signalling pathways involved in stimulating cell death (281). Raj et al 2001 have shown previously that AAV-induced cell death occurred via apoptosis since cells were positive for Annexin-V (220). As such we wanted to characterise apoptotic cell death via caspase activation. Saos-2 cells were infected with AAV and whole cell lysates were examined for caspase activation by looking for the generation of cleavage products since caspase cleavage is indicative of caspase activation. Figure 3.1.9A shows western blot analysis of caspase 3 and highlights the generation of lower molecular weight products that correspond to caspase 3 cleavage products. Caspase 3 is one of the core executioner caspase molecules activated in cells undergoing apoptosis. However, we also observed the activation of caspase 6, whose activity is associated with fewer inducers of cell death. A panel of cells were examined for caspase-6 activation (Figure 3.1.9B). Saos-2 cells, U2OS cells and U2OS cells transduced with shRb were infected with AAV and whole cell lysates were examined via western blotting. We observed that while caspase-6 was clearly activated in Saos-2 cells, U2OS cells did not activate caspase-6 in response to AAV infection. However when pRb levels were diminished in U2OS cells via shRb we saw that upon AAV infection, caspase-6 activation was observed. The pattern of caspase-6 activation is consistent with the cells that we have thus far demonstrated to be susceptible to AAV-mediated cell death.

Figure 3.1.9

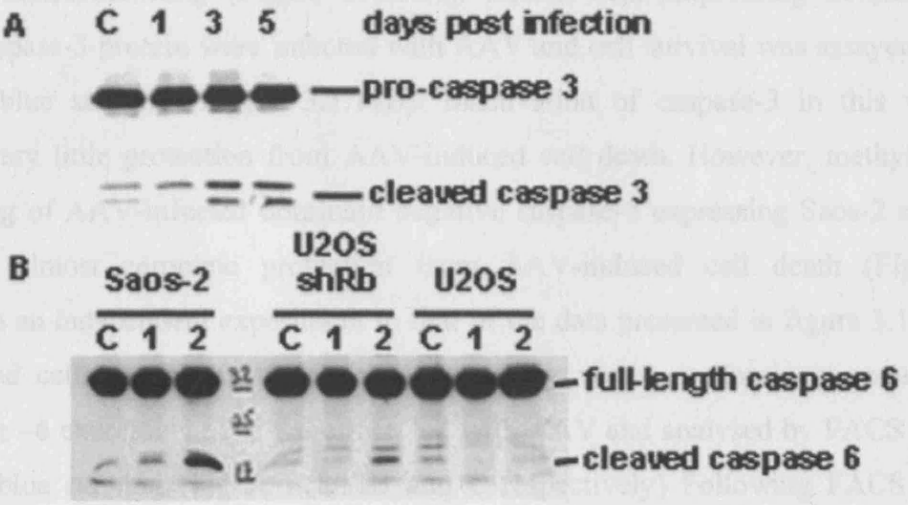


Figure 3.1.9. AAV infection leads to the activation of caspase-3 and caspase-6 in cells susceptible to AAV induced cell death. *(A)* Caspase 3 western blot analysis of lysates of Saos-2 at various days post-AAV infection *(B)* Caspase 6 western blot analyses of lysates of Saos-2, U2OS cells expressing shRb and wild-type U2OS cells, 1 and 2 days post infection with AAV. Uninfected cell lysates are labelled as "c".

Caspase-6 is the principle caspase involved in mediating cell death in response to AAV

To clarify whether caspase-6 was a fundamental mediator of cell death in response to AAV-induced DNA damage signalling as opposed to it's activation being merely a consequence of cell death, we employed point mutant dominant negative caspase molecules to inhibit caspase activity. Saos-2 cells were transfected and selected to express dominant-negative caspase-6 or dominant-negative caspase-3 protein as shown by immunoblotting (Figure 3.1.10A). Saos-2 cells expressing dominant-negative caspase-3 protein were infected with AAV and cell survival was assayed by methylene blue staining (Figure 3.1.10B). Inactivation of caspase-3 in this way conferred very little protection from AAV-induced cell death. However, methylene blue staining of AAV-infected dominant negative caspase-6 expressing Saos-2 cells highlighted almost complete protection from AAV-induced cell death (Figure 3.1.10C). In an independent experiment to that of the data presented in figure 3.1.10, untransfected cells, cells transfected with the empty vector or dominant negative caspase-3 or –6 expressing cells were infected with AAV and analysed by FACS and methylene blue staining (Figure 3.1.11B and C respectively) Following FACS the measure of cells with a sub-G1 DNA content were quantified and this is annotated against each profile. Following AAV infection between 70 and 90% of Saos-2 or Saos-2 empty vector control cells underwent cell death as determined by the percentage of cells with sub-G1 DNA content. Conversely, only 20% of cells underwent cell death when expressing the dominant-negative caspase-6 protein compared with 30% of cells expressing dominant negative caspase-3. Long-term cell survival assays and FACS analysis both yielded the same conclusion; that caspase-6 be the fundamental caspase involved in mediating cell death in response to AAV. Since caspase-6 is activated by a more limited group of pro-apoptotic agents our search for the pro-apoptotic mediator of AAV-induced cell death could be more refined.

Figure 3.1.10

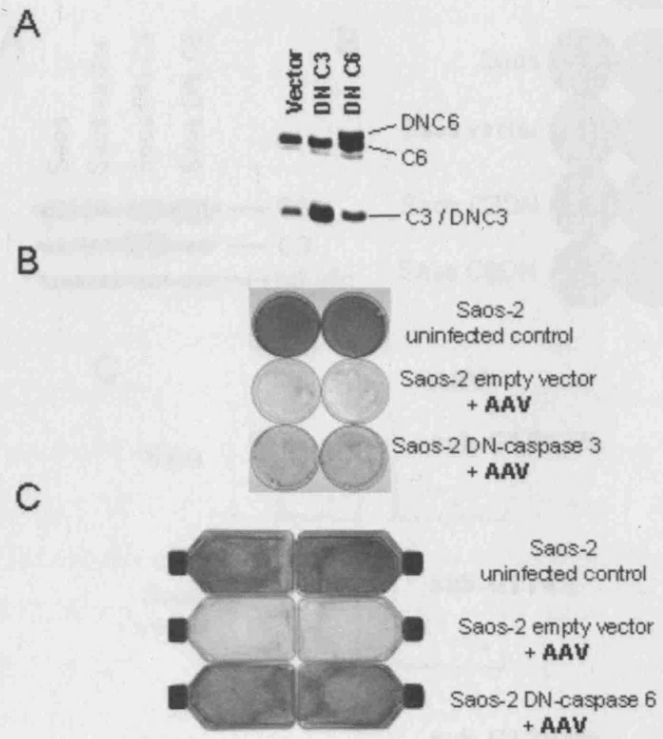


Figure 3.1.10. Dominant negative caspase-6 expressing Saos-2 cells are rescued from AAV-induced cell death. *(A)* Western blot analysis using anti-caspase 3 and anti-caspase 6 of lysates from Saos-2 cells that were engineered to express either dominant-negative caspase 6 (DN C6), which possesses a tag sequence, or dominant-negative caspase 3 (DN C3), which is a point mutant of the same length as the wild-type protein. *(B)* Duplicate methylene blue staining of uninfected Saos-2 cells, Saos-2 cells containing the empty vector and Saos-2 cells expressing dominant-negative caspase 3, seven days post-infection with AAV. *(C)* Duplicate methylene blue staining of uninfected Saos-2 cells, Saos-2 cells containing the empty vector and Saos-2 cells expressing dominant-negative caspase 6, seven days post-infection with AAV.

Figure 3.1.11

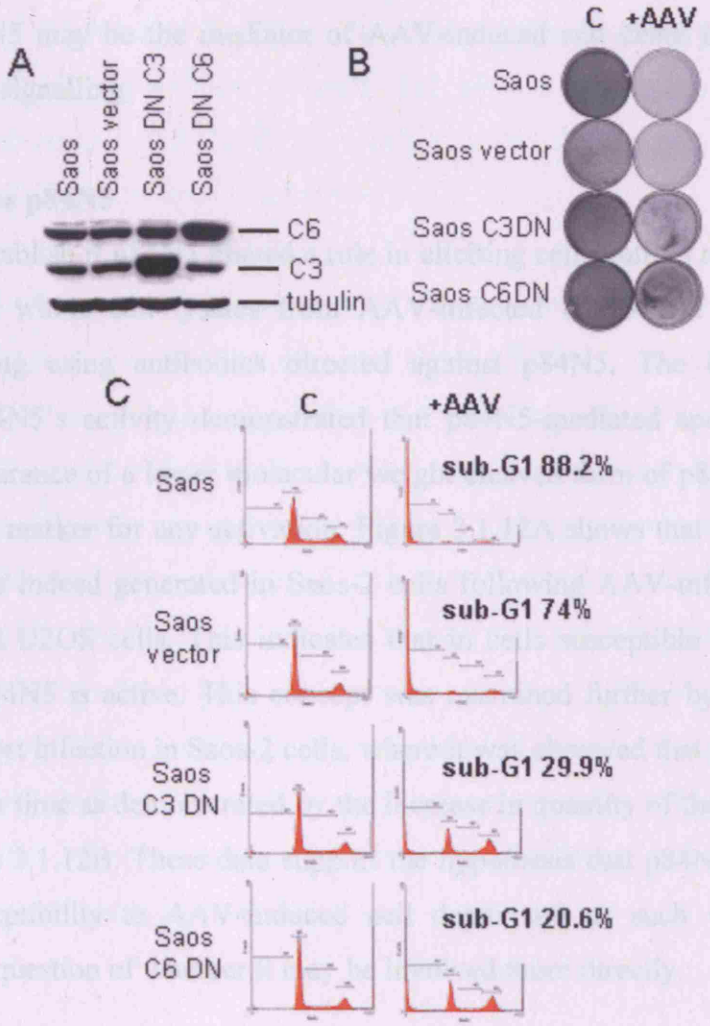


Figure 3.1.11. FACS analysis of AAV-induced cell death in Saos-2 cells expressing dominant-negativecaspase 3 / 6. *(A)* Western blotting of lysates of untransduced Saos-2 cells, Saos-2 cells transduced with an empty vector and Saos-2 cells that had been transduced with either a dominant negative caspase-3 or a dominant negative caspase-6 expression construct. Expression is indicated by the increase in amount of caspase-3 or –6 respectively. Expression of tubulin is shown to demonstrate equal loading. *(B)* Saos-2 cells, Saos-2 cells transduced with an empty vector or Saos-2 cells transduced with the dominant negative capsase-3 or dominant negative caspase-6 construct were infected with AAV (+AAV) or mock-uninfected (c). Plates were stained with methylene blue five days post-infection to show surviving adherent cells. *(C)* In parallel cells were harvested for FACS analysis to allow quantification of relative amounts of cell death following AAV infection by quantification of cells with sub-G1 DNA content.

One protein that has been demonstrated to activate caspase-6 is the pro-apoptotic protein p84N5 (77). The activity of p84N5 was of particular interest since its activity has been associated with pRb in that it has been shown that p84N5's pro-apoptotic propensity is inhibited by active, dephosphorylated pRb. As such we considered whether p84N5 may be the mediator of AAV-induced cell death in the absence of p53-p21-pRb signalling.

AAV activates p84N5

In order to establish if p84N5 played a role in eliciting cell death in response to AAV, we examined whole cell lysates from AAV-infected U2OS and Saos-2 cells by immunoblotting using antibodies directed against p84N5. The limited literature regarding p84N5's activity demonstrated that p84N5-mediated apoptosis coincides with the appearance of a lower molecular weight cleaved form of p84N5. As such we used this as a marker for any activation. Figure 3.1.12A shows that the cleaved form of p84N5 was indeed generated in Saos-2 cells following AAV-infection but not in AAV-infected U2OS cells. This indicates that in cells susceptible to AAV-induced cell death, p84N5 is active. This concept was examined further by looking at later time points post infection in Saos-2 cells, where it was observed that p84N5 activation increased over time as demonstrated by the increase in quantity of the cleaved form of p84N5, figure 3.1.12B. These data support the hypothesis that p84N5 is important in lending susceptibility to AAV-induced cell death and as such we continued to approach the question of whether it may be involved more directly.

Inactivation of p84N5 relieves susceptibility to AAV-mediated cell death

In order to examine the functional importance of p84N5 activity with regard to AAV-mediated cell death, we examined whether inactivation of p84N5 would prevent cell death in Saos-2 cells, which lack the activity of the p53-p21-pRb pathway. The p84N5 protein contains putative death domains (77) considered to be of importance in terms of the proteins pro-apoptotic activities. We used a retroviral construct to express a truncated form of the protein lacking the death domain regions. This mutant has previously been characterised and shown to function as a dominant-negative, thus inhibiting the activity of the wild-type protein (75). Saos-2 cells were transduced to express the dominant-negative protein as shown in figure 3.1.12C. These and control cells transduced with the empty retroviral vector were subjected to antibiotic selection

and subsequently infected with AAV. Results are shown in duplicate in figure 3.1.12D. Tissue culture plates were stained with methylene blue to highlight surviving cells. The results clearly show that while control Saos-2 cells underwent cell death upon AAV infection, leaving empty tissue culture plates, Saos-2 cells expressing the dominant negative p84N5 protein fully populated the plates following AAV infection. These observations support the concept that p84N5 is the pro-apoptotic factor involved in promoting cell death in response to an AAV-induced DNA damage response in cells that lack the p53-p21-pRb.

