

E2F1 induction following DNA-damage and oncogene activation

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Abstract

The transcription factor E2F1, a critical target of the tumour suppressor pRb, is deregulated in most human cancers. Oncogenes have been shown to deregulate E2F1 through inhibition of pRb and deregulation of E2F1 is an event that occurs in most human cancers. The essential role of E2F1 in apoptosis is well documented and deregulated E2F1 can enhance drug induced death. E2F1 is induced by various chemotherapeutic drugs and this induction, in addition with oncogenic stress, contributes to increased chemosensitivity.

Cells expressing the adenovirus early region 1A (*E1A*) oncogene have been used as a tool to identify cellular regulatory pathways that modulate chemosensitivity. E1A sensitises cells to the induction of apoptosis by diverse stimuli, including many chemotherapeutic drugs. These E1A activities are mediated through binding the RB family proteins (pRb, p107 and p130) and via the E1A N-terminal domain that interacts with different cellular protein complexes including the p300/CBP transcriptional activator and p400/TRRAP chromatin-remodeling complex.

The results presented here illustrate novel mechanisms of E2F1 induction both by oncogenes and chemotherapeutic drugs. Two minimal domains of E2F1 are described that are induced following DNA damage via mechanism(s) not previously identified. In addition, data are presented which show that E1A expression not only deregulates E2F1, but also elevates E2F1 levels. E1A is dependent on interaction with RB protein to induce E2F1 levels and this elevation contributes to cell death. Using previously described protein binding deficient truncations of E1A, we demonstrate that E1A binding to the p400/TRRAP protein complex is also critical for the induction of E2F1. E1A binding to p400/TRRAP was also critical in sensitizing these cells to drug induced apoptosis. Suppression of p400 using siRNA had similar affect on E2F1 induction and caused an increase in drug sensitivity indicating that E1A inhibits p400 function.

These results contribute to the understanding of how activation of the E2F1 pathway may be targeted therapeutically to enhance chemotherapy-induced tumour cell death.

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Author's declaration

I am the sole author of this thesis. All of the references have been consulted by myself in the preparation of this manuscript. The work presented in this thesis was performed personally.

Abbreviations

A	Adenosine
AA	Amino Acids
ATP	adenosine 5'-triphosphate
C	cytosine
Cdk	cyclin dependent kinase
cDNA	DNA complementary to mRNA
dCTP	deoxycytodine-5'-triphosphate
DBD	DNA Binding Domain
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylene diamine triacetic acid
ECL	Enhanced Chemiluminescence
FCS	Foetal calf serum
EtBr	Ethidium Bromide
G	guanine
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
H	hour/s
H ₂ O	water
HEPES	4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid
HRP	Horseradish Peroxidase
kDa	kilodalton
LB	Leuria-Bertani medium
Mins	minutes
mRNA	messenger RNA
NLS	nuclear localisation signal
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethanesulphonyl fluoride
RPE	Retinal Pigment Epithelia
RNA	ribonucleic acid
RNase	Ribonuclease
Rpm	revolutions per minute
rRNA	ribosomal RNA
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
siRNA	short interfering RNA
T	thymine
TBS	Tris buffered saline
TBST	TBS-Tween
TE	Tris-EDTA buffer
Tris	Tris(hydroxymethyl)aminomethane
UV	ultra violet

Chapter 1: Introduction

1 Chapter 1: Introduction

It is estimated that cancer affects one in three persons in the developed world and half of those affected will die from the disease (Evan, 1997). Treatments can include surgery, radiotherapy, chemotherapy or a mixture of these.

Chemotherapy involves using anti-cancer drugs to destroy cancer cells. Many commonly used cytotoxic drugs cause DNA damage and affect rapidly growing cells by interfering with cell division in various possible ways. However, most forms of chemotherapy target all rapidly growing cells and are therefore have effects on certain normal cells in addition to cancer cells. Although some degree of specificity may come from the inability of many cancer cells to repair DNA damage, cancer cells are often difficult to eliminate because they have impaired cell death pathways and are therefore resistant to many drugs.

Rationally designed therapy utilizing a specific cancer-associated molecular target has therefore become a very active research area.

Drugs that restore the normal cell death pathways have the potential for effectively treating cancers that depend on aberrations of the apoptotic pathway to survive. The transcription factor E2F1 is almost always deregulated, though rarely mutated, in human cancers (Gorgoulis et al., 2002; Nevins, 2001). E2F1 is a potent inducer of apoptosis and can be activated following chemotoxic-induced DNA damage. The E2F1 induced apoptotic pathway is therefore an attractive target for tailored therapy which can lead to increased chemotherapy and better cancer treatment.

1.1 Tumourigenesis and cell death

Humans are multicellular organisms that need proper cellular function of every tissue type to survive. Normal cells proliferate, differentiate and die in order to maintain homeostasis within the body. It is estimated that around 100 thousand billion (10^{14}) cells comprise the human body. During the course of a human life these cells undergo a total of some 10^{16} cell divisions that are governed by a complex series of extracellular stimuli and intracellular pathways (Evan, 1997). These pathways have many internal checkpoints and safety nets. Normal cells can respond to DNA damage by undergoing cell cycle arrest to allow time for the repair of damaged DNA. Successful repair allows the cell to reverse the growth arrest and subsequently divide. For unicellular organism, repair of the damaged DNA is the only sensible way to ensure survival. However, in multicellular organisms it may be safer for the organism as a whole to eradicate damaged cells. Programmed cell death (apoptosis) is therefore prudent option when severe DNA damage occurs or repair is unsuccessful.

1.1.1 *Cancer formation*

Corruption of the machinery that senses or implements DNA damage can allow accumulation of mutations and potentially lead to tumour formation. A series of key aberrations can result in disruption of the intracellular pathways that control cell survival and therefore greatly predispose to cancer formation. The complexity of the pathways controlling cell survival and homeostasis reflect the diversity of genetic alternations leading to tumours. Alterations can vary and are often specific to tumour type but can also be different between individuals with the same tumour. It is therefore the focus of many researchers to identify and dissect the common regulatory pathways that are altered in most human cancers to validate targets with therapeutic potential in a wide range of human tumours.

Several lines of evidence indicate that tumourigenesis in humans is a multistep process. These steps reflect genetic alterations in pathways that drive transformation of normal cells into highly malignant cancer cells (Foulds, 1954). Six essential alterations in cell physiology have been defined to collectively contribute to tumour formation. In addition to being able to evade programmed

cell death, cells must acquire self-sufficiency in growth signals, insensitivity in growth-inhibitory signal, limitless replicative potential, sustained angiogenesis, and the ability to invade tissue and metastasise (Hanahan and Weinberg, 2000). It is believed that these six capabilities are shared in common by most, if not all human cancers, despite their diversity and heterogeneity.

1.1.1.1 Cell proliferation pathways are deregulated in human cancers

All tumours share the ability to proliferate beyond the constraints limiting growth in normal tissue. Therefore it is no surprise that each of the pathways that are involved in proliferative responses in normal cells is perturbed in most cancers. Activating mutations of the mitogen-responsive receptor tyrosine kinases (RTKs) or G-protein signal transducers such as Ras, or mutations affecting one of the many intermediary molecules in the pathway, allow cells to short circuit the normal requirement of somatic cells for external mitogenic signals (Evan and Vousden, 2001). Mutations that target the cell-cycle checkpoint pathway, regulated by the retinoblastoma tumour suppressor pRb are also common (Harbour and Dean, 2000). Defects in this pathway, which may be universal in human cancers, include deletion or mutation of the *RB* gene itself and deregulation of the cyclin dependent kinases (CDKs) that phosphorylate and functionally inactivate pRb, either through direct over-activation of CDKs or through genetic loss of their inhibitors (Fig. 1.1) (Bookstein et al., 1990; Friend et al., 1986; Fung et al., 1987; Harbour et al., 1988; Lee et al., 1988; Sherr, 1996; T'Ang et al., 1988). Inactivation of pRb in tumours relieves inhibition of the activating members of the E2F family of transcription factors (E2F1-3a) which express many target genes involved in cell cycle progression (Bell and Ryan, 2005; Goodrich et al., 1991; Hiebert et al., 1992; Kowalik et al., 1995). Viral oncoproteins such as type 5 adenovirus early region 1A (E1A), simian virus 40 (SV40) large T, and E7 protein from high-risk human papiloma virus (HPV) all target pRb leading to de-regulated E2F activity and cell cycle progression (Chellappan et al., 1992; DeCaprio et al., 1988; Dyson et al., 1989; Whyte et al., 1988).

E1A, one of the best studied viral oncoproteins, binds to several host proteins including the p300/CBP transcriptional activator and p400/TRRAP chromatin-remodelling complex (Frisch and Mymryk, 2002; Fuchs et al., 2001; O'Connor et

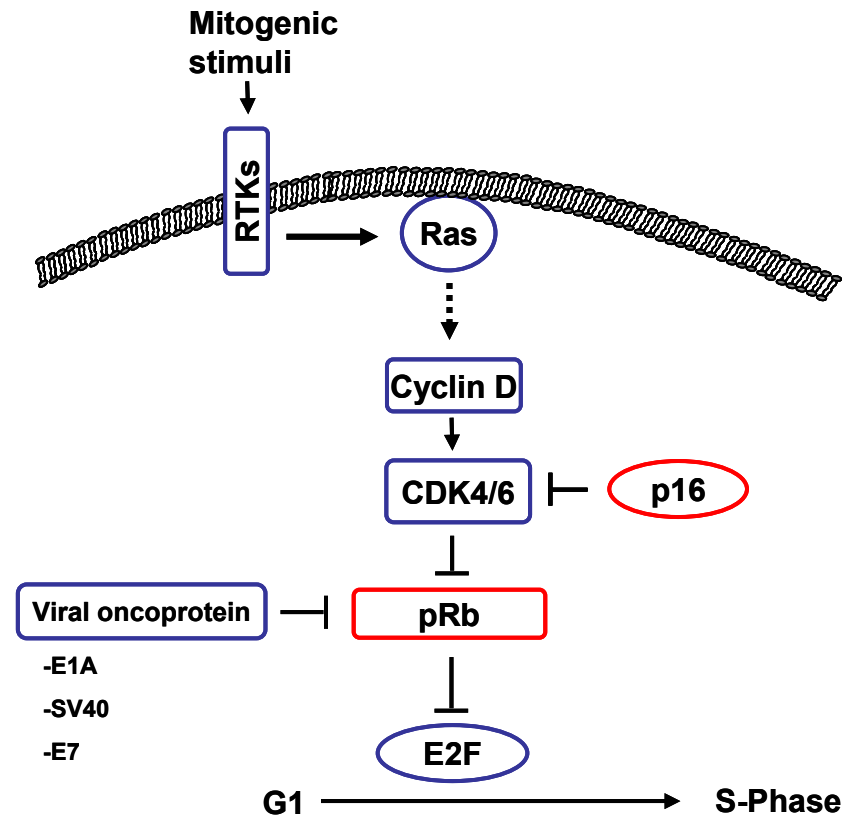


Figure 1-1 The pRb pathway is deregulated in most human cancers. This can occur through activating mutations of the mitogen receptor tyrosine kinases (RTKs) or signal transducers such as Ras. pRb itself is often mutated or lost in cancers. The cyclin dependent kinases (such as CDK4/6) that phosphorylate and inactivate pRb are activated in many tumours, either through direct over-activation or through loss of their inhibitors such as p16. Inactivation of pRb relieves inhibition of the activating E2Fs leading to increased cell cycle progression. Viral oncoproteins can also target and inactivate pRb leading to increased E2F activity. Tumour suppressor proteins are outlined in red, with oncoproteins in blue. Dashed line indicates effect not requiring direct association.

al., 1999). E1A has been shown to induce Myc expression in a manner dependant on pRb and p300/CBP binding (Baluchamy et al., 2007). Myc, like E2F, is a transcription factor and has the ability to drive cell proliferation. Myc expression is tightly controlled by mitogen availability in normal cells, but it is usually expressed in a deregulated or elevated manner in tumour cells (Baudino and Cleveland, 2001).

The presence of mutations in these pathways in most human cancers indicates the importance of their role in driving proliferation in tumours. However, many of these growth control mechanisms are linked to apoptosis so that excessive or inappropriate proliferation concomitantly signals cell death. Overexpression of growth promoting oncogenes as E2F1, E1A and Myc sensitizes cells to apoptosis (Evan et al., 1992; Qin et al., 1994; Shan and Lee, 1994; White et al., 1991). Ras, E2F1, E1A (via E2F1) and Myc all induce ARF, an alternate product of the *INK4a* locus which binds and inactivates Mdm2, a key part of the ubiquitin ligase

that targets the tumour suppressor p53 for degradation (Fig. 1.2) (Lowe, 1999). Both E2F1 and E1A induce upregulation of Apoptotic Protease Activating Factor-1 (Apaf-1) and pro-caspase 9 (Fearnhead et al., 1998; Moroni et al., 2001). Elevation of Apaf-1 increases the sensitivity of apoptosome activation to cytochrome *c* release from the mitochondria (Green and Evan, 2002; Zou et al., 1997).

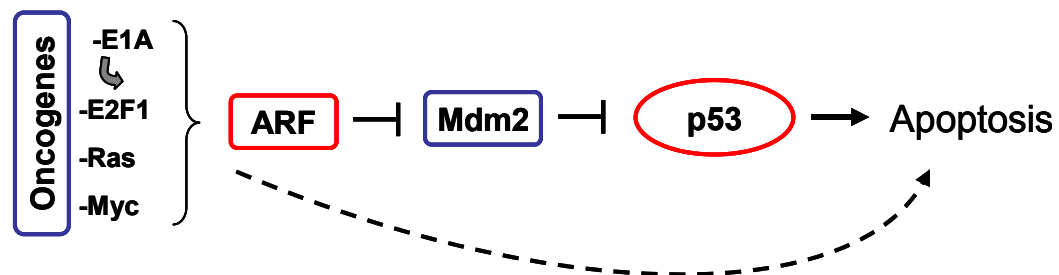


Figure 1-2 Signals that promote cell proliferation can also promote apoptosis. Overexpression of growth promoting oncogenes as E2F1, E1A and Myc sensitize cells to apoptosis. E2F1, E1A (via E2F1), Ras and Myc all induce ARF which binds and inactivates Mdm2, a protein that binds p53 and targets it for degradation. This oncogenes activation therefore leads to p53 stabilization and induction of apoptosis. Other pathways are also involved in oncogene-induced cell death sensitization (dotted line).

Tumour progression therefore, in addition to oncogenes activation, requires the expression of anti-apoptotic proteins or inactivation of essential pro-apoptotic proteins often encoded by tumour suppressor genes (Bissonnette et al., 1992; Harrington et al., 1994; Sabbatini et al., 1995).

1.1.1.2 Cell death and survival pathways are deregulated in human cancers

The tumour suppressor p53 is the most frequently mutated gene in human cancers. 50% of all tumours carry p53 mutations and the protein may be functionally inactive in many more (Hollstein et al., 1994; Hollstein et al., 1991). p53 exists at low levels in normal cells but can be stabilized and activated by various cellular stresses including DNA damage (Lu and Lane, 1993; Ryan et al., 2001). The N-terminal domain of p53 is phosphorylated by the DNA damage sensing kinases Ataxia Telangiectasia Mutated protein (ATM), ATM and Rad3-related protein (ATR), Chk2 and Chk1 (Banin et al., 1998; Canman et al., 1998; Shieh et al., 2000). Once stabilised, p53 mediates its tumour suppressor effects by inducing cell cycle arrest and apoptosis (Choisy-Rossi and Yonish-Rouach, 1998). p53 is a transcription factor but is able to induce apoptosis through a variety of mechanisms including both transcription dependent and

transcription independent pathways (Attardi et al., 1996; Chipuk et al., 2004; Erster et al., 2004; Erster and Moll, 2005; Marchenko et al., 2000; Mihara et al., 2003; Moll et al., 2005; Schuler and Green, 2001; Yonish-Rouach et al., 1996). The fact that p53 is both stress-responsive and is activated following DNA damage to mediate programmed cell death means that there is a strong selection for tumour to lose p53 function (Woods and Vousden, 2001).

In addition to tumours with mutated p53, many tumours containing wild type p53 have developed other mechanisms to evade p53 mediated tumour suppression. Overexpression and/or upregulation of Mdm2 has been found in approximately 8% of human cancers with upregulation of Mdm2 leading to p53 degradation (Momand et al., 1998). Tumours containing amplification of the mdm2 gene itself as well as p53 mutations are rare, suggesting Mdm2 is the primary regulator of p53 stability (Cordon-Cardo et al., 1994). The E6 protein from oncogenic Human Papilloma Virus (HPV) acts in a similar way to Mdm2 by binding and targeting p53 for ubiquitin-mediated proteolysis (Fig. 1.3) (Scheffner et al., 1990). Nearly all cervical cancers infected with HPV contain wild type p53 indicating that E6 can substitute for p53 mutation in human cancers (Mantovani and Banks, 2001).