As such we surmise the following model, which dictates the outcome of AAV infection in terms of cell death or survival. The AAV DNA, as a consequence of its unusual structure, is recognised as a DNA lesion. As such the AAV DNA initiates a DNA damage response, which likely involves many biochemical-signalling cascades. Here we focus upon two (i) the activation of p53 leading to the increased expression of p21 and ultimately the activation of pRb and (ii) the activation of p84N5, which together determine the fate of the cell in response to AAV infection. In the presence of a functionally intact p53-p21-pRb signalling pathway, pRb will be successfully activated. The activation of pRb by dephosphorylation permits its interaction with and inhibition of p84N5 from activation, and as such prevents the initiation of apoptosis. However, if pRb activation cannot take place, p84N5 can be activated and cleaved leading to apoptosis via caspase-6.

Figure 3.1.12

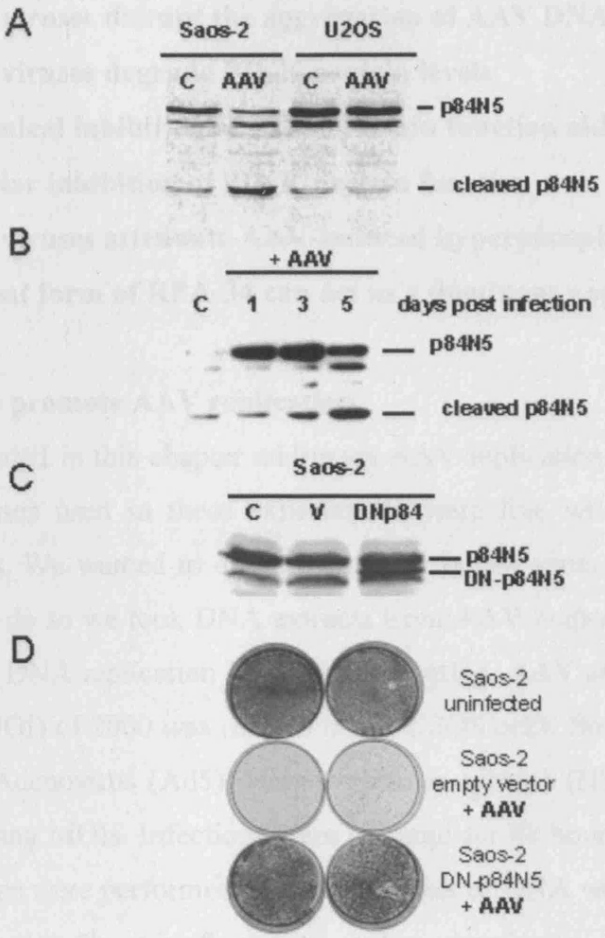


Figure 3.1.12. Expression of dominant negative p84N5 rescues Saos-2 cells from AAV induced cell death. *(A)* Western blotting of lysates of Saos-2 or U2OS uninfected (c) cells and cells 2 days post infection with AAV using antibodies against p84N5 protein. *(B)* p84N5 western blot analysis of lysates from Saos-2 cells that were harvested at various days post-AAV infection *(C)* Western blot analysis using antibodies against p84N5 of untransduced cells (c), cells transduced with empty vector (V) or with vector expressing dominant-negative p84N5 protein (DNp84), which is a shortened form (lacking the death domains) of the wild type protein. *(D)* Duplicate methylene blue staining of uninfected Saos-2 cells, Saos-2 cells transduced with empty vector or Saos-2 cells expressing dominant-negative p84N5 protein, ten days post AAV infection.

Chapter 2 AAV-induced DNA damage signalling as a potential factor restricting autonomous replication of AAV

- **Helper viruses promote AAV replication**
- **Helper viruses disrupt the aggregation of AAV DNA into nuclear foci**
- **Helper viruses degrade PIKK protein levels**
- **Biochemical inhibition of PIKK protein function aids AAV replication**
- **Molecular inhibition of PIKK protein function**
- **Helper viruses attenuate AAV-induced hyperphosphorylation of RPA**
- **A mutant form of RPA-34 can act as a dominant negative**

Helper viruses promote AAV replication

The data presented in this chapter addresses AAV replication restriction and as such all of the viruses used in these experiments were live wild-type AAV, not UV-irradiated virus. We wanted to directly examine helper virus-mediated help to AVV replication. To do so we took DNA extracts from AAV-helper virus coinfections and analysed AAV DNA replication by southern blotting. AAV at a constant multiplicity of infection (MOI) of 2000 was used to infect U2OS cells. Simultaneously cells were infected with Adenovirus (Ad5), Herpes simplex virus-1 (HSV1) or Vaccinia virus (Vv) in increasing MOIs. Infections were cultured for 48 hours following which total DNA extractions were performed. Equal quantities of DNA were loaded onto agarose gels and subsequently transferred to nylon membrane for southern analysis. Membranes were probed using a radiolabelled AAV DNA probes (Figure 3.2.1). Figure 3.2.1 illustrates the pattern of bands observed for the different sized products of AAV replication intermediates. The lower band corresponds to the single-stranded (ss) AAV DNA. The middle band corresponds to the monomeric replicative form (Rf*m*) and the upper band to the duplex replicative form (Rf*d*). In the absence of helper virus, AAV DNA from infected U2OS cells was difficult to detect by southerm blot. However in the presence of co-infecting helper virus, (even at lowest MOI) AAV DNA and its replication intermediates were observed. The ratio of different species of AAV DNA and replication intermediates was different for each helper virus. Furthermore this ratio and the amount of DNA reflects the MOI of helper virus used. In the case of Adenovirus, we observed that with regard to the three MOIs used,

the quantity of ss AAV DNA increased with increasing MOI. The quantity of Rf*m* remained fairly constant and the Rf*d* form was only visible upon longer exposure. The MOI values of co-infecting HSV1 highlighted a considerable increase in ss AAV DNA, which peaked at the mid MOI used with a corresponding peak in Rf*m* and Rf*d* species. At lower or higher MOI of coinfecting HSV1 there was considerable replication and indeed at all MOI values used HSV1 appeared to aid AAV replication more than Ad5 coinfection as determined by relative abundance of ss AAV DNA and replicative intermediates. It appears that Vv conferred the least replicative help to AAV when compared with Ad5 and HSV1. Again the mid value MOI perhaps gave the optimum output of newly-synthesised AAV DNA, the three replicative forms of which appeared in approximately equal ratios. In summary figure 3.2.1 provides a visual representation of helper virus-mediated AAV DNA replication.

Helper viruses disrupt the aggregation of AAV DNA into nuclear foci

We have already demonstrated that in non-permissive conditions, i.e. in the absence of helper viruses, that AAV DNA rapidly accumulates into intranuclear foci that colocalise with DNA damage-related proteins. These foci are reminiscent of DNA damage-induced nuclear foci. We wanted to examine whether similar foci accumulated upon AAV and helper virus coinfection. As such U2OS cells grown on glass coverslips were co-infected with Ad5, HSV1 or Vaccinia virus with AAV and cultured for 24 hours. *In situ* hybridisation techniques were employed to visualise both AAV DNA and helper virus DNA. Figures 3.2.2, 3.2.3 and 3.2.4 provide a comparison of AAV infection alone with that of helper virus coinfections.

Figure 3.2.2 compares AAV infection alone with that of an Ad5 coinfection. When AAV infects cells the DNA appears restricted to intranuclear foci, which we have shown in previous experiments, directly colocalise with DNA damage related proteins (see Figure 3.1.1). However when cultured in coinfection with Adenovirus, the AAV DNA was no longer confined to punctuate foci but rather appeared throughout the nucleus. The number of cells infected with AAV appeared less than infection with AAV alone. Since similar amounts of AAV were used it is likely that the number of cells infected with AAV was the same here as AAV infection alone. Yet it seems that fewer cells demonstrate AAV DNA foci even in the absence of any apparent

replication. Figure 3.2.3 compares AAV infection with HSV1-AAV co-infections. In a similar way to Adenovirus co-infections, we see that in the presence of HSV1, AAV was no longer confined to discrete intranuclear foci but rather AAV DNA was found throughout much of the nucleus. Furthermore, while the number of cells demonstrating significant replication of AAV DNA was considerable for HSV1-AAV coinfections (greater than in the context of Ad5-AAV coinfections, in aggrement with the southern blot data of figure 3.2.1) even cells that appeared to have low quantities of AAV DNA did not have observable intranuclear foci. Figure 3.2.4 compares AAV infections with Vv-AAV co-infections. It is clear that the help to AAV replication by Vv was not as significant as the help derived from HSV1 or Ad5 coinfection and this is again in agreement with the data shown in the southern blot of figure 3.2.1. However, when replication was observed, AAV DNA was not confined to discrete intranuclear foci but rather is dispersed diffusely within the nucleus. It is noteworthy also that in addition to the lower levels of AAV replication when compared to HSV1 or Ad5 coinfected cells, there were considerably more cells that while positive for both Vv DNA and AAV DNA did nevertheless display AAV DNA in nuclear foci in the absence of apparent replication. This may be a reflection of the fact that unlike Ad5 or HSV1, Vv replication is cytoplasmic, associating with endoplasmic reticulum compartments (279). There are few demonstrations of in situ hybridisation of Vv DNA during replication and while reportedly strictly cytoplasmic the data presented here appears to demonstrate some nuclear localisation of Vv DNA in U2OS cells.

It is also of note that when the localisation of AAV DNA and helper virus DNA is compared, one appears almost totally excluded from the region of the other. This is apparent in the combined in situ hybridisation fluorescence images of coinfections highlighted in figures 3.2.2, 3.2.3 and 3.2.4. The localisation of helper virus replication centres and AAV replication centres is of some dispute. In the past, some evidence has supported the notion that AAV and helper virus replication compartments overlap (115, 262, 297). However a more recent study using indirect visualisation techniques has suggested that AAV and HSV1 replication compartments are distinct (102). Here we show direct evidence for exclusion of three different helper viral DNAs from AAV DNA by direct *in situ* hybridisation.

The observation that in permissive conditions, AAV DNA is not retained in nuclear foci led us to the hypothesis that elements of the DNA damage-like foci and the DNA damage response may be factors that restrict AAV's autonomous replication. Since the primary sensors of DNA damage are the PIKK proteins, ATM, ATR and DNA-PK we wanted to examine whether signalling from these enzymes plays a role in inhibiting AAV replication.

Figure 3.2.1

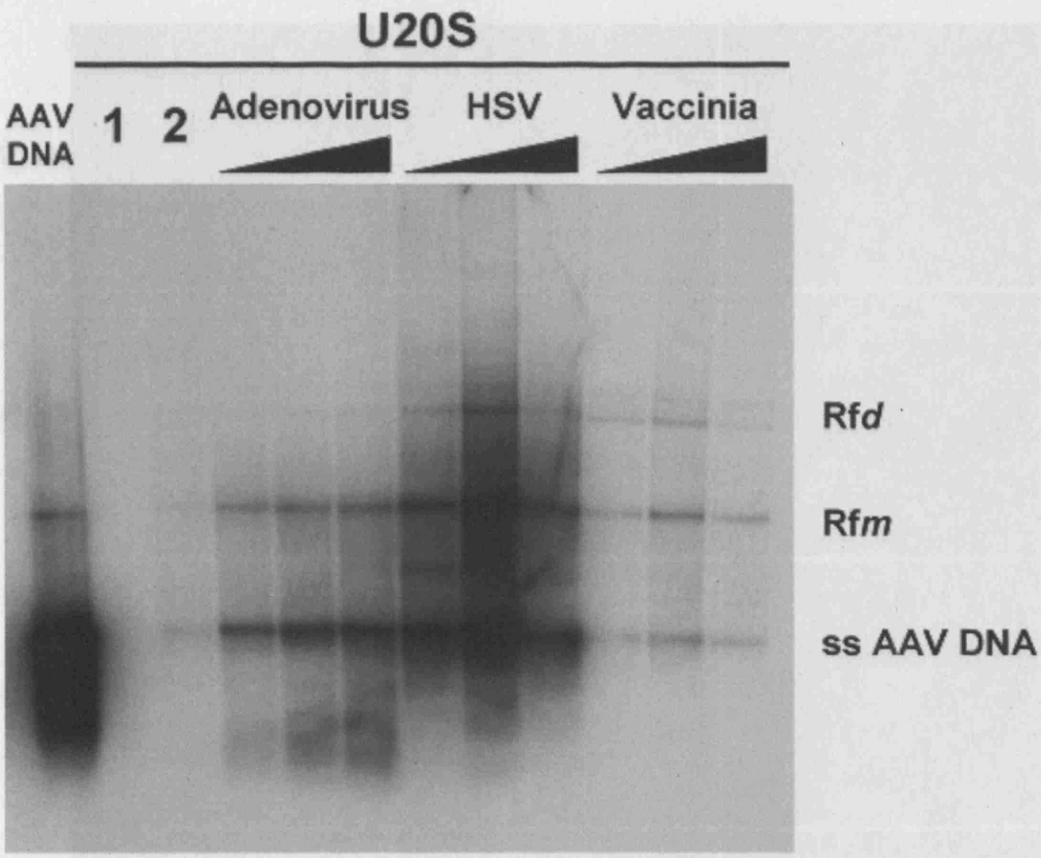


Figure 3.2.1. Adenovirus, Herpes simplex virus (HSV) and Vaccinia virus facilitate AAV replication. Lanes 1 and 2 indicate mock infected U2OS and AAV infected U2OS respectively. All other lanes were infected with AAV at an MOI of 2000 in combination with increasing amounts of helper virus at MOIs of 5, 15 and 30. The membrane was probed with a radiolabelled anti-AAV DNA probe. Single-stranded AAV DNA (ssAAV DNA), monomeric replicative forms (RFm) and duplex replicative forms (RFd) are highlighted.