Many oncogenes can upregulate ARF which binds Mdm2 and inhibits Mdm2-mediated degradation of p53. Inactivation of ARF through methylation of the ARF promoter occurs in many colorectal cancers making the tumour cells permissive for the presence of activated oncogenes even in the context of wild type p53 (Esteller et al., 2000; Robertson and Jones, 1998)

Multiple mechanisms underline the induction of apoptosis by oncogenes. Nevertheless, many of them are p53-dependant and induce apoptosis predominantly through the intrinsic pathway involving mitochondria (see section 1.1.2.2). Following mitochondrial outer membrane permeabilization (MOMP), cytochrome *c* is released from the mitochondrial intermembrane space (Green and Reed, 1998). The pro-apoptotic proteins Bax and Bak are required for MOMP, while the antiapoptotic Bcl-2 proteins, including Bcl-2 and Bcl-x_L, prevent MOMP (Green, 2006). Different BH3-only proteins also promote apoptosis and are presumed to be important mediators of the apoptotic response to genotoxic damage. Bax is a transcriptional target of p53 and two of the BH3-only family,

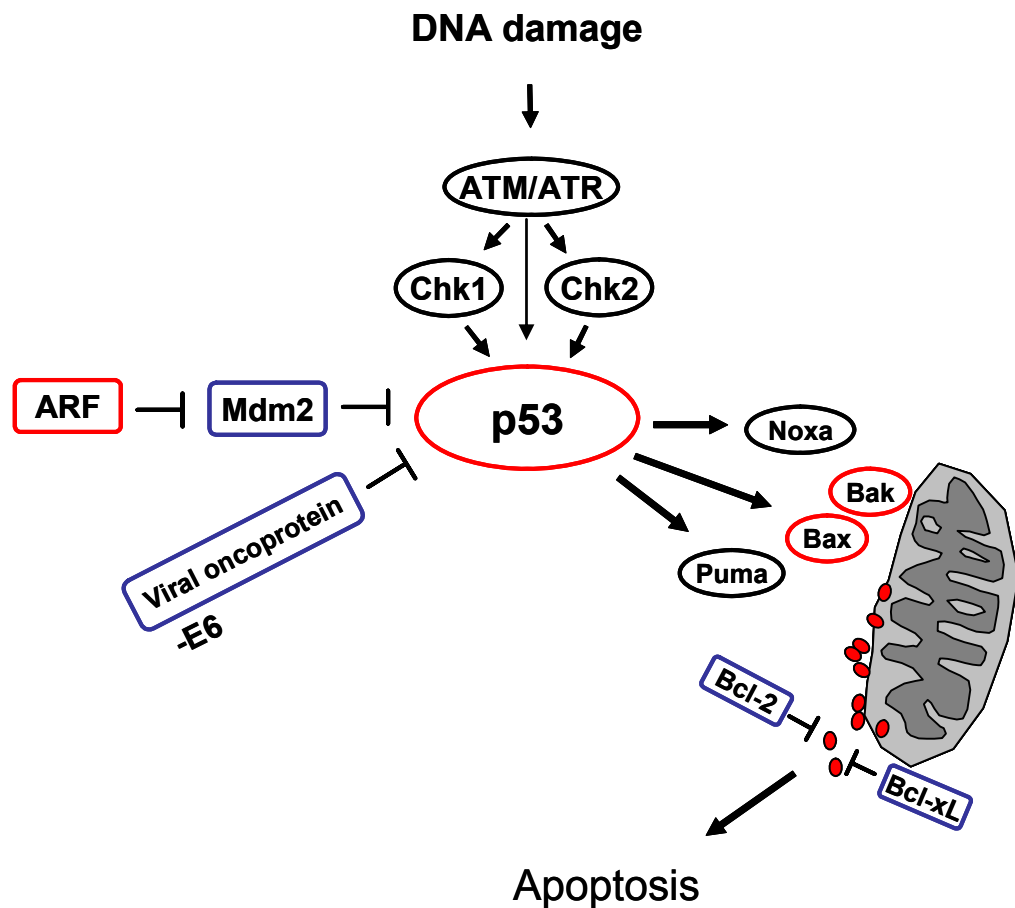


Figure 1-3 Cell death and survival pathways are deregulated in human cancers. The tumour suppressor p53 is mutated in around 50% of tumours. Mdm2 is upregulated in many tumours leading to p53 degradation and nearly all cervical cancers have the human papilloma virus oncoprotein E6 which also inhibits p53 activity. ARF, which inhibits Mdm2 mediated p53 degradation, is inactivated through methylation in many colorectal cancers. The pro-apoptotic Bax and Bak have decreased expression in many tumours, whereas the anti-apoptotic proteins Bcl-2 and Bcl-xL are overexpressed in several tumours. Proteins outlined in red are mutated or downregulated in tumours, and overexpressed proteins are outlined in blue.

Noxa and Puma, have also shown to be a target of p53 (Miyashita et al., 1994; Nakano and Vousden, 2001; Oda et al., 2000; Yu et al., 2001). A decreased expression or mutation of Bax and Bak has been reported in several tumours. In addition, both anti-apoptotic proteins, Bcl-2 and Bcl-xL are overexpressed in several tumour types (Crisan, 1996; Kelekar and Thompson, 1998; Krajewska et al., 1996; Krajewski et al., 1995; Packham, 1998).

1.1.2 Apoptosis

Regulated or programmed cell death is essential to control cell numbers and tissue maintenance. It serves to remove dangerous cells that threaten homeostasis. Discovered and rediscovered several times by various scientists, programmed cell death acquired a number of names over the past two centuries

(Hengartner, 2000). In fact, naturally occurring cell death did not receive widespread recognition until 1972 when Kerr et al. described the morphological features of an ordered form of death they called 'apoptosis' (Kerr et al., 1972). Apoptosis (a term derived from the Greek word describing the falling off of petals from a flower or leaves from a tree), or natural cell death, were recognised after that as wide-spread phenomenon not restricted to embryogenesis (Fadeel et al., 1999).

The anatomy of apoptosis occurs in a sequence of cellular morphological events. First, the cell undergoes nuclear and cytoplasmic condensation with blebbing of the plasma membrane. Eventually, the cell breaks up into membrane-bound fragments termed apoptotic bodies containing structurally intact organelles, as well as portions of the nucleus. Subsequently, the apoptotic bodies are rapidly recognised, ingested and degraded by phagocytic cells (Kerr et al., 1972). This is in contrast to necrosis, a pathological or accidental mode of cell death, characterised by irreversible swelling of the cytoplasm and organelles, including the mitochondria. Eventually there is a loss of membrane integrity resulting in cell lysis. Necrosis occurs when cells are subjected to toxic stimuli such as hyperthermia, hypoxia and direct cell trauma (Kerr et al., 1972). However, recently necrosis has been shown to have a genetic component. In response to alkylating DNA damage, cells undergo necrosis as a self-determined cell fate, which does not require the central apoptotic mediators p53, Bax/Bak or caspases (Zong et al., 2004).

Apoptosis is found throughout the animal kingdom. In vertebrates, programmed cell death has been observed in almost all tissues and has been studied most extensively in the developing nervous system and in the immune system. In the nematode *Caenorhabditis elegans*, where divisions and deaths of individual cells can be observed easily in living animals, apoptosis has been shown to occur in normal development in many different cell types (Ellis et al., 1991). Many human homologues of proteins involved in cell death in nematodes have been found and the remarkable degree of conservation of the cell death pathway from nematodes to humans suggests that core death machinery exists in every cell (Horvitz, 1999; Zou et al., 1997).

Apoptosis in mammalian cells is mediated by a family of cysteine proteases, which are homologous to each other and are a part of a large protein family known as the caspases. Caspases bring about most of the visible changes that characterize apoptotic cell death and are therefore thought of as the central executioners of the apoptotic pathway. Caspases are initially expressed in cells as inactive pro-caspase precursors and are processed into their active form through cleavage at two or more critical aspartate residues. Caspases have been sub-classed into “initiator” and “effector” caspases. The initiator caspases include caspase 8 and -9 (Thornberry and Lazebnik, 1998). They are activated through protein-protein interactions and involved in two alternative death pathways. Caspase-8 is the key initiator caspase in the death-receptor pathway (extrinsic pathway, see section 1.1.2.1) while caspase-9 is triggered by the mitochondrial pathway (intrinsic pathway, see section 1.1.2.2). The effector caspases are usually activated proteolytically by the initiator caspases. Once an initiator caspase is cleaved and activated, downstream effector caspases such as caspase-3, -6 or -7 can cleave a variety of cellular substrates, resulting in chromatin condensation, nuclear fragmentation and DNA cleavage in the nucleus, blebbing of the cell membrane and the fragmentation of the cytoplasm, all characteristics of an apoptotic cell (Cryns and Yuan, 1998; Salvesen and Dixit, 1997; Thornberry and Lazebnik, 1998).