Figure 3.2.2

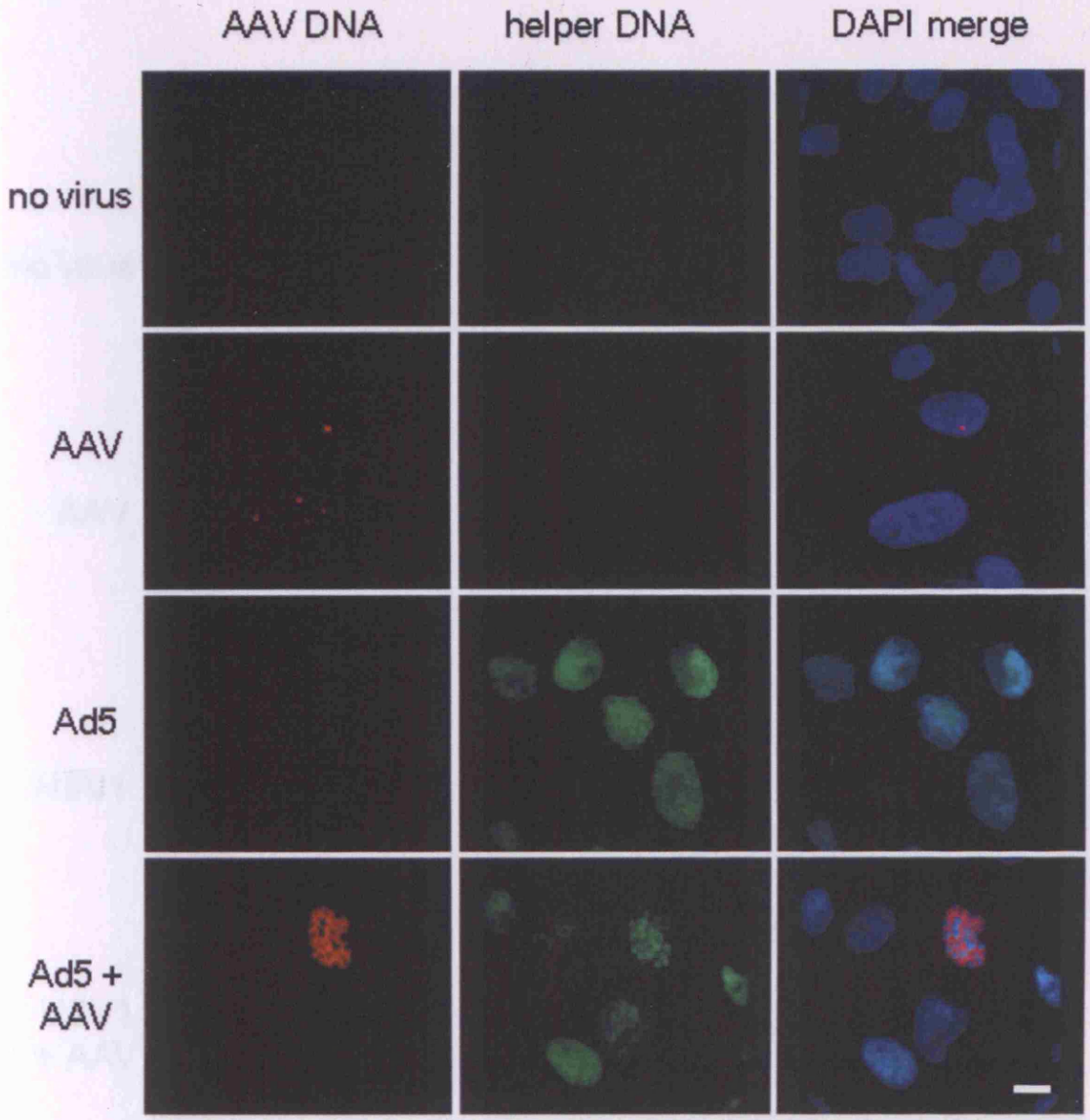


Figure 3.2.2. AAV DNA is not restricted to discrete foci in Adenovirus (Ad5) and AAV coinfections. U2OS cells were cultured on glass coverslips and infected with AAV (MOI 2000) alone or AAV (MOI 2000) and Ad5 (MOI 15) together. Cells were cultured for 24 hours and harvested and processed for *in situ* hybridisation. Biotinylated AAV probes were used to detect AAV DNA and detected using streptavidin conjugated Alexa-594 in combination with Vaccinia virus specific dig-labelled probes that were visualised using FITC conjugated anti-Dig immunolabelling. DAPI stain highlights DNA and the scale represents 10 μm.

Figure 3.2.3

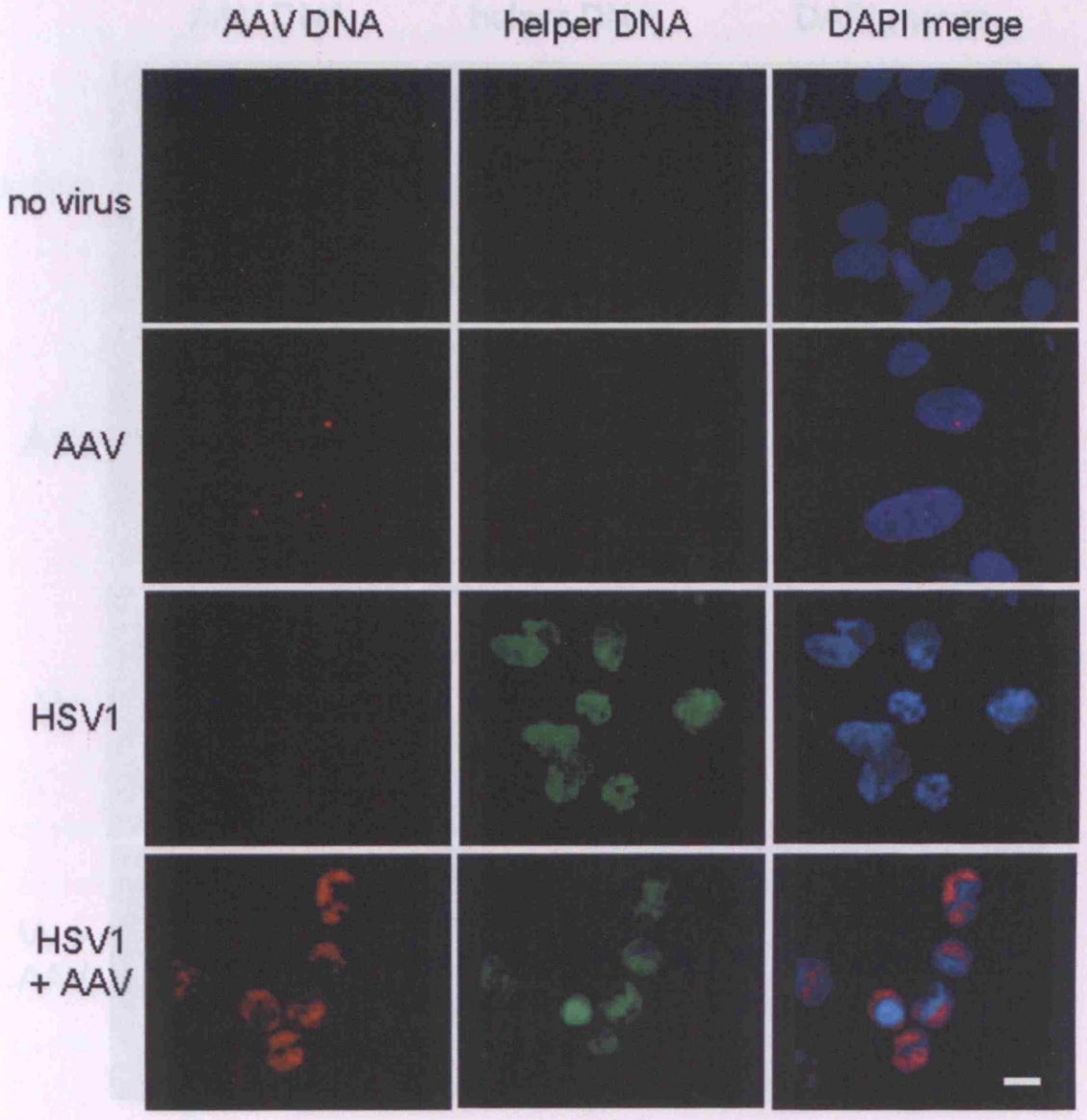


Figure 3.2.3. AAV DNA is not restricted to discrete foci in Herpes simplex virus (HSV) and AAV coinfections. U2OS cells were cultured on glass coverslips and infected with AAV (MOI 2000) alone or AAV (MOI 2000) and HSV (MOI 15) together. Cells were cultured for 24 hours and harvested and processed for *in situ* hybridisation. Biotinylated AAV probes were used to detect AAV DNA and detected using streptavidin conjugated Alexa-594 in combination with HSV specific dig-labelled probes that were visualised using FITC conjugated anti-Dig immunolabelling. DAPI stain highlights DNA and the scale represents 10 μm.

Figure 3.2.4

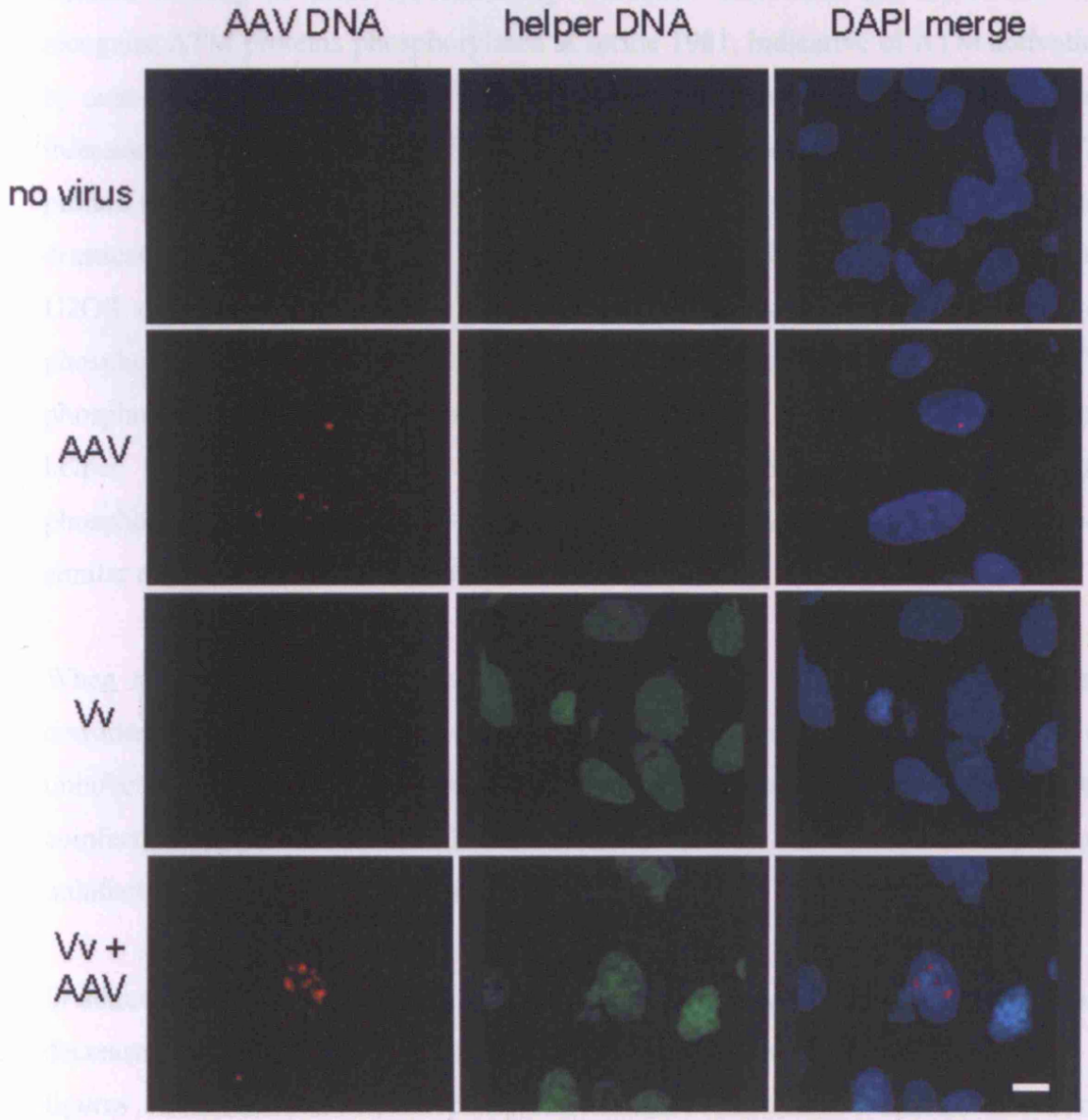


Figure 3.2.4. AAV DNA is not restricted to discrete foci in Vaccinia virus (Vv) and AAV coinfections. U2OS cells were cultured on glass coverslips and infected with AAV alone (MOI 2000) or AAV (MOI 2000) and Vv (MOI 15) together. Cells were cultured for 24 hours and harvested and processed for *in situ* hybridisation. Biotinylated AAV probes were used to detect AAV DNA and detected using streptavidin conjugated Alexa-594 in combination with Vv specific dig-labelled probes that were visualised using FITC conjugated anti-Dig immunolabelling. DAPI stain highlights DNA and the scale represents 10 μm.