1.1.2.1 The extrinsic apoptotic pathway

The two pathways by which caspase activation can be triggered are the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway (Fig.1.4). The extrinsic pathway is a receptor-linked pathway that requires the engagement of death receptors by their ligands on the surface of a cell. It is triggered by the death-receptor superfamily (such as Fas/CD95, tumour necrosis factor (TNF) receptor 1, TRAIL-R1/DR4 and TRAIL-R2/DR5) which have universal death domains (DDs) located within their cytoplasmic tails. The binding of a ligand to its receptor, such as FasL to Fas/FasCD95 or TRAIL to TRAIL-R induces receptor clustering and formation of a death-inducing signalling complex (DISC) (Ashkenazi and Dixit, 1998; Budihardjo et al., 1999). This complex recruits, via the adaptor molecule FADD (Fas-associated death domain protein), multiple pro-caspase-8 molecules, resulting in caspase-8 activation through induced proximity. Upon caspase-8 cleavage and subsequent activation, caspase-8

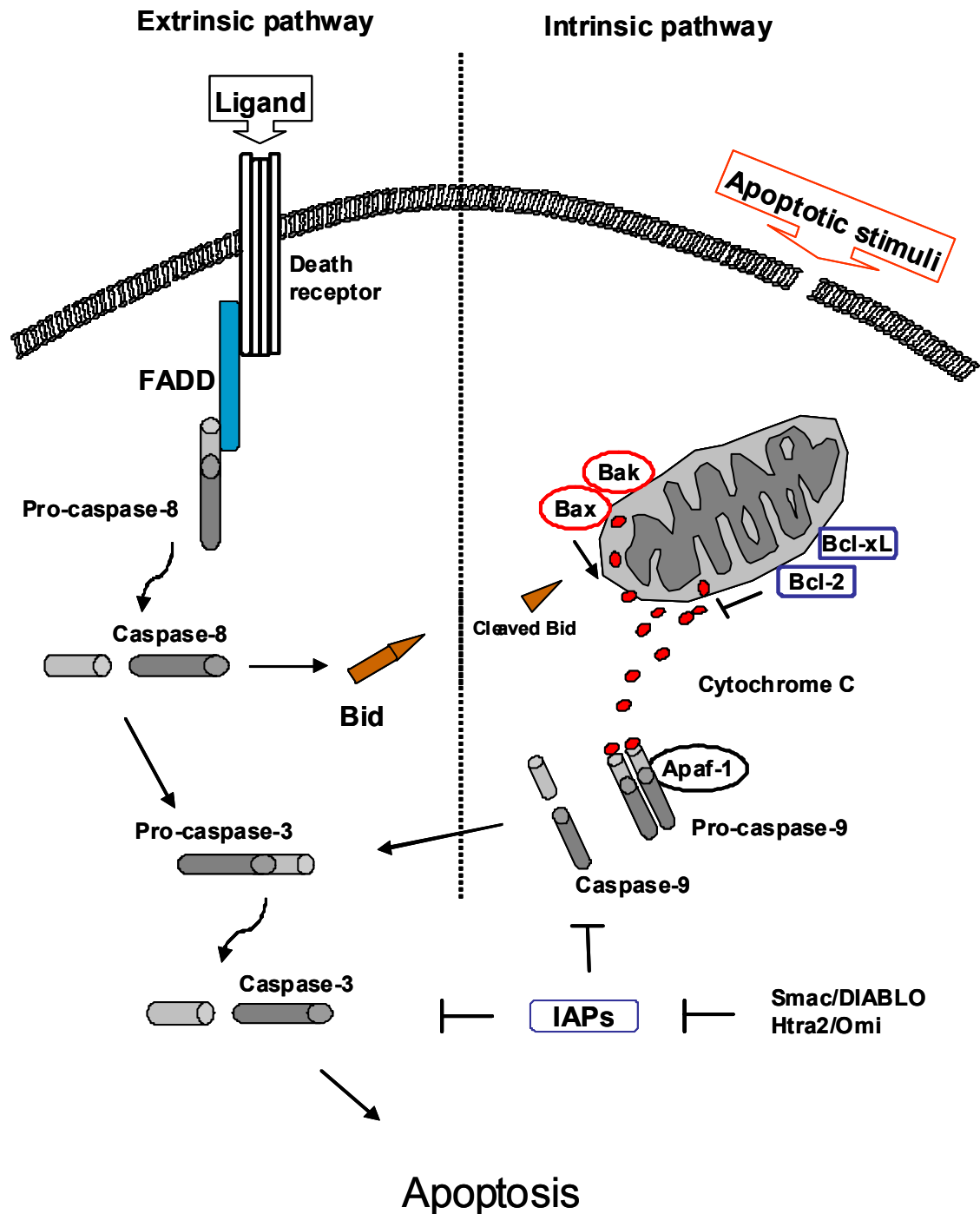


Figure 1-4 Pathways to cell death. Apoptosis can be triggered by two alternative pathways, the extrinsic (death receptor) and the intrinsic (mitochondrial) pathway. In both circumstances induction of apoptosis leads to activation of initiator caspases (caspase-8 in the extrinsic, caspase-9 in the intrinsic pathway) that are cleaved and activate the effector caspases. Active effector caspases finally cleave death substrates which eventually leads to apoptosis. The extrinsic pathway is activated following ligand binding to the death receptor family, which recruit FADD and caspase-8. The intrinsic pathway occurs via the mitochondria where cytochrome C release is regulated by the Bcl-2 family of proteins. There is a cross-talk between the two pathways, for example the Bcl-2 family protein, Bid, is cleaved by caspase-8 following activation of the extrinsic pathway, leading to activation of the intrinsic pathway, cytochrome C release and apoptosis.

initiates the proteolytic cascade through the effector caspases that cleave cellular targets and results in apoptosis. Caspase-8 can also cleave downstream caspases indirectly by cleaving Bid, a member of the Bcl-2 family, which then translocates to the mitochondria and induces outer membrane permeabilization and cytochrome *c* release from the mitochondrial intermembrane space (Crichton and Ryan, 2004; Hengartner, 2000; Li et al., 1998; Luo et al., 1998; Wang et al., 1996).

1.1.2.2 The intrinsic apoptotic pathway

The intrinsic pathway occurs via the mitochondria and is used extensively in response to extra and intra cellular stresses such as growth factor withdrawal, DNA damage and oncogene activation. These diverse response pathways induce apoptosis, often through activation of a pro-apoptotic member of the Bcl-2 family, by inducing mitochondrial outer membrane permeabilization and release of cytochrome *c*. Cytochrome *c* normally resides in the space between the inner and outer mitochondrial membranes where it plays a role in electron transport. Once released, cytochrome *c* associates with Apaf-1 and then pro-caspase-9 to form a multiprotein caspase-activating complex, the “apoptosome” (Cain et al., 2002; Chinnaiyan, 1999). Fully assembled apoptosome recruits pro-caspase-3 which is then cleaved and activated by active caspase-9. Active caspase-3 can then initiate the apoptotic cascade involving other effector caspases resulting in DNA fragmentation and cell death (Green and Reed, 1998).

Cytochrome *c* release from the mitochondria is controlled by members of the Bcl-2 family proteins (defined by their sharing of, from one to four Bcl-2 homology (BH) domains). Pro- and anti-apoptotic Bcl-2 family members meet at the surface of the mitochondria, where they regulate cytochrome *c* release. Bcl-2 and Bcl-x_L suppress apoptosis by preventing MOMP and cytochrome *c* release and therefore caspase-9 activation. In contrast, pro-apoptotic Bcl-2 family members, such as Bax and Bak, appear to be essential for MOMP, and probably directly form a pore in the outer membrane (Goping et al., 1998; Mikhailov et al., 2003; Nechushtan et al., 2001). Different pro-apoptotic BH3-only proteins act to interfere with the function of the anti-apoptotic Bcl-2 members and/or activate Bax and Bak, but the precise manner in which they do so is not clear (Green, 2006; Green and Evan, 2002).

Although cytochrome *c* is necessary for activation of the apoptosome, downstream activation of the effector caspases can be inhibited by the IAPs (inhibitor of apoptosis proteins). IAPs act as endogenous caspase inhibitors, possibly through their function as E3-ligases, and target active caspases for rapid degradation (Suzuki et al., 2001b). X-linked IAP (XIAP), and probably other IAPs, can bind active caspase-9 and inhibit mitochondrial cell death. XIAP has also been shown to inhibit and bind to caspase-3 downstream of caspase-8 in the extrinsic pathway. IAPs are in turn regulated by at least two proteins released from the mitochondria upon MOMP. Smac/DIABLO and Htra2/Omi, which both contain IAP binding motifs, can bind IAPs and prevent IAP-mediated inhibition of caspases and therefore induce caspase-dependent death (Du et al., 2000; Hegde et al., 2002; Suzuki et al., 2001a; Verhagen et al., 2000; Verhagen et al., 2002).

There are other proteins within the mitochondrial intermembrane space that control cell death in caspase-independent ways. Apoptosis inducing factor (AIF) translocates to the nucleus after its release, where it appears to induce chromatin condensation and DNA fragmentation (Susin et al., 1999). In addition, Endonuclease G is thought to directly mediate nuclear DNA fragmentation upon its release from the mitochondrial intermembrane space (Li et al., 2001).

1.1.3 DNA damage induces apoptosis

The genome is constantly exposed to exogenous DNA damaging events such as solar radiation, viral infection and chemicals. Eukaryotic organisms have evolved a highly conserved signalling pathway, called the DNA damage response, to protect against genomic damage. Within cells, sensor proteins detect various forms of damage and signal via a complex pathway regulated by protein phosphorylation, stabilization and transcriptional regulation. The DNA damage response can cause cell cycle arrest and induction of DNA repair function. However, the response of many cells with more severe damage is to induce apoptosis (Gasser and Raulet, 2006).

Following DNA damage, the PI3-kinase-related serine/threonine protein kinases ATM (Ataxia Telangiectasia, mutated) and ATR (ATM- and Rad3-related) cooperate with other proteins to initiate the DNA damage response. ATM is mutated in the genetic disorder ataxia-telangiectasia (AT), causing defective

cell cycle checkpoint activation, a reduced repair of DNA double strand breaks and abnormal apoptosis (Lavin and Kozlov, 2007).

Double strand breaks preferentially activate ATM (although other stimuli are also capable of ATM activation), whereas stalled DNA replication induces ATR activity. The exact mechanism of activation remains unknown, but the MRN complex (Mre11/Rad50/Nbs1) plays an important role both in the recruitment of ATM to the sites of DNA damage and in the efficient activation of ATM (Mirzoeva and Petrini, 2001; Petrini and Stracker, 2003). In responses to many genomic insults, however, both kinases are eventually activated, ultimately triggering the activation of their downstream substrates.

DNA damage, caused by various DNA damaging agents such as ionizing radiation (IR), UV and chemotherapeutic drugs, leads to p53 activation and stabilization. ATM directly phosphorylates p53 on serine 15 and a number of other sites which contributes to p53 stabilization following DNA damage (Lavin and Kozlov, 2007). ATR has also been shown to play a role in the activation of p53 and phosphorylates p53 on serine 15 and 37 in cells exposed to UV light (Appella and Anderson, 2001). Phosphorylation at serine 15 and 37 has been shown to decrease the interaction of p53 and Mdm2, the ubiquitin ligase that normally targets p53 for rapid turnover (Shieh et al., 1997). Mdm2 has also been shown to be phosphorylated by ATM/ATR in response to DNA damage (Kastan and Bartek, 2004; Maya et al., 2001). These modifications of p53 and Mdm2 contribute to the stabilization and activation of the p53 protein. Other targets involved in apoptosis have also been shown to respond to ATM/ATR activity. The transcription factor E2F1 is phosphorylated at serine 31 following DNA damage by ATM kinase, which leads to induced E2F1 accumulation (Lin et al., 2001).

Downstream of ATM/ATR are the serine/threonine effector kinases Chk2 and Chk1. ATM-mediated phosphorylation preferentially triggers the activation of Chk2 where Chk1 is mainly phosphorylated by ATR. ATM phosphorylates and activates Chk2 following double-strand breaks caused by IR, whereas ATR is activated by a broader spectrum of genotoxic stimuli such as lesions caused by UV and inhibitors of DNA replication. Both kinases have been shown to be phosphorylated following treatment with the topoisomerase II inhibitor adriamycin (doxorubicin), a clinically important chemotherapeutic drug (Ho et

al., 2005). Phosphorylation of both proteins leads to their activation and subsequent regulation of proteins that are involved in the DNA damage response.

In response to IR, Chk2 phosphorylates p53 on serine 20. This site is known to interfere with Mdm2 binding and therefore leads to p53 stabilization (Hirao et al., 2000). Chk2 has also been shown to phosphorylate E2F1 following treatment with the chemotherapeutic drug etoposide. This results in stabilization of the E2F1 protein and its subsequent increased transcriptional activity (Stevens et al., 2003).

Another way of activating the transcription factors p53 and E2F1 in response to DNA damage is through acetylation. p53 is acetylated at lysines 320, 373 and 382 in the C-terminus of the protein. Following IR or UV induced DNA damage, CREB binding protein (CBP)/p300 acetylates p53 at lysine-373 and lysine-382, whilst p300/CBP-associated factor (P/CAF) can acetylate p53 at lysine-320, resulting in increased affinity of p53 for DNA (Gu and Roeder, 1997; Liu et al., 1999; Sakaguchi et al., 1998). It has been implicated that p53 phosphorylation at serine 37 and 15 recruit CBP/p300 to p53 and control p53 acetylation, therefore suggesting that p53 activation is controlled by a combination of phosphorylation and acetylation (Lakin and Jackson, 1999). E2F1 has also been shown to be a target of P/CAF, and to a lesser extent p300/CBP, *in vitro*. Acetylation by P/CAF on lysines 117, 120 and 125, which lie adjacent to the E2F1 DNA-binding domain, stabilize E2F1 and increase its DNA binding ability and transactivation potential (Martinez-Balbas et al., 2000).

A number of genes involved in apoptosis have been demonstrated to be targets of p53 and E2F1 following activation or in response to DNA damage. p53 stimulates increased expression of the pro-apoptotic protein Bax, which can induce apoptosis via the intrinsic pathway (Miyashita et al., 1994). In addition, Noxa and Puma have been shown to be targets of activated p53. Both proteins can interact with anti-apoptotic Bcl-2 resulting in the release of cytochrome *c* from the mitochondria and caspase-9 activation (Nakano and Vousden, 2001; Oda et al., 2000; Yu et al., 2001). Several other genes involved in the intrinsic apoptotic pathway have also been shown to be targets of E2F1 when overexpressed, for example caspase 7 and Apaf-1 (Furukawa et al., 2002). However, it was only recently shown that DNA damage induced E2F1 activates

specific subset of target genes, for example, in response to adriamycin-induced DNA damage, E2F1 is acetylated and subsequently activates genes involved in apoptosis, such as p73 (Pediconi et al., 2003).

1.1.4 Activated oncogenes sensitize cells to apoptosis

Many mitogenic oncogenes, such as Ras, E2F1, Myc and E1A force uncontrolled proliferation of cells. However, although the processes of cell proliferation and cell death appear to be opposing, much evidence now exists to indicate that the two processes are linked. Many oncogenes have now been shown to enhance apoptosis in addition to induced cell division. c-Myc (one member of the Myc family of proteins) was one of the first oncoproteins to show pro-apoptotic activity. c-Myc is a transcription factor and requires the N-terminal transactivation domain and the DNA binding domain for its apoptotic potential (Evan et al., 1992). c-Myc is dependent on Max, its dimerization partner, to induce both proliferation and apoptosis and is able to induce both p53-dependent and p53-independent apoptosis (Amati et al., 1993; Sakamuro et al., 1995). c-Myc is able to activate p53 directly through induced ARF expression, resulting in induced cell death (Zindy et al., 1998).