Helper viruses decrease PIKK protein levels

We considered the levels of ATM, ATR and DNA-PK as well as the activation status of ATM (via serine 1981 phosphorylation) by western blotting. Whole cell lysates of AAV, Ad5, HSV1 or Vv infected cells were taken at 24 and 48 hours as well as coinfected cultures at the 48-hour time point. Figure 3.2.5 displays the result of western blotting of these lysates using anti-ATM antibodies and antibodies that recognise ATM proteins phosphorylated at serine 1981, indicative of ATM activation by auto-phosphorylation. Levels of ATM protein appeared considerably raised upon infection with AAV while coinfections did not show a dramatic increase of ATM protein and in the case of an HSV1 and AAV coinfection, the levels of ATM were drastically decreased below that of control, uninfected U2OS cells. AAV-infected U2OS cells showed significant activation of ATM as interpreted by levels of phosphorylation, though uninfected U2OS cells also appeared to exhibit ATM phosphorylation. While the reasons for this are not clear, it is important to note that helper virus-AAV coinfected cultures of Ad5 or Vv showed little if any phosphorylation of ATM. HSV-AAV coinfections appeared to activate ATM to a similar degree to that of AAV alone.

When we consider ATR (Figure 3.2.6) we observed that levels of ATR were considerably decreased in all coinfected cultures when compared with AAV alone or uninfected controls. Similarly, levels of DNA-PK were profoundly decreased by AAV coinfection with Ad5 or HSV1 when compared with AAV-infected U2OS and uninfected controls (Figure 3.2.6).

In summary we see that in the context of helper virus-AAV coinfections there is a decrease in at least one if not all of the PIKK protein levels or activation shown in figures 3.2.5 and 3.2.6 when compared to AAV infections alone or uninfected controls. This suggests that one unified way in which helper viruses may modulate the cellular environment to render it permissive for AAV replication is by manipulation of PIKK proteins and / or signalling.

Figure 3.2.5

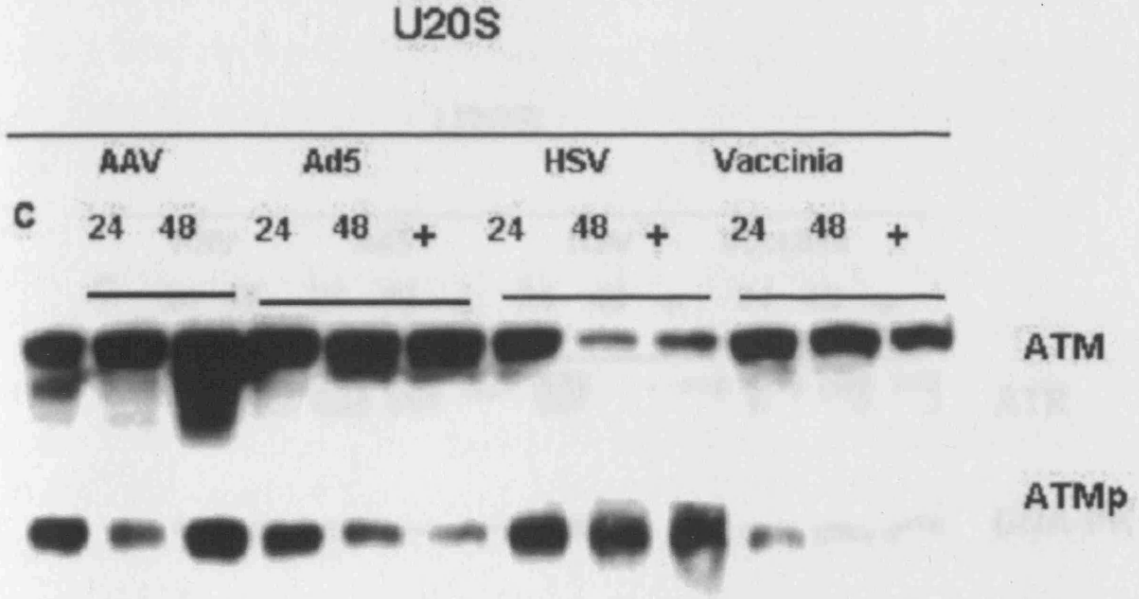


Figure 3.2.5 – Effect of AAV, Adenovirus, HSV, Vaccinia virus and helper virus-AAV coinfections on ATM and ATM phosphorylation of serine 1981 at 24 and 48 hours post infection. The first lane represents an uninfected control. Lanes labelled '+' denote 48 hour coinfections of the respective helper virus with AAV.

Figure 3.2.6

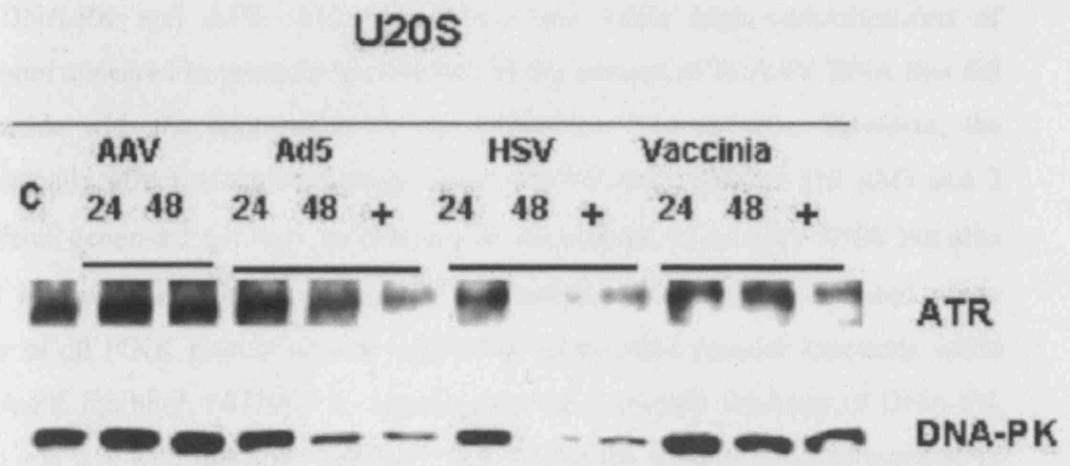


Figure 3.2.6 – Effect of AAV, Adenovirus, HSV, Vaccinia virus and helper virus-AAV coinfections on ATR and DNA-PK at 24 and 48 hours post infection. The first lane represents an uninfected control. Lanes labelled '+' denote 48 hour coinfections of respective helper virus with AAV.

Biochemical inhibition of PIKK protein function aids AAV replication

To establish whether ATM, ATR and DNA-PK signalling were repressors of AAV replication we chose to examine whether chemical inhibitors of their kinase activity would augment AAV replication. We used the chemical wortmannin, NU7027 (Calbiochem) a DNA-PK inhibitor, and caffeine, to inhibit a combination of activities (Figure 3.2.7). Wortmannin is used at the lower concentration of 20 μM to inhibit ATM and DNA-PK. At the higher concentration of 150 μM wortmannin inhibits ATM, DNA-PK and ATR (41). We found that while high concentrations of wortmannin appeared to promote an increase in the amount of ss AAV DNA this did not coincide with the appearance of any replicative intermediates. However, the physiologically effective concentration range of DNA-PK inhibitor (10 μM) and 2 μM caffeine generated not only an increase in the amount of ss AAV DNA but also resulted in the generation of replicative intermediates. Caffeine is a broad range inhibitor of all PIKK protein activity and other unspecified cellular functions while the DNA-PK inhibitor, NU7027 is considered to be a specific inhibitor of DNA-PK function and it is with this drug treatment that we see the most significant increase to the quantity of AAV DNA. The inclusion of DNA extracts from Ad5-AAV coinfections was shown as a positive control and appears significantly over-exposed since it was necessary to expose the other portions of the blot for considerably longer.

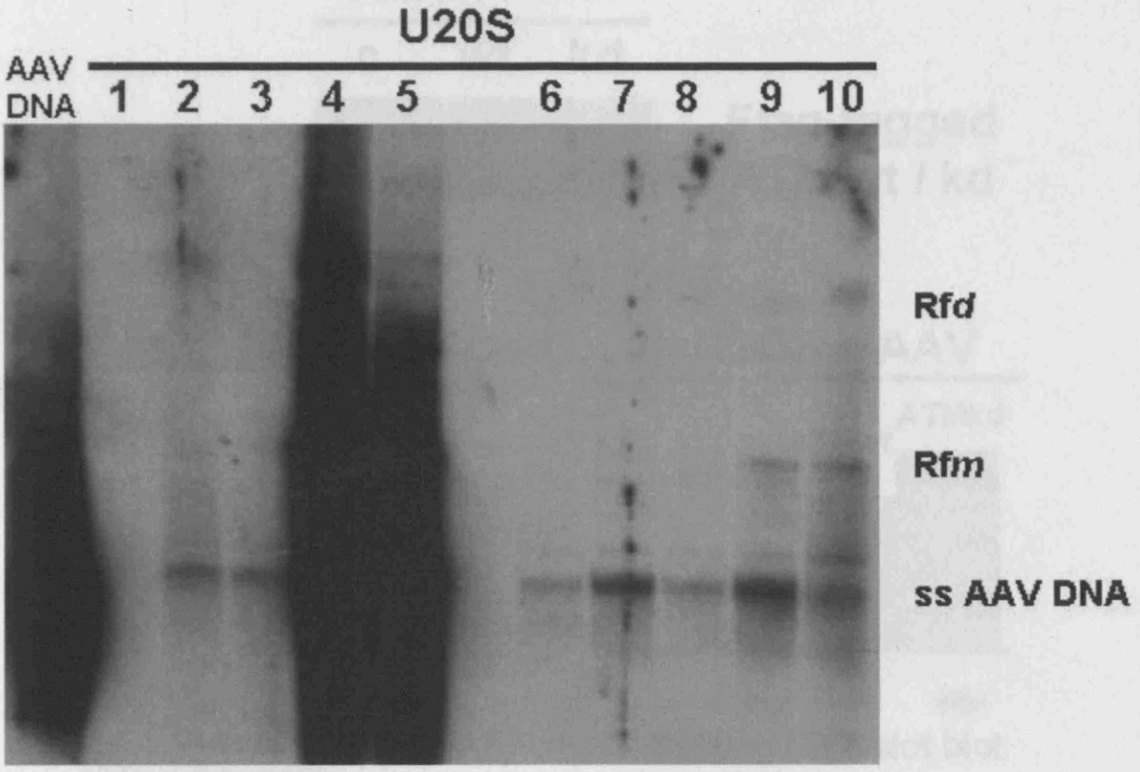
Molecular inhibition of PIKK protein function

While the biochemical inhibition of PIKK protein signalling, particularly the inhibition of DNA-PK appears to permit some replication of AAV we wanted to ensure that observed replication was not a consequence of drug-induced genotoxic stress for example. As such we sought to employ short interfering RNA (siRNA) and Tetracycline inducible systems to either silence expression or promote expression of kinase dead dominant-negative proteins respectively with the aim to inhibit ATM, ATR and DNA-PK function. Difficulty was encountered in terms of siRNA-mediated expression silencing of both ATM and ATR. Furthermore the expression of kinase dead ATM also required some optimisation and the results are far from satisfactory (figure 3.2.8A). While the results for siRNA mediated silencing of DNA-PK have proven more fruitful the results from these experiments remain preliminary and are only indicative.

Figure 3.2.8A demonstrates the expression of either flag-tagged wild-type ATM (ATMwt) or kinase dead ATM (ATMkd). Although the levels of the proteins were not as high as we had hoped for, these cells were infected with AAV and cultured for 48 hours. Total DNA extractions were performed and equal quantities of DNA were loaded onto either an agarose gel or slot blot apparatus. DNA was transferred to nylon membrane and radiolabelled anti-AAV probes were used to probe for AAV DNA species. Figure 3.2.8B shows the appearance of the monomeric replicative form of AAV DNA in the presence of ATMkd. This suggests that inhibition of ATM may help AAV replication. This is reinforced by slot-blot data in figure 3.2.8C. The slot blot image has been subject to phosphorimager analysis and the intensity of signal is plotted graphically. As demonstrated in the graph, there is some increase in AAV DNA in both ATM wt and kd expressing cells though a greater increase in the quantity of AAV DNA occurs in the presence of ATMkd protein. It is clear that ATMwt and ATMkd experiments require further optimisation and for the experiment to be repeated under more ideal conditions before any conclusions be drawn.