E1A is another growth promoting oncoprotein that sensitizes cells to cell death. E1A is expressed by adenoviruses which also express the antiapoptotic E1B genes, which oppose the cytotoxic effects of E1A, for successful viral replication (White et al., 1991). Both the growth-promoting and apoptotic functions of E1A map mainly to the N-terminal half of the protein. E1A stimulates cell cycle progression by binding to proteins that are critical in normal cell cycle control. E1A can mediate these effects by binding to the RB family (pRb, p107 and p130) through a motif located in a conserved region 2, (CR2) and to a lesser extent CR1 (Dyson et al., 1992). The RB family proteins control normal cell cycle by binding and inhibiting the E2F proteins. E1A inhibits the normal activity of RB and thereby de-regulate the E2Fs to constitutively activate genes involved in cell proliferation and to activate the viral E2 promoter (Chellappan et al., 1992; Fattaey et al., 1993).

However, RB inhibition is not the only way E1A promotes uncontrolled cell cycle progression. The N-terminus of E1A binds and interferes with the function of

p300 and CBP, two highly related transcriptional activators that enhance expression of genes involved in growth inhibition (Arany et al., 1995; O'Connor et al., 1999). E1A is also able to target p400 and TRRAP, using overlapping sequences in the N-terminus. p400 and TRRAP are unrelated proteins that interact with each other and additional proteins involved in chromatin remodelling (Fuchs et al., 2001). TRRAP has also been shown to interact with c-Myc N-terminus and the transactivation domain of E2F1, suggesting that TRRAP is an essential cofactor for other oncogenic transcription factors (Deleu et al., 2001; McMahon et al., 1998). The C-terminus of E1A has also been shown to be able to bind the cellular proteins CtBP, which has been proposed to recruit histone de-acetylases to promoters and promote negative chromatin remodelling (Sundqvist et al., 1998).

The way that these co-factors work and their gene specificity is not well understood, however, their interactions appear to be important for the apoptotic activity of both E1A and Myc. E1A can cause an increase in the levels of p53 and induction of p53-dependent apoptosis. By using deletion mutants of E1A it has been shown that deletion of a portion of CR1 prevents apoptosis, suggesting that binding of p300 and/or pRb is necessary for E1A-induced apoptosis. The ability of E1A to induce p53 levels is also dependent on p300 and pRb binding (Mymryk et al., 1994; Querido et al., 1997). However the mechanism through which E1A binding to p300 affects p53 stability is not clear. As discussed previously, CBP/p300 can lead to increased p53 activity by acetylating p53 following DNA damage. However, it has been suggested that E1A binding to p300-Mdm2-complexes disrupts p53 degradation, possibly by blocking poly-ubiquitination of p53 and inhibiting its normal turnover (Grossman et al., 2003; Grossman et al., 1998). In another study it was shown that E1A's ability to induce p53 was dependent on the pRb signaling pathway and ARF induction (de Stanchina et al., 1998). E1A has also been shown to induce p53-independent apoptosis. E1A is dependent on binding to pRb and p300 to increase expression of p73 mRNA and protein through E2F1 activation. E1A can also induce the p53 target Noxa in p53-deficient cancer cell lines, suggesting that E1A can promote apoptosis without p53 activation (Flinterman et al., 2005).

1.1.4.1 E1A expression sensitizes cells to drug induced apoptosis

Many anticancer drugs cause DNA damage leading to induced apoptosis, suggesting that chemosensitivity of tumour cells is influenced by the efficiency of the drug to activate cellular apoptotic programs. This suggests that tumours with oncogenes that can signal to apoptosis, such as Myc, are more susceptible to apoptosis than normal tissue and should be responsive to drug treatment. In contrast, tumours unable to efficiently engage apoptosis due to inactivation of tumour suppressor gene such as p53 become more resistant to drug treatment.

E1A expression has been shown to sensitize cells to apoptosis induced by IR, 5-fluorouracil, etoposide and adriamycin, in a p53-dependant manner (Lowe et al., 1993). The p53 target Bax contributes to apoptosis in E1A expressing cells and can therefore function as an effector of p53 in chemotherapy-induced apoptosis (McCurrach et al., 1997). As cells can tolerate ectopic E1A expression but become extremely prone to apoptosis, E1A expressing cells have been used as a simple model to further investigate cellular processes that modulate chemosensitivity. This allows analysis of induced chemosensitivity following oncogene activation in genetically normal cells outside the context of adenoviral infection. Using retrovirally delivered E1A into cells, selective induction of p53 and increased apoptosis following adriamycin treatment was shown to be dependent on E1A binding to pRb (but not p107 and p130) and p300/CBP (Samuelson and Lowe, 1997). However, in this study an N-terminal deletion mutant was used that was not refined enough to exclude other targets such as the p400/TRRAP complex. Later, it was showed by the same group, that E1A-p300/CBP interaction is largely dispensable for E1A to induce ARF, p53 and apoptosis following adriamycin treatment. Instead it was shown that E1A interacting with pRb and the p400/TRRAP complex is essential for these effects (Samuelson et al., 2005).

The interaction between E1A and pRb deregulates E2F1 which could contribute to the increase in chemosensitivity. However, E2F1's contribution to apoptosis in this context is not clear. E2F1's involvement in apoptosis will be discussed later in this chapter.

1.2 Cancer therapy

The diversity of different cancer types and the signaling complexity underlying tumourigenesis means that the design of successful therapies becomes very complex (Bell and Ryan, 2005). However, some of the most promising potential therapies may lie with targeting the apoptotic pathways. Defects in apoptosis can lead to tumourigenesis and contributes to drug resistance since many of the current chemotherapeutic drugs depend on the cellular apoptotic pathway for death. Reactivation of the apoptotic pathway in cancer cells or stimulation downstream of defects might therefore prove to be a successful way to kill tumours and could be used in combination with existing therapies.

1.2.1 The apoptotic pathway as a therapeutic target

Most drugs currently used in anti-cancer therapy kill cancer cells by apoptosis, both through the extrinsic and intrinsic apoptotic pathway. Possible drug targets that activate both of these pathways have now been discovered (Fesik, 2005; Nicholson, 2000).

In an attempt to target the extrinsic apoptotic pathway in cancers, agonistic antibodies against the TNF-related apoptosis inducing ligand (TRAIL) receptors have been made. These antibodies induce apoptosis in TRAIL-sensitive tumour cells whereas normal cells are not affected (Chuntharapai et al., 2001; Ichikawa et al., 2001; Takeda et al., 2004). Another approach for activating TRAIL receptors involves the use of soluble truncated version of TRAIL. It has been shown to induce apoptosis independently of p53 expression in a number of cell lines without affecting normal cells. Chemotherapeutic drugs such as etoposide and adriamycin significantly increased TRAIL-induced apoptosis in cancer cells through upregulation in the TRAIL receptors DR4, DR5, Bax and Bak, and induction of caspase activity. This suggests that both ex- and intrinsic pathways can synergize to enhance cell death following drug treatment and TRAIL activation (Shankar et al., 2005). However, varying degrees of TRAIL sensitivity or resistance have also been observed. Targeting the anti-apoptotic proteins Bcl-2 and IAPs (X-IAP or survivin) with RNA interference (RNAi) resulted in increase in apoptosis when used in combination with TRAIL (Chawla-Sarkar et al., 2004). Additional evidence supports the importance of mitochondrial

regulation in TRAIL-induced death. The pro-apoptotic protein Bax is crucial for mitochondrial changes and caspase activation and is essential in TRAIL-mediated death in cancer cells (LeBlanc et al., 2002).

The Bcl-2 family members play a crucial role in the intrinsic apoptotic pathway and serve as a potential targets for cancer therapy. The anti-apoptotic protein Bcl-2 is overexpressed in many cancers, confers resistance to treatment with radiotherapy and traditional cytotoxic chemotherapy and correlates with poor survival and progression of the disease (Fesik, 2005). Therefore, targeting Bcl-2 in tumours could be a good therapeutic option. Two main approaches have been taken to target the Bcl-2 proteins. One is to reduce their expression levels using antisense oligonucleotides and some data show that reducing the expression levels synergizes with many therapeutic agents against various tumours (Klasa et al., 2002). Another approach is to use synthetic peptides to mimic the activity of the pro-apoptotic BH3-only proteins. Small molecular inhibitors that bind and inhibit Bcl-2 and Bcl-x_L have therefore been made and have been shown to enter cells and induce apoptosis (Holinger et al., 1999; Wang et al., 2000; Zangemeister-Wittke et al., 2000).

The IAPs are inhibitors of caspases and are therefore important regulators of apoptosis. The interaction between IAPs and caspases can be inhibited by Smac/DIABLO which is released from the mitochondria. XIAP is the most potent member of the IAP family in terms of caspase and apoptosis inhibition. The XIAP protein is overexpressed in various cancers and its expression correlates with cell death resistance (Fesik, 2005). Reduced expression of XIAP by antisense oligonucleotides induces apoptosis in lung cancer cells and sensitizes cells to death following treatment with chemotherapeutic drugs such as adriamycin and etoposide (Hu et al., 2003). Both peptides and small molecular inhibitors of XIAP have also been made in order to mimic Smac/DIABLO binding, activate caspases and induce apoptosis (Arnt et al., 2002). The XIAP inhibitors can induce caspase activation and sensitize cells to drug induced apoptosis suggesting that XIAP is a viable target for cancer therapy (Arnt et al., 2002; Oost et al., 2004).

Numerous studies have focused on activating p53 as a strategy for cancer therapy. Many different approaches have been established, such as using gene

therapy to introduce wild type p53 into tumours or use small molecular drugs to re-activate p53 in tumours with mutated p53 (Romer et al., 2006). A chemical compound called PRIMA, (p53 reactivation and induction of massive apoptosis) is able to restore native confirmation and wild type function to mutant p53, activate p53 target genes and induce apoptosis in cell lines with mutant p53 while having little effect on cells with wild type p53 (Bykov et al., 2002; Li et al., 2005; Yu, 2006).

An alternative approach is to use small inhibitors that inhibit the interaction of p53 with its negative regulator Mdm2. Inhibitors like nutlin and RITA mimic the binding of p53 to the p53-binding pocket of Mdm2 and inhibit p53 degradation leading to p53 accumulation and apoptosis (Issaeva et al., 2004; Vassilev et al., 2004). Nutlins have been shown to activate apoptosis in wild type p53 cancer types but not in mutant p53 cancer cells, indicating that they have therapeutic potential in tumours with Mdm2 overexpression (Fesik, 2005; Levesque and Eastman, 2007; Romer et al., 2006).

There are many other peptides and compounds that induce apoptosis and have therapeutic potential. Demethylating agents and/or histone deacetylase (HDAC) inhibitors have been used to target the silencing mechanism and reactivate pro-apoptotic proteins (Marks et al., 2001). In addition, antisense approaches to decrease expression of a variety of anti-apoptotic proteins, including Ras and Mdm2, are in clinical development (Cunningham et al., 2001; Johnstone et al., 2002; Tamm et al., 2001). Disruption of pRb, leading to activation of E2F1 in tumors, has also been investigated and will be discussed further later in this chapter.

1.3 E2F family proteins

1.3.1 Identification of E2F

In 1986 Kovesidi et al. identified a cellular factor that interacts with the adenovirus E2 promoter. The DNA binding activity of this factor increased following E1A infection and was involved in transactivation of the E1A viral protein itself (Kovesdi et al., 1986a; Kovesdi et al., 1986b). They later termed this factor E2F (E2 promoter binding factor) and showed it was a transcription

factor responsible for E1A-mediated stimulation of the E2 gene as well as the E1A gene, but not involved in the activation of the other E1A-inducible promoters (Kovesdi et al., 1987). That same year, La Thangue and co-workers identified a cellular activity called differentiation regulated transcription factor 1 (DRTF1). It was involved in the control of gene expression during cell differentiation (La Thangue and Rigby, 1987). This factor was found in several protein complexes which have the same DNA specificity. DRTF1 was able to interact with the retinoblastoma (pRb) tumour suppressor gene product that is a target of the transforming proteins of several DNA tumour viruses such as E1A and simian virus large T antigen (Whyte et al., 1988). E1A causes dissociation of the pRb protein from this complex which requires conserved regions 1 and 2 (CR1, CR2) of E1A that are known to be essential for efficient cell transformation by E1A (Dyson et al., 1992; Fattaey et al., 1993; Whyte et al., 1989). This suggested that the pRb protein forms a complex and regulates transcription of a DNA bound transcription factor, or the E2F (Bandara and La Thangue, 1991; Chellappan et al., 1992; Chellappan et al., 1991).

The mechanism of how cell proliferation is regulated by pRb was established in 1991 when pRb was found in a complex with E2F. The interaction of pRb with E2F was suggested to be an important mechanism involved in the control of cellular proliferation. The dissociation of the complex by E1A was shown to inactivate pRb function (Chellappan et al., 1991). Other viruses were also shown to be able to interact and inhibit pRb by using shared amino acid sequences that were the same as those required for the transforming activity of the viral proteins. It was suggested that the ability of E1A, SV40 large T and E7 (from high risk human papilloma virus) proteins to dissociate the E2F-Rb complex had evolved to stimulate cell proliferation in infected quiescent cells to allow efficient viral replication. These findings, in addition to the discovery that either the E2F-Rb complex was absent or pRb was mutated in various human cervical carcinoma cell lines, were the first indications that E2F might be associated with cancer (Chellappan et al., 1992).

Since the detection of the E2F factor many discoveries about the importance of this factor in cell cycle regulation have emerged. The regulation is complex and E2F activity has been shown to involve many proteins. Tight cell cycle regulation occurs due to the combined action of the E2F family members.

1.3.2 E2F family members

The family of E2F transcriptional factors consists now of eight human components. The protein products from these eight genes heterodimerize with members of the DP family (DP1, DP2 and DP4) to give a rise to functional E2F complexes. All possible combinations of E2F-DP complexes exist *in vivo* (Helin et al., 1993b; Krek et al., 1993; Wu et al., 1995). All of the E2Fs have core domains that mediate DNA binding and in some cases dimerization with DP. The DNA binding specificity is determined by the E2F through an evolutionary conserved DNA binding domain. The E2F family is believed to be central regulators of the cell cycle and regulate overlapping sets of many target genes. The family can be further divided into distinct subgroups on the basis of their sequence homology and transcriptional properties.

1.3.2.1 The activating E2F's

E2F1 was the first family member to be cloned and characterised as a protein that interacts with pRb (Helin et al., 1992). The transcriptional activity of E2F1 is inhibited by direct binding of pRb whereas a naturally occurring pRb mutant is unable to inhibit this activity (Helin et al., 1993a). Subsequently, two additional E2F-like proteins were characterised. Both are able to bind to wild-type, but not mutant E2F recognition sites and specifically interact with the pRb protein (Helin et al., 1993b). This suggested that many of previously described observations resulted from combined action of a family of E2F proteins rather than a single E2F protein.

E2F1, E2F2 and E2F3a are now termed the “activating” sub-group members of the E2F family (Fig. 1.5). They are all able to activate transcription and bind to pRb. E2F1-3a are highly homologous proteins, especially in the domains that are responsible for DNA binding, DP dimerization and pRb binding. Over-expression of E2F1-3a induces S phase entry in quiescent cells and overcomes G1 arrest mediated by the p16INK4 tumour suppressor protein (Lukas et al., 1996; Mann and Jones, 1996). Over-expression of E2F1 can overcome inhibition of proliferation mediated by type B transforming growth factor (TGF- β) (Schwarz et al., 1995). In addition, over-expressed E2F1 can induce p53-mediated apoptosis. This can be suppressed by wild-type pRb but not the naturally