Figure 3.2.9A demonstrates the expression silencing of DNA-PK in U2OS cells. Cells in which siDNA-PK had been employed to reduce DNA-PK expression as well as siCONTROL transfected cells were infected with AAV. Cells were infected 24 hours post transfection and cultured for 48 hours. Total DNA extractions were performed and equal quantities of DNA were loaded onto agarose gels and transferred to nylon membrane for southern blotting as described above (Figure 3.2.9B). Southern blotting illustrates a considerable increase in quantities of the ss AAV DNA also with the appearance of monomeric replicative forms in siDNA-PK treated cells. This implies a significant role for DNA-PK in the inhibition of AAV replication.

Figure 3.2.7



1 mock control
2 AAV infection alone
3 DMSO AAV infection control
4 Ad5 (high m.o.i) + AAV
5 Ad5 (low m.o.I) + AAV
6 20 μM wortmannin + AAV
7 150 μM wortmannin + AAV
8 1 μM DNA-PK inhibitor + AAV
9 10 μM DNA-PK inhibitor + AAV
10 2 μM caffeine + AAV

Figure 3.2.7. Caffeine treatment and DNA-PK inhibitor of U2OS cells promotes AAV replication. Lane numbers represent treatments highlighted above. Cells were drug treated for 24 hours prior to AAV infection. Cells were cultured in the presence of AAV and drug for a further 48 hours. Total DNA extractions were perfomed and equal quantities of DNA loaded onto a 0.625% agarose gel. DNA was transferred to nylon membrane and subject to southern blotting with radiolabelled, anti-AAV probes. Single stranded AAV DNA (ssAAV DNA), monomeric replicative forms (RFm) and duplex replicative forms (RFd) are highlighted accordingly.

Figure 3.2.8

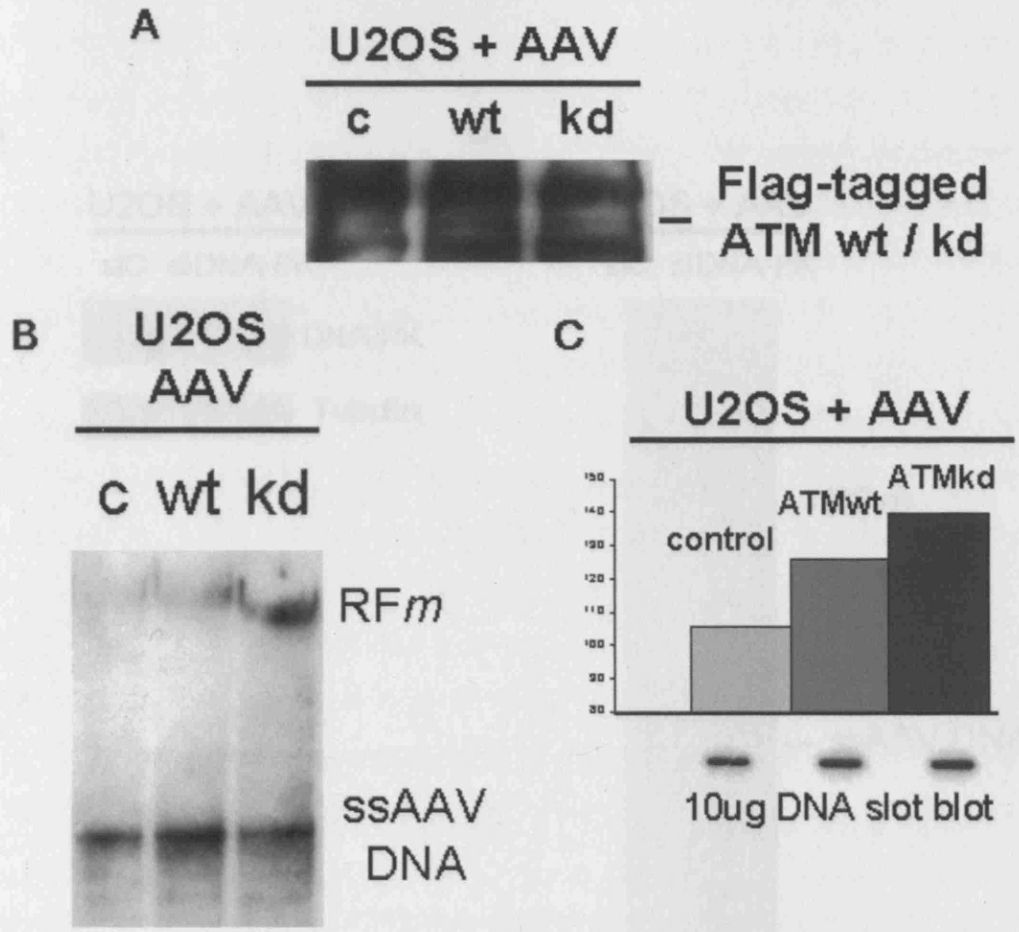


Figure 3.2.8. Expression of ATMkd in U2OS cells enhances replication of AAV. *(A)* U2OS cells were transfected with an empty expression vector (c) or a vector harbouring the wild-type ATM gene (ATMwt) or a kinase dead mutant ATM (ATMkd). One day post transfection, cells were infected with AAV and cultured for 48 hours. Half of the cells were lysed and subject to protein extraction and quantification; an equal quantity of protein was loaded onto each lane. Protein was transferred to nitrocellulose membrane and subject to western blotting with anti-FLAG antibodies. *(B)* The other half of cells were subject to total DNA extraction and an equal quantity of DNA was loaded onto each lane of a 0.625% agarose gel. DNA was transferred to nylon membrane and subject to southern blotting with a radiolabelled anti-AAV DNA probe. *(C)* In parallel equal quantities of DNA were loaded onto a slot blot and transferred to nylon membrane subject to southern blotting using a radiolabelled anti-AAV DNA probe. RF*m* indicates the monomeric replicative form and ssAAV DNA represents the single-stranded AAV DNA.

Figure 3.2.9

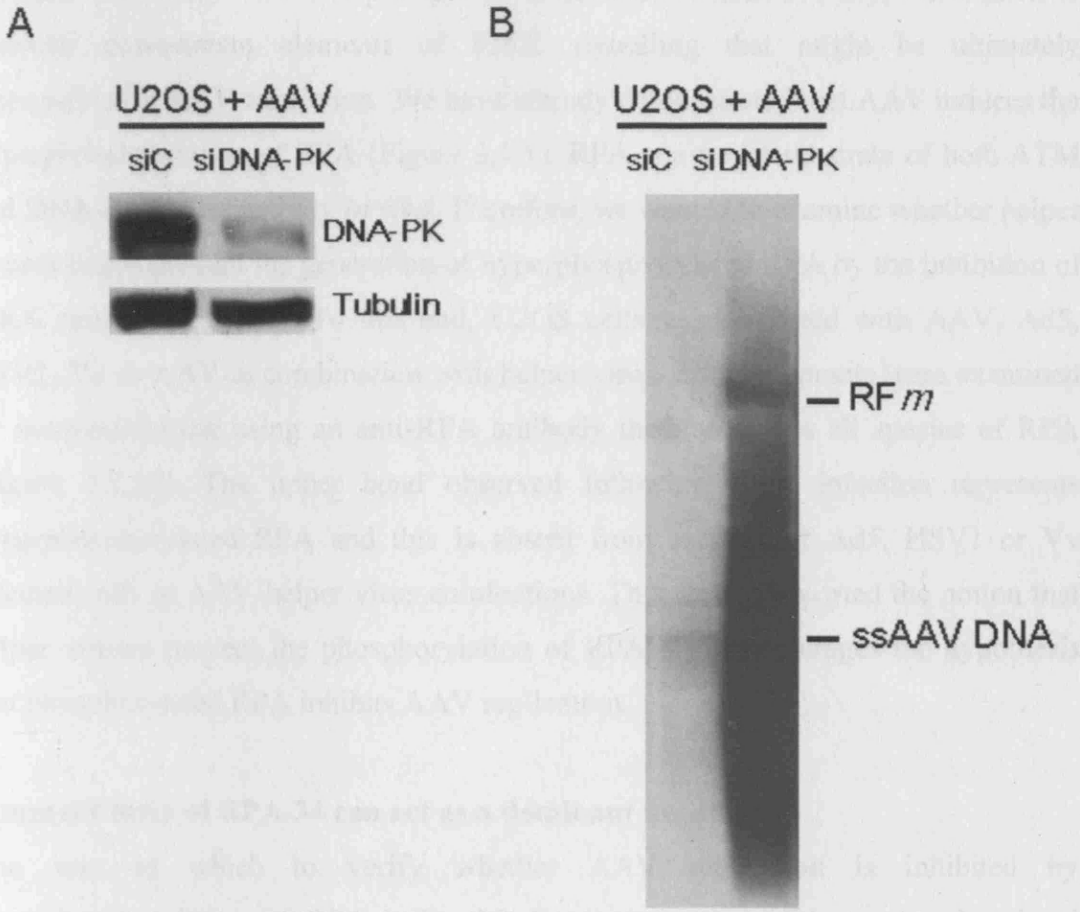


Figure 3.2.9. Expression of siDNA-PK in U2OS cells enhances replication of AAV. *(A)* U2OS cells were transfected with siCONTROL DNA or siRNA directed against DNA-PKcs. One day post transfection, cells were infected with AAV and cultured for 48 hours. Half of the cells were lysed and subject to protein extraction and quantification; an equal quantity of protein was loaded onto each lane. Protein was transferred to nitrocellulose membrane and subject to western blotting with anti-DNA-PK antibodies and tubulin to demonstrate equal loading. *(B)* The other half of cells were subject to total DNA extraction and an equal quantity of DNA was loaded onto each lane of a 0.625% agarose gel. DNA was transferred to nylon membrane and subject to southern blotting with a radiolabelled anti-AAV DNA probe. RF*m* indicates the monomeric replicative form and ssAAV DNA represents the single-stranded AAV DNA.

Helper viruses attenuate AAV-induced hyperphosphorylation of RPA

While data addressing the question of whether the PIKK proteins inhibit AAV replication remain preliminary, it appears to point in the direction DNA-PK and perhaps ATM as inhibitory factors. These initial experiments, both biochemical and molecular, pinpoint DNA-PK as being of particular importance. If helper viruses do facilitate AAV replication, in part by the inhibition of PIKK activity, we wanted to consider downstream elements of PIKK signalling that might be ultimately responsible for AAV restriction. We have already demonstrated that AAV induces the hyperphosphorylation of RPA (Figure 3.1.1). RPA is a target substrate of both ATM and DNA-PK kinase activity *in vivo.* Therefore, we wanted to examine whether helper viruses might prevent the generation of hyperphosphorylated RPA by the inhibition of PIKK protein signalling. To this end, U2OS cells were infected with AAV, Ad5, HSV1, Vv or AAV in combination with helper virus. Protein extracts were examined by immunoblotting using an anti-RPA antibody that recognises all species of RPA (Figure 3.2.10). The upper band observed following AAV infection represents hyperphosphorylated RPA and this is absent from extracts of Ad5, HSV1 or Vv infected cells or AAV-helper virus coinfections. This result supported the notion that helper viruses prevent the phosphorylation of RPA. This encourages the hypothesis that phosphorylated RPA inhibits AAV replication.

A mutant form of RPA-34 can act as a dominant negative

One way in which to verify whether AAV replication is inhibited by hyperphosphorylation of RPA is by the direct prevention of hyperphosphorylated RPA species' generation in the context of an AAV infection. AAV requires unphosphorylated RPA for replication and so it is not possible to simply prevent expression of RPA. Furthermore, RPA is absolutely required for cell survival also. RPA functions as a heterotrimeric complex and it is the N-terminus 32 kDa subunit that is particularly phosphorylated in response to DNA damage. To date there is no known dominant negative form of RPA that can inhibit RPA phosphorylation in cells. We wanted to know if expression of a mutant form of the 32 kDa subunit lacking the region of the N-terminus that is the substrate for phosphorylation would prevent the generation of the hyperphosphorylated RPA complex following DNA damage. As such we sought to express a mutant form to act in a dominant negative manner. In 2004 Binz et al. produced a series of RPA-32 mutants to express in bacteria and

analysed their DNA binding affinities (30). One such mutant, RPA32Δ33 lacked the phosphorylation domain but retained much of its DNA binding capabilities. As such we considered that this mutant might retain its ability to function in the context of the cellular RPA heterotrimer in terms of replication but may prevent the hyperphosphorylation of RPA in response to DNA damage. The mutant DNA sequence of RPA32 was amplified from the original construct using PCR. Restriction sites were added at either end to facilitate directional cloning into a mammalian expression vector. When expressed in U2OS cells, the protein was only tolerated at low levels. Cells were transfected with either 1 μg (+) or 2.5 μg (++) of RPA32Δ33 and then irradiated with 5 J/m^2 UV (Figure 3.2.11). The figure clearly demonstrates that following UV irradiation, control cells generated considerable levels of hyperphosphorylated RPA. However, expression of the RPA32Δ33 mutant protein prevented this. This suggests that this mutant can function as a dominant-negative *in vivo*, preventing the generation of hyperphosphorylated RPA in response to DNA damage. Furthermore, while cells irradiated with 5 J/m^2 UV appeared arrested or dying, the RPA32Δ33 continued to cycle. We must further verify if cells do indeed continue to replicate their DNA in the presence of DNA damage when expressing this mutant. If this is the case, this mutant will prove a powerful tool for examining the inhibitory role of hyperphosphorylated RPA separately from dephosphorylated RPA, which we know to be required for AAV replication (200). AAV infection of cells expressing such a mutant will allow us to determine the involvement of RPA phosphorylation in AAV replication.