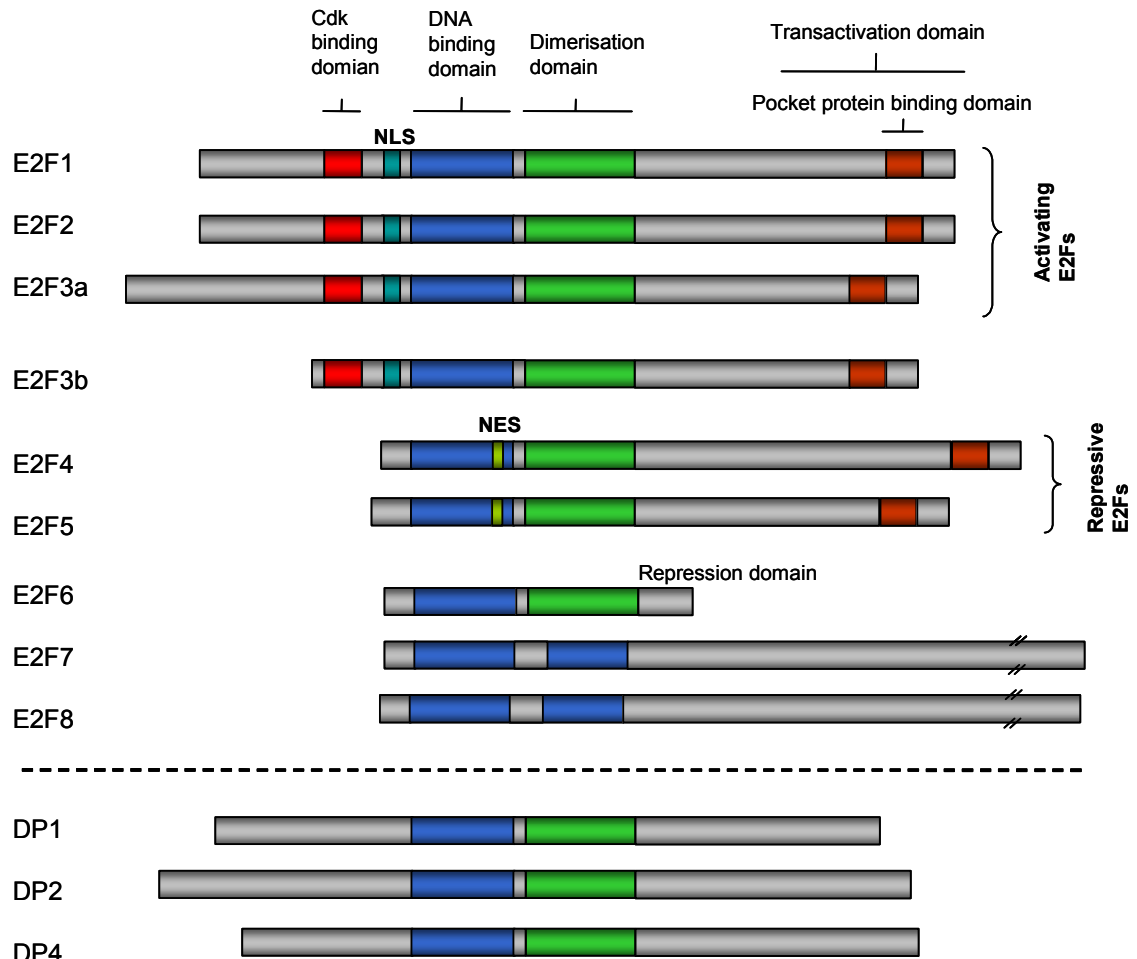


Figure 1-5 All of the E2F family members have sequence homology in the DNA binding and dimerization domain. E2F1-3a activate transcription and have a cdk2-cyclin A binding site, transactivation domain and binding domain for pocket proteins. E2F3b, E2F4 and E2F5 repress activation but also have pocket protein binding domain. E2F1-3 have a nuclear localization signal (NLS) but E2F4-5 have a nuclear export signal (NES). E2F6 lacks the transactivation domain and can act as a transcriptional repressor. E2F7 and E2F8 are also transcriptional repressors. They have two DNA binding domains but lack a transactivation domain. E2F1-6 all bind DNA as heterodimers with DP family proteins, while E2F7 and E2F8 appear to associate with DNA independent of DP proteins.

occurring loss-of-function pRb mutant (Qin et al., 1994). The combined effect of all three activating E2Fs was tested by conditional gene targeting and revealed that the combined loss of E2F1-E2F3a completely abolishes S phase entry and cell cycle progression of cells, underlining their essential role in cell cycle progression (Wu et al., 2001).

1.3.2.2 The repressive E2F's

The second subclass of the E2F family includes E2F4 and E2F5. These E2Fs were originally identified and cloned by virtue of their association with the RB family members p107 and p130 (Beijersbergen et al., 1994; Dyson et al., 1993; Hijmans et al., 1995). The sequences of these proteins diverge from the activating E2Fs

and are regulated differently. E2F4 and E2F5 expression is mainly detected in quiescent (G_0) cells, where E2F1, E2F2 and E2F3a are primarily restricted to actively dividing cells (Ikeda et al., 1996; Moberg et al., 1996). The repressive E2F4 binds to each of the pocket proteins at different points in the cell cycle, but E2F5 is mainly regulated by p130. In contrast to the activating E2Fs, E2F4 and E2F5 are poor transcriptional activators and are unable to drive quiescent cells to re-enter the cell cycle (Muller et al., 1997). The different activity of the two E2F subgroups, when not bound to DP, results from differences in their subcellular localisation. E2F1-3a are constitutively nuclear, whereas E2F4 and E2F5, lacking basic nuclear localization signal (NLS), are predominantly cytoplasmic (Verona et al., 1997).

Inactivation of E2F4 and E2F5 in mice suggests that they play individual roles during cell differentiation of certain cell lineages. Mice simultaneously nullizygous for both the E2F4 and E2F5 genes result in neonatal lethality, suggesting their functions overlap during mouse development. Fibroblasts from these mice proliferate normally but fail to arrest in G1 in response to p16INK4 (Gaubatz et al., 2000).

Other E2F family members (E2F3b and E2F6-8) have been discovered more recently and also appear to function as transcriptional repressors. E2F3b, an alternative form of the activating E2F3a, is produced from E2F3 mRNA via the use of an alternative translational start site and lacks the conserved N-terminal region found in E2F3a (He et al., 2000). E2F3b interacts with pRb and is expressed equivalently in quiescent and proliferating cells (Leone et al., 2000).

E2F6 shares homology with the domains of E2F1-5 that mediate dimerization (both the leucine zipper domain and region of homology known as the marked box) and DNA binding properties. However, it lacks the sequences that bind to the pRb, p107 and p130. E2F6 can act as a transcriptional repressor, but through a distinct, pocket-protein-independent manner (Trimarchi et al., 1998). E2F6 represses transcription through its ability to recruit the polycomb transcriptional repressor group (Trimarchi et al., 2001). E2F6 has also been found in a complex that contains Max which can bind to Myc binding sites, suggesting that E2F6 can recruit chromatin modifiers and contribute to the silencing of E2F- and Myc-responsive genes (Ogawa et al., 2002). E2F6 deficient

mice exhibit no defects in assays of proliferation or quiescence and but display similar skeletal transformations observed in polycomb mice, suggesting that the main function of E2F6 is to recruit polycomb proteins to specific target promoters during development (Storre et al., 2002).

E2F7 and E2F8 have two distinct DNA-binding domains but lack the dimerization domain as well as the transactivation and pRb-binding domain. They bind to E2F DNA consensus sites independently of DP co-factors. Both have properties of a transcriptional repressor capable of negatively influencing cellular proliferation (de Bruin et al., 2003; Di Stefano et al., 2003; Logan et al., 2004; Logan et al., 2005). The novel structure of E2F7 and E2F8 may suggest a unique role for this new sub-set of E2Fs in regulating cellular proliferation.

1.3.3 The DP family of proteins

The DP family (DP1, DP2 and DP4) function as binding partners for E2F transcription factors. DP1 (DRFT1-polypeptide-1) was first identified in 1993 as a partner for E2F1 (Girling et al., 1993). DP1 and E2F share similar DNA binding domains as well as the ability to recognize the same DNA sequence. The association of phosphorylated DP1 with E2F enhances both the DNA binding affinity and the transactivation function of the heterodimer (Bandara et al., 1993; Helin et al., 1993b).

In 1995 the second member to the family was isolated (Ormondroyd et al., 1995; Zhang and Chellappan, 1995). Like DP1, DP2 binds to E2F proteins enhancing binding and transcriptional function and modulates the function of E2F in cell cycle regulation (Wu et al., 1995). A new member of the family, DP4 was recently characterised. Like the other DP proteins it forms heterodimer with E2F, binds to E2F sites and associates with pocket proteins including pRB. However, in contrast with DP1 and DP2, DP4 reduces DNA binding activity when bound to E2F and can interfere with E2F1-dependent transcription and delay cell cycle progression (Milton et al., 2006a).

1.4 The role of E2Fs in cell cycle control

E2Fs involvement in cell cycle control is mediated by its interaction with the pocket proteins (pRb, p107 and p130). In normal cells, the activating E2Fs (E2F1-E2F3a) are specially regulated by their association with pRb, but not the related pocket-proteins (Lees et al., 1993). The ability of pRb to bind to the E2F1-E2F3a is regulated by its cell cycle dependent phosphorylation (Mittnacht, 1998). During the G₀ and early G₁ stages of the cell cycle pRb is unphosphorylated, and in this form it binds to E2F and inhibits activation. In response to growth factor stimulation, cyclin-dependent kinase (cdk) complexes are activated (Fig. 1.6).

D-type cyclins are induced in resting cells following growth factor stimulation and are expressed throughout G₁ in cycling cells, whereas cyclin E expression is induced in mid-late G₁. Subsequently the activated cdks, cdk4/cdk6-cyclin D and cdk2-cyclin E, phosphorylate pRb leading to pRb-E2F dissociation and release of E2F inhibition. Cdk2-cyclin E is unable to phosphorylate