Figure 3.2.10

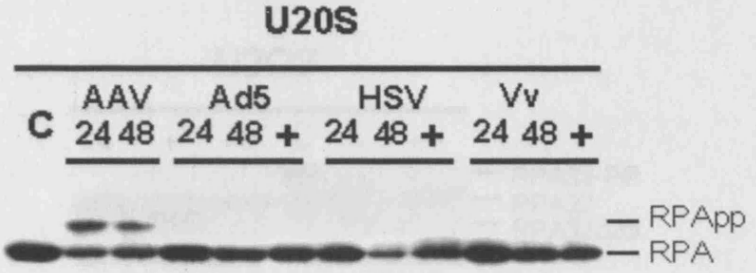


Figure 3.2.10. AAV infection results in the hyperphosphorylation of RPA protein. Cells were infected and harvested at 24 and 48 hours post infection and total protein extraction performed. Equal quantities of total protein were loaded onto each lane. The first lane represents an uninfected control. Lanes labelled '+' denote a 48 hour coinfection with AAV. The membrane was probed using an anti-RPA antibody that recognises all RPA species. RPApp denotes the hyperphosphorylated form of the protein.

Figure 3.2.11

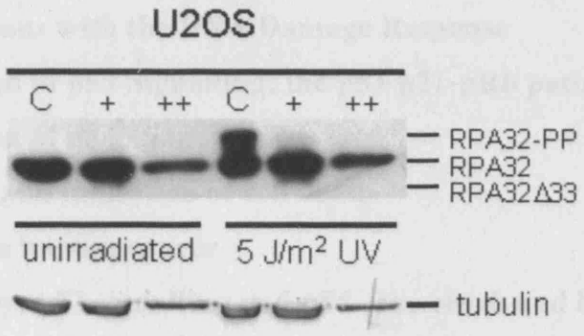


Figure 3.2.11. Transfection with RPA32Δ33 mutant prevents hyperphosphorylation of RPA in response to UV irradiation.
Western analysis of RPA. U2OS cells were transfected with an empty vector (c) or a vector harbouring the RPA32Δ33 mutant in two quantities, 1μg (+) or 2.5 μg (++). One-day post transfection cells were irradiated with 5J/m^2 UV or mock treated. Tubulin immunoblotting demonstrates loading of the protein.

Section 4 Discussion

Chapter 1 Cells lacking the p53-p21-pRb pathway are susceptible to AAV-induced cell death via p84N5

- **Viral Interactions with the DNA Damage Response**
- **AAV Activation of p53 Signalling; the p53-p21-pRb pathway**
- **AAV Activation of p84N5**
- **p53 signalling and inhibition of cell death**
- **p84N5's role in human cancer**
- **Cancer therapy: p53 signalling and p84 - the check and balance**

Viral Interactions with the DNA Damage Response

We are only just beginning to understand the complex cellular response that takes place in the event of DNA damage. Damage to the cellular DNA can take place as a consequence of both exogenous and endogenous events; from UV-induced DNA lesions to mistakes during replication. However, when we consider multicellular organisms we must consider that each cell takes responsibility for the fidelity with which it passes on its vital genetic information to daughter cells. To do this cells employ a complex array of signalling proteins and pathways to detect, repair and replicate their DNA. In the event that the faithful transfer of the cellular genome to progeny cells be jeopardised, these cells also have the potential to undergo programmed cell death. The strict coordination of these pathways is pivotal to normal life.

The cellular DNA damage response is carefully honed to be able to detect not simply DNA damage, but to distinguish between lesion types and respond with appropriate repair mechanisms. The complexity required to perform these functions whilst ensuring the accurate replication of the cellular genome is of course phenomenal. It is not surprising then that the presence of viral DNA and replication intermediates in the nucleus of a host cell would not go un-noticed. It has only been relatively recently that the interactions between viruses and cellular DNA damage responses have been truly appreciated. For some viruses, DNA damage responses appear to be detrimental and

as such, some viruses have evolved methods by which to evade such responses while conversely other viruses appear to benefit from DNA damage repair and signalling components. There are few reports of RNA virus interactions with DNA damage response though retroviruses, and particularly their integration steps have warranted several reports focusing on elements of DNA recombination machinery. However, there are numerous reports regarding DNA viruses and their interactions with DNA damage machinery. Adenovirus targets several components of the MRN complex for degradation since the MRN complex in conjunction with other components of the cellular DNA repair machinery recognise the linear, double-stranded DNA genome of the virus as broken DNA ends to be recombined (261). If Adenovirus did not produce proteins to manipulate the localisation and degradation of Mre11, newly replicated Adenovirus genomes would be processed by cellular repair factors leading to circularised concatemer molecules that cannot be packaged into viral genomes. As our knowledge and understanding of cellular DNA damage responses increases, so too does our recognition of viral interactions with them.

Unlike viruses whose interactions with cellular DNA damage responses may to some degree be seen as part of the viral lifecycle, the AAV-induced DNA damage responses that we discuss here do not involve viral proteins that arise from viral transcription or incoming viral particles (220). The ability of AAV DNA to induce G2 arrest, activate p53 signalling or induce p84N5-dependent apoptosis is entirely dependent upon the structure of the AAV DNA alone.

AAV DNA is a single-stranded structure with hairpin loop formation at either end as a consequence of palindromic repeat sequences (28). The AAV DNA enters the nucleus of a cell by a process that is not fully understood but requires viral uncoating and translocation through the nuclear envelope. Although the precise time frame within which the AAV DNA engages the cellular DNA damage machinery has not yet been investigated, it is clear that within 24 hours a DNA damage response is activated. An ATR and Chk2-mediated G2 arrest is observed within 24 hours (129, 220). This response coincides with an inhibition of cyclin B/cdc2 activity and the activation of p53 and its downstream targets including p21 and pRb as demonstrated here. When *in situ* hybridisation was employed to visualise AAV DNA within the nucleus during this period, the AAV DNA was seen to be present in foci that also contained RPA and

TopBP1 (Figure 3.1.1A and 3.1.1B). Since the formation of such foci and the hyperphosphorylation of RPA are both consistent with an activated DNA damage response, our observations imply that AAV DNA is the stimulus for such responses.

AAV Activation of p53 Signalling; the p53-p21-pRb pathway

The DNA damage-induced activation of p53 was one of the discoveries that prompted p53's christening as 'guardian of the genome' (Lane 1992). Many forms of cellular stress lead to the activation of p53, which is essentially determined by the measurable stabilisation of cellular levels of p53 and by the phosphorylation status of key residues of p53 protein. Activation of p53 in response to DNA damage occurs as a consequence of several routes of signalling, ATM and ATR signalling via Chk1 and Chk2 for example. In p53 serine 15 and serine 20 are considered key sites of ATM and ATR-dependent phosphorylation. Here we demonstrate that the AAV-induced DNA damage response leads to the activation of p53 as demonstrated by the increased phosphorylation of serine 15 (Figure 3.1.3). Since serine 15 phosphorylation of p53 is considered to be indicative of ATM and ATR activity this data reinforces previous observations that the AAV-induced G2 arrest is at least an ATR-mediated DNA damage response (129). The activation of p53 is crucial for the cell to survive this. Cells that lack p53 will also arrest at the G2/M transition but they do not emerge from this arrest. Instead they undergo apoptosis. Although activated p53 in these cells stimulates the expression of several of its target genes such as 14-3-3σ and p21, only p21 appears to be crucial for protecting cells from cell death (220).

There are several reports of p21 playing a protective role in response to DNA damage (286, 287). Protection from AAV-induced cell death by p21 may in part be a reflection of the role of p21 in maintaining G2 arrest (31, 276), Blagosklonny et al 2000). In the absence of p21, cells may still be capable of undergoing arrest but this arrest may not be sustained (36). Since arrest may be a period in which cells have the opportunity to repair DNA damage, p21's protective role may be to allow for DNA repair. In the absence of p21's ability to sustain G2/M phase arrest cells may enter into mitosis in the presence of DNA damage leading to mitotic catastrophe; an unprogrammed form of cell death (95). p21 may also exert its protective properties by preventing DNA replication in the light of DNA damage. This hypothesis stems from the observation that following DNA damage, cells without p21 may appear to

undergo cell cycle arrest at G2 but continue to synthesise their DNA without intervening mitosis leading to cells with abnormal DNA content (aneupolidy) (121, 286). In such cases cell death invariably occurs. This may be deemed as a consequence of mitotic catastrophe, as cells enter mitosis with DNA damage or genomic imbalance (43) or apoptosis in response to genomic instability (276). Indeed, the loss of p21 has been shown to sensitize tumours to radiation (300) and cisplatin cytotoxicity (311).

We observe a response induced by AAV that is very much reminiscent of the well-characterised p53-mediated signal transduction associated with DNA damage responses i.e. the induction of *p21* gene transcription. Initial experiments demonstrate the importance of p53 and p21 in preventing cell death following AAV infection. Although the apparent protective role of p53 in this context may be contrary to preconceived ideas regarding p53 activity, the demonstration that p21 may prevent cell death supports the protective role of p53 in response to DNA damage. However, it was also clear that neither p53 nor p21 alone were sufficient to protect cells from AAV-induced apoptosis. As such we examined the signalling components downstream of p53 and p21.

p21 inhibits CDKs and thus cell cycle progression. One of the fundamental targets of cyclin-CDK complexes is pRb (1). We demonstrate that AAV activates not only p53 leading to the increased expression of p21 but that this also leads to the activation of pRb. By inhibition of pRb expression in the context of functional p53 and p21 the data highlighted in figure 3.1.8 demonstrates that pRb is also required for the resistance of cells to AAV-induced cell death. While the activation of pRb is important in terms of restricting progression through the cell cycle, such activation may also function to suppress E2F from inducing apoptosis (65). However, in addition to pro-apoptotic targets of E2F transactivation, E2F also stimulates the transcription of repair proteins (224). As such it is unlikely that pRb exerts its protective role through E2F transcription inhibition. The ability of pRb to suppress cell death may be via its ability to inhibit the activity of c-Jun N-terminal kinase (JNK) (143). This infers a further role for p53 signalling as p53 itself has been shown to directly inhibit JNK-mediated cell death (165). In summary, while p53 signalling is a well-characterised in terms of induction of apoptosis, we know that several aspects

of p53-p21-pRb pathway may function in the protection against cell death in response to DNA damage. The importance of p53 signalling and more specifically the competent signal transduction that can lead to the activation (dephosphorylation) of pRb following the activation of p53 became clear with regard to preventing AAV-induced cell death. From this observation it was our objective to understand the mechanism of cell death induction and how this may be connected to p53 signalling to confer a protective role to this pro-apoptotic mediator.

AAV Activation of p84N5

The process of apoptosis in mammalian cells in mediated almost exclusively by two well-characterised pathways, the intrinsic and the extrinsic pathways. The fundamental difference is that the intrinsic pathway is activated by a stimulus from within the cell while the extrinsic pathway is activated in response to external stimuli. Despite their differences both pathways are carried out by defined groups of caspase proteins in distinct cascades. The family of caspase enzymes are cysteine proteases that facilitate the proteolytic cleavage of cellular proteins upon activation of an apoptotic programme (226) The intrinsic pathway is mediated by the mitochondria (289) and involves the activation of caspase 9 and subsequently a cascade of caspase activation that converges on the activation of caspase-3. However the binding of extracellular death ligands leads to the activation of the initiator caspase, caspase-8 then followed by the activation of the effector caspase, caspase-3 (167). In mammals the initiator caspases and effector caspases are grouped as follows, caspases-2, -8, -9, and 10 represent initiator caspases while caspases-3, -6 and –7 are known as the effector caspases. The initiator caspases are auto-activated leading to the activation of downstream effector caspases. Caspase activation coincides with the cleavage of pro-caspase proteins.

In our efforts to identify the apoptotic mediator of AAV-induced cell death, we examined caspase activation since, as described, the profile of caspase activation can be indicative of the nature of apoptotic cell death. Based on the identification of the activation of at least caspase-3 and –6 in response to AAV, and the demonstrated presiding importance of caspase-6, it was possible to refine the search for apoptotic mediator to a few significant points. The role of caspase-6 in terms of apoptosis is not

well defined. It remains unclear whether capsase-6 is activated prior to or as a consequence of caspase-3 activation. Our results suggested a role for caspease-6 that may not require caspase-3 and as such it allowed further refinement of the search for a the apoptotic mediator of AAV-induced cell death. The reports of caspase-6 activation and targets are few but of particular significance is the involvement of caspase-6 in cell death mediated by death effector domain (DED)-containing proteins (235). The DED is a protein/protein interaction domain found predominantly in proteins involved in apoptotic signalling. One such protein known as DEDD has been shown to localise to nucleoli substructures, activate caspase-6 and inhibit RNA polymerase I. We considered other such proteins, in particular the p84N5 protein.

The literature base for p84N5 is relatively small but there are several key observations that have been made regarding p84N5-induced apoptosis, which highlights a remarkable similarity to AAV-induced cell death. Here we show that AAV induces a G2 arrest, which precedes apoptosis and that apoptosis is associated with the activation of caspase-6. In the case of p84N5-induced cell death it was observed that prior to the onset of apoptosis, a G2/M phase cell-cycle checkpoint was also observed (76). p84N5 contains a region of significant similarity to the death domain region containing proteins that are involved in apoptotic signalling and indeed the ability of p84N5 to induce apoptosis is dependent upon the intact nature of such a death domain. Furthermore, direct evidence associates the activation of caspase-6 with p84N5-induced apoptosis (77). The process of p84N5-mediated apoptosis coincides with the cleavage of full-length p84N5. While the process of p84N5 cleavage has not been fully investigated it is hypothesised to be a consequence of caspase-dependent proteolysis by consideration of specific caspase cleavage sites (271).

However, one of the most profound connections, which allowed us to bridge the gap between the apparent protective role of p53 signalling and AAV-mediated cell death was the report that activated pRb is a potent inhibitor of p84N5's apoptotic activity (75). Upon further investigation we found that AAV does indeed activate p84N5 and furthermore in the absence of p84N5 activity, cells demonstrate resistance to AAV-induced cell death. As such the data supported a model in which two pathways are activated by the AAV-induced DNA damage response. The balance of these two pathways dictates life or death for the cell. The model details that upon AAV

infection, the AAV DNA is introduced to the nucleus. The cell recognises the AAV DNA as a DNA lesion and as such a DNA damage response takes place. Consequently at least two pathways are activated. Firstly, p53 activation takes place leading to the increased expression of p21, which in turn promotes the activation of pRb by dephosphorylation. In parallel a second signalling pathway is set in motion to activate the apoptotic activity of p84N5.

In cells in which the p53-p21-pRb signalling pathway is complete, this stream of signalling causes transient cell cycle arrest and p84N5 activation is prevented by hypophosphorylated pRb. However, in the absence of functional p53 signalling that would normally result in the activation of pRb, p84N5 activation is unrestrained and as such is able to initiate an apoptotic cascade that leads to the activation of caspase-6 and subsequently cell death. However it must be highlighted that there are certain unresolved issues that remain to be addressed. These include a full analysis of all key caspases and their potential role in the cell death observed here. Furthermore a thorough analysis of the phosphorylation status of pRb in Saos cells and the ability of activated pRb to inhibit p84N5 has not been addressed here but rather in previous work by other groups (75). In the interest of completion it would be beneficial to more thoroughally address these issues in accompaniment to the data presented here.

Furthermore, while UV-AAV was used for the experiments described above to prevent viral transcription it would be interesting to consider whether a artificially manufactured non-UV irradiated hairpin DNA or similar structure to the AAV genome lacking coding regions may produce a similar effect.

p53 signalling and inhibition of cell death

Cellular stress such as DNA damage can lead to genetic changes, which by their very nature will be passed on from generation to generation of the cell. This is the very foundation upon which cancer builds; the cumulative amass of mutations. As such it is pivotal that 'incidents' that could generate mutation are dealt with at the point when still detectable i.e. at the point of DNA damage. This is the fundamental role of p53 as a tumour suppressor, to respond to DNA damage and deal with it accordingly. In the light of potential damage to the cellular genome it is without a doubt that the safest option is to remove the potentially compromised cell by inducing cell death, thus

protecting the whole organism in the long-term. This is the long-standing, well-documented pro-apoptotic activity of p53. Experimental evidence supports the notion of p53's over-riding pro-apoptotic propensity by observing the outcome of drug-treatment or radiation-induced lesion generation. It is without a doubt that such treatments yield multiple lesions of various types to the cellular genome. In these instances p53 activation leads to cell death, but what is the outcome of p53 activation when levels of damage are low? Can p53 modulate its activity to accommodate varying degrees of damage and thus risk? The possibility that this may be the case has recently emerged and there is increasing evidence that p53 may promote cell survival in the light of DNA damage. Our data support such a notion in which p53 signalling may prevent apoptosis. It is important to appreciate the complex modulation of life and death that p53 co-ordinates. p53's role as a pivot between the two warrants more thorough investigation. To do so, it is likely that we will need to refine our methods for inducing a DNA damage response. As such AAV represents an interesting tool since introducing AAV DNA as the substrate for a DNA damage response does not cause damage to the cellular genome in any way and can be titrated to introduce a approximately defined number of 'lesions'. As a tool to generate a DNA damage response, AAV can be more easily controlled within an experimental context.

The outcome of p53 signalling is likely to be dependent on the balance of transcription-dependent and more controversially, the transcription-independent roles of p53. Various groups have considered the role of p53 transcription in determining the outcome of DNA damage signalling in terms of survival versus apoptosis. Speidel et al 2006 considered the up-regulation of pro-apoptotic mediators such as Bax, PIG3 and PUMA as well as anti-apoptotic targets upon p53 activation. They found that within the scope of their study, the transcriptional targets of p53 did not change regardless of dose of damaging agent but that the outcome was swayed towards apoptosis by events independent of p53 transcription. Their work and that of others in which the role of p21 in terms of cell survival has been further considered support the concept that p53 activation sets a balanced stage that pivots the outcome for cell mortality between survival and death. The non-transcription roles of p53 may also contribute to the balance between life and death. For instance p53 has been shown to induce apoptosis independently from its ability to affect gene expression. It is reported that p53 is able to localise to the mitochondria and directly bind apoptotic

inhibitors and as such may be actively involved in apoptotic signalling from the mitochondria (173). Alternatively, p53 may harbour direct roles in DNA repair via inherent exonuclease activity (194) and an ability to bind damaged DNA in a manner distinct from its transactivational DNA binding activity (146, 221, 299).

While both the transcriptional and non-transcriptional roles of p53 may determine the outcome of DNA damage responses there is clear evidence for cell type specificity in determining the outcome of p53 signalling. For example it has been observed that p53 is not induced to the same degree between tissue types and furthermore even when p53 is stabilised to the same degree between two different cell types the levels of cell death may not be the same between the two (168). Such tissue specific responses to p53 is considered to be in part, due to differences in expression of downstream factors involved in p53's regulation of cell fate (32). There is also evidence for tissue-specific expression of p53 inhibitors that may also manipulate signalling toward apoptosis in certain tissues (231).

Cell-cycle arrest in response to DNA damage is considered an opportunity to repair DNA damage and poses a mechanism by which p53 may be actively participating in promoting cell survival. Such a notion is compounded by observations that p53-dependent expression of genes involved in DNA repair such as GADD45 and ERCC3 (86, 252), may promote cell survival via repair in response to DNA damage (251). The concept that p53's ability to induce cell-cycle arrest is a method by which p53 can encourage cell survival is supported by the work presented here in which we demonstrate that it is the p21-inducing activity of p53 that actually prevents apoptosis by p84N5.

p84N5's role in human cancer

As described earlier, the literature available regarding p84N5 is somewhat limited. However one might expect that this may change as we begin to understand more thoroughly the links between DNA damage and apoptosis. Making such connections can often be hindered by variations in nomenclature. p84N5 is also known as Thoc1 or Hpr1, whose role in transcription has been extensively investigated. As might be suggested by the inconsistency of identification, the two roles of p84N5 as both

apoptotic mediator and alternatively as RNA processing factor have thus far been considered rather independently. However, it is likely that these two roles are linked and perhaps p84N5 is pivotal in integrating signalling between the process of transcription and the DNA damage surveillance machinery. The following section will discuss the current evidence for such a premise and highlight the role that such a hypothesis may generate for p84N5 in terms of tumorigenesis.

Thoc1 / Hpr1 or p84N5 as we shall continue to refer to it here is part of the evolutionarily conserved RNA TREX (Transcription/Export) complex. In yeast, the TREX complex links transcription with messenger ribonucleoprotein biogenesis, RNA processing and RNA export for a subset of genes (152). The TREX complex is required to aid the export of spliced mRNA from the nucleus where maturation takes place, to the cytoplasm to allow mRNA translation (49). DNA damage causes DNA lesions that stall not only DNA replication but also RNA transcription. Blockage to transcription elongation has been shown to activate p53 in an ATR and RPA-dependent manner (69). Such studies suggest that ATR and RPA may also be recruited to regions of stalled RNA polymerase II, facilitating a transcription stress response (163). Although initially conceptual, the idea that the transcription machinery may be involved in DNA damage surveillance and the suppression of mutagenesis is proving to be new chapter in our understanding of DNA damage recognition. Indeed, the data presented here very pointedly highlights an area in which DNA damage responses and transcription control mechanisms overlap. In terms of the dual role of p84N5 one might consider the ability of p84N5 to function in terms of both RNA processing and apoptotic signalling as a function of the cleavage status of p84N5. For example, it may be that cleaved p84N5 demonstrates different protein interaction properties that mediate apoptotic function as oppose to RNA processing. Full-length p84N5's presence in an RNA-processing complex may function in a similar way to ATR's presence at the replication fork. In the absence of DNA damage, full-length p84N5 aids in transcription and RNA export. Upon encountering a DNA lesion that stalls RNA polymerase, p84N5 may undergo post-translational modification, such as cleavage. As such p84N5 may no-longer function in RNA processing mechanisms but rather as an apoptotic mediator. The ability of only dephosphorylated pRb to prevent p84N5-mediated apoptosis suggests that p84N5 may be primed for apoptotic activity during S phase, monitoring replication and DNA

integrity through transcription The suggestion that transcription might act as a DNA damage dosimeter (164) is an interesting hypothesis and perhaps relies on elements of the transcriptional machinery recognising DNA damage and signalling to DNA-damage response pathways. The identification here of a pro-apoptotic factor, p84N5, that integrates transcription control via the TREX complex with DNA damage signalling via p53 represents a candidate mediator of such function. Particularly since we show here that functional p53 signalling is required to prevent apoptosis via p84N5.

Such a role for p53 is certainly contrary to the dogma of p53 function, but as our appreciation for the 'fine-tuning' of responses to DNA damage evolves so must our perception of the elements involved in executing such function. The role of p53 is of course pivotal to maintaining genome integrity, without which cancer is invariably the outcome. Perhaps this intermediary role of p84N5 provides a mechanism by which mutagenesis can be avoided when principle 'guardians' of integrity fail i.e. in the absence of functional p53. Particularly if we consider the role of tumour genotype-dependent therapy and the fact that p53-/- tumours can be rather resistant to treatment when many of our chemotherapeutic agents rely heavily upon the competency of the DNA damage response machinery. The proposition that moving our chemotherapeutic focus toward the transcription machinery may provide alternative, clinically relevant solutions has already been made (68). Indeed, the increased efficacy of certain treatments that also inhibit transcription has been observed but specific targets have not been thoroughly examined. Should this be the case, p84N5 and other members of the transcription regulatory machinery may find themselves in the spotlight for cancer therapeutics.

The status of p84N5 in human cancers has only recently been considered. One study has linked the human TREX complex to breast carcinoma (107). The work suggests that increased p84N5 expression is linked to an increase in tumour size, progression and metastatic state. The authors demonstrated that siRNA-mediated silencing of p84N5 abrogates nuclear export of mRNA and decreases cellular proliferation. Such a result is not entirely unexpected since growth and proliferation are intrinsically linked to a requirement for protein production and thus efficient transcription and translation, presumably facilitated by the TREX complex and thus p84N5. Perhaps an increase in

p84N5 expression reflects the disrupted growth pattern inherent in a tumour and its microenvironment or perhaps it is causally related to tumorigenesis.

To begin to address the question of how p84N5 may contribute to a cancer state, the requirements for p84N5 by isogenic normal cells and oncogene-transformed cells has been compared (151). The data delineates increased apoptosis of neoplastic cells depleted of p84N5 when compared to normal cells. Interestingly, normal cells become resistant to transformation upon depletion of p84N5. Such data suggests that cancer cells not only require the increased expression of p84N5 for survival but also that p84N5 be required for the very initial stages of transformation.

Our data suggests that upon activation of a DNA damage response induced by AAV, the levels of p84N5 appear to rise (Figure 3.1.12). Perhaps this is akin to the stabilisation of p53 protein upon activation of a DNA damage response. As such it may be the case that the increased expression of p84N5 in metastatic breast carcinoma cells reflects the presence of DNA damage signalling. The activation of DNA damage signalling in early tumorigenesis is a recently highlighted phenomenon (20, 104, 283) thought to act as an early barrier to tumorigesis. It is likely to be activated by aberrant DNA replication or the attenuated to repair DNA lesions that may occur through replication. It is of course important to understand the dynamics of expression of proteins such as p84N5 whose expression appears to be increased in response to tumorigenesis. In doing so we will have a clearer picture of how to approach manipulation of p84N5 or integrated pathways in terms of beneficial therapeutic techniques.

Cancer therapy: p53 signalling and p84 - the check and balance

The data presented here describes a check and balance system between p53 signalling and p84N5's pro-apoptotic activation that directly demonstrates a mechanism in which p53's induction of cell cycle checkpoint activators can prevent apoptosis. One might speculate that this mechanism prevails when damage signalling is low and thus additional signals are not present to sway the balance in favour of apoptosis by alternative mechanisms. This reinforces the view of p53 as pivot and provides an added dimension for understanding how p53 may orchestrate its role as 'guardian of the genome'.

Furthermore, understanding the role of p84N5 as a member of the DNA damage surveillance pathways and its involvement in response to damage signalling will undoubtedly provide insight into the somewhat undeveloped area of how transcription, RNA processing and translation machinery are incorporated in the cell to survey and preserve genome integrity.

Our observations may not have been possible if it were not for the subtle way in which AAV can generate DNA damage signalling in a cell without causing DNA damage and without modulating the emerging DNA damage response. These observations are important in that they may pave the way for the generation of drugs that specifically target the activation of p84N5, particularly in the context of tumours lacking functional p53 or pRb. These observations may also provide some insight into the frequently observed increase in apoptosis of cells lacking p53, p21 or pRb, the principle that underlies many of the current chemotherapeutic agents (48). However, the status of p53 protein is rarely considered in terms of therapy administration. Perhaps this is because of the unclear outcome of p53 signalling in response to DNA damage in terms of tissue context and overlapping pro-apoptotic and pro-survival roles as discussed above. The many instances in which the absence of p53 may sensitise cells to cell death may complicate the clinical relevance of p53 status in terms of cancer therapeutic intervention (45, 92, 113, 182, 220, 302, 308). However a clearer appreciation of how p53 can protect cells from cell death may provide more appropriate therapeutic targets that could circumvent our reliance on p53 activity for therapeutic efficacy.

The development in our understanding of how AAV can selectively mediate the death of cells that lack functional p53-p21-pRb signalling may also add some appeal to the pursuit of developing AAV as a delivery system for a DNA damage response in cancer therapy directly. The pursuit of oncolytic viruses once had considerable momentum until certain tragic events lead to shunning of such avenues by many of the large pharmaceutical companies (180). The principle of oncolytic viruses lies in the ability of the engineered virus to replicate only in cancer cells leading to cell death of the infected, cancerous cell. The benefits of such a therapy theoretically lie in the transmissibility and selectivity of the virus and lysis. Viruses that have been

engineered for use in such a way include Adenoviruses and Vaccinia virus derivatives. These viruses are commonly recognised as pathogenic entities and the reservations regarding their use in human cancer therapeutics are reasonable. However, the potential use of AAV's DNA damage inducing potential and selective killing of p53-p21-pRb defective cells has not been considered. The potential uses of AAV in terms of oncolytic therapy will be considered more in the following chapter.

Chapter 2 AAV-induced DNA damage signalling as a potential factor restricting autonomous replication of AAV

- **Helper virus-mediated AAV replication and PIKK protein disruption**
- **Hyperphosphorylated RPA as a key inhibitor of viral replication**
- **Viral restriction and oncolytic therapy**

Helper virus-mediated AAV replication and PIKK protein disruption

It has long been recognised that AAV replication requires coinfection with a helper virus to augment replication. While there are specific helper viral proteins that are considered required for this, there appears to be no common mechanism that lends help or rather, the cellular restriction factors that ultimately inhibit AAV replication have not been identified. The autonomous Parvoviruses must wait for cells to enter S phase when the cellular factors required by the viruses for replication become available. It is clear that among the necessary helper virus functions the ability of proteins such as Adenovirus E1A to push cells into S phase, providing cellular replication factors does indeed 'help' AAV. However, the question remains, what prevents AAV from replicating as the autonomous Parvoviruses during the cells' S phase, without a helper virus?

AAV DNA is very quickly recognised by the cellular DNA damage machinery. As one might expect, the single-stranded DNA-binding protein RPA binds to the AAV genome but most importantly appears in the hyperphosphorylated form. RPA is a heterotrimeric protein consisting of three subunits, RPA70, 32 and 14. Phosphorylation of the 32 kDa subunit occurs in a cell cycle dependent manner as a consequence of CDK activity (72). RPA phosphorylation correlates with the G1/S transition, replication and M phase and appears dephosphorylated at the end of M phase. Additional sites within the N-terminus of the 32 kDa subunit are phosphorylated following DNA damage to generate hyperphosphorylated RPA. RPA is a substrate for DNA-damage dependent phosphorylation by the PIKK proteins; the family which includes ATM, ATR and DNA-PK, all of which have been shown to phosphorylate RPA *in vitro* and/ or *in vivo* (30). Hyperphosphorylation appears to be involved in the signalling of DNA damage and recruitment of other repair factors. Indeed RPA has been implicated in the activation of ATR (320) and interacts with several cellular proteins including p53, BRCA1 and nucleolin (54, 83, 290). As such

hyperphosphorylation can play a role in the direction of DNA-damage induced foci formation. We show directly that both AAV DNA and hyperphosphorylated RPA colocalise and we also demonstrate the colocalisation of TopBP1 to RPA in similar foci following AAV infection (Figures 3.1.1A and 3.1.1B). The DNA damage related proteins that associate with AAV DNA in infected cells has been further characterised to also include DNA polymerase delta, ATR and the 9/1/1 complex (129).

In non-permissive cells AAV DNA appears restricted to punctate intranuclear foci and we wanted to consider if such foci formation was a contributing factor to inhibition of AAV replication. When we examined permissive helper virus-AAV coinfected cells, AAV DNA did not appear restricted to discrete foci and the number of cells exhibiting such foci was significantly reduced, even in cells that did not exhibit a significant increase in the quantity of AAV DNA. Since PIKK proteins are pivotal in the generation of hyperphosphorylated RPA, and also are members of DNA damage-induced foci we considered whether the helper viruses manipulated ATM, ATR and DNA-PK levels or the activation of ATM. Adenovirus has previously been shown to redistribute and degrade components of the MRN complex and very recently such degradation has been implicated in helping replication and transduction of recombinant AAV (238). Furthermore the Herpes virus protein ICP0 has been shown to decrease the expression of the catalytic subunit of DNA-PK: DNA-PKcs (147). As such it seemed reasonable to examine whether Ad5, HSV1 and Vv affect the levels of the PIKK proteins. We found that infection with either of these very different helper viruses had some impact on a combination of ATM, ATR or DNA-PK levels and the activation of ATM.

These observations reinforced the hypothesis that PIKK proteins may restrict AAV replication. As such we undertook a series of biochemical studies, the result of which highlighted the importance of DNA-PK in particular. Whilst in the process of verifying these observation using direct molecular approaches, the involvement of DNA-PK in inhibiting AAV replication has been further emphasised. The observation that HSV1, in these studies, demonstrates the most profound augmentation of AAV replication may be a reflection of the ability of ICP0 to degrade DNA-PK specifically. DNA-PK has been shown to be involved in AAV genome processing and viral

persistence (53). Choi *et al* found that when using self-complimentary recombinant AAV (scAAV), the activity of ATM and DNA-PKcs was required for efficient conversion of the genome into a double-stranded circular form in non-dividing cells (53). Perhaps the ATM and DNA-PK enhanced circularisation activity inhibits AAV genome amplification or perhaps promotes genome integration. A focus of further work will be to examine these possibilities more thoroughly.

Hyperphosphorylated RPA as a key inhibitor of viral replication

We observed that all of the helper viruses examined in this study promoted a decrease in levels of a combination of PIKK family protein or activation. We questioned whether helper virus disruption of PIKK proteins ultimately prevents the hyperphosphorylation of RPA and that hyperphosphorylated RPA may be the key element in restricting AAV replication and indeed a restriction factor that helper viruses have evolved to overcome. Like cellular DNA replication, RPA is required for AAV replication, but it is conceivable that hyperphosphorylated RPA may be restrictive since hyperphosphorylated species function less efficiently in DNA replication and instead promote repair and recombination events (206). We examined the phosphorylated species of RPA generated in response to AAV infection as oppose to helper virus infection or coinfection. We found that hyperphosphorylated RPA was generated in response to AAV infection (reiterating the immunofluorescence data already shown) but not in response to Ad5, HSV or Vv infection or helper virus-AAV coinfections. This lends gravity to the proposition that helper viruses prevent the generation of hyperphosphorylated forms of RPA and that this may represent a restriction factor for AAV replication. We have generated a novel system for effective inhibition of hyperphosphorylated RPA in response to DNA damage whilst retaining the replicative functions of RPA. This tool will prove crucial in ultimately answering the question of whether hyperphosphorylated RPA inhibits AAV replication.

The possibility of a dual role for DNA-PK and RPA in terms of AAV restriction must not be dismissed. RPA has been also shown to interact with key members of the non-homologous end-joining machinery including DNA-PK. RPA dissociates from DNA-PK in the event of DNA damage (241). It is thought that RPA may remain bound to single strand DNA, such as that generated during replication, and that encountering a double strand break may activate DNA-PK resulting in phosphorylation of RPA

leading to dissociation of RPA from the Ku-DNA-PKcs complex, perhaps promoting non-homologous end-joining. AAV DNA may present an ideal substrate for the activation of DNA-PK and thus the hyperphosphorylation of RPA since it is both a single-stranded hairpin loop with a double-stranded portion. Furthermore, since AAV DNA is almost invariably processed into a double stranded form even in the absence of significant replication, blunt double-strand break-like structures may also represent a substrate for the activation of DNA-PK. As such the activation of DNA-PK may simultaneously lead to detrimental viral processing and the generation of RPA species that are not beneficial to DNA replication.

Viral restriction and oncolytic therapy

Understanding the factors that restrict AAV replication may seem to be interesting from a purely academic viewpoint. However, when we consider the potential for oncolytic therapy in cancer treatment one might envisage how the two chapters discussed here may intertwine. On one hand we have presented a mechanism by which a non-pathogenic virus can cause a DNA-damage response that culminates in death of cells that, like many cancer cells, harbour defects in the p53-p21-pRb pathway. On the other hand we discuss factors that restrict that same virus from replicating. When we consider that one of the fundamental stumbling blocks to cancer therapy and particularly oncolytic cancer therapy is transmission and penetration within a target tissue it is clear that wild-type AAV could not function as a transmissible entity (this issue is quite separate from the potential uses of recombinant AAVs in gene therapy). However, in terms of tissue penetration it has been shown that AAV may be able to penetrate a solid tissue mass considerably more efficiently than Adenovirus for example (87). This is likely a consequence of the size of AAV, one of the smallest DNA viruses reported.

However, if AAV could also replicate to some degree, and as demonstrated here, selectively induce cell death in cancer cells, AAV may in this way prove a novel tool for penetrating and destroying cancerous tissue. Our current experiments will clarify the role of both DNA-PK and / or hyperphosphorylated RPA in terms of inhibition of AAV replication. There are numerous DNA-PK inhibitors available that inhibit DNA-PK activity specifically with considerable efficacy (see Calbiochem). The implication

of their use to enhance AAV replication may be translated to the concept of their restricted use in the locale of cancerous cells in combination with AAV infection. Alternatively, the inhibition of hyperphosphorylation of RPA may yield a more potent augmentation of AAV replication. Experiments conducted in the course of this work have shown that very low levels of caffeine can be used to inhibit the hyperphosphorylation of RPA in response to DNA damage (2 μM) while higher concentrations appear to actually cause hyperphosphorylation, perhaps as a cause of replicative stress (Figure 4.2.1). Caffeine inhibition of RPA hyperphosphorylation most likely occurs as a consequence of inhibition of PIKK kinase activity. Low-level caffeine treatment to facilitate AAV replication may be of little detrimental physiological consequence. This combined with the restricted replication of a non-pathogenic virus, transporting a DNA damage response to selectively kill cancerous cells via p84N5 may be an intriguing avenue to pursue in the fields of oncolytic therapy.

Our data thus far also highlights the potential role of PIKK signalling and / or the hyperphosphorylation of RPA in inhibition of viruses such as Adenoviruses, Herpes viruses and the Pox family virus, Vaccinia virus. These viruses remain of pathological significance and so perhaps exploring the mechanisms by which these viruses may overcome inhibition by PIKK proteins or hyperphosphorylated RPA may provide novel anti-viral targets. Furthermore, several derivates of Adenovirus for example have already been generated for use in oncolytic therapy. Perhaps caffeine treatment or DNA-PK inhibitors for example would be useful adjuvants along side such therapy to increase treatment efficacy.

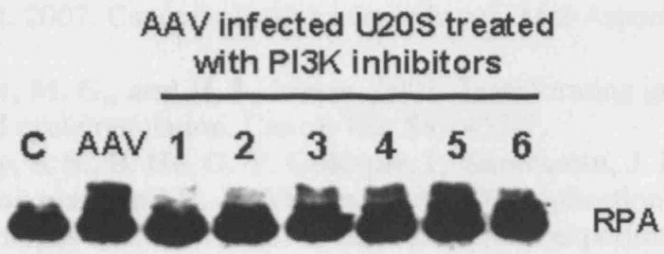


Figure 4.2.1. AAV causes the hyperphosphorylation of RPA, which can be inhibited using 2mM caffeine. The first lane to the left is an uninfected control. The adjacent lane shows the hyperphosphorylation of RPA that occurs following AAV infection. The following lanes were treated as follows with PI3K inhibitor drugs for 3 hours prior to infection.
Lane 1 – 2mM caffeine. ***Lane 2*** – 4mM caffeine. ***Lane 3*** – 6mM caffeine. ***Lane 4*** – 4mM caffeine + 30μM wortmannin. ***Lane 5*** - 4mM caffeine + 10 μM DNA-PK inhibitor. ***Lane 6*** 4mM caffeine + 30μM wortmannin + 10 μM DNA-PK inhibitor.

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Appendix I

Research Article

Cells with Defective p53-p21-pRb Pathway Are Susceptible to Apoptosis Induced by p84N5 via Caspase-6

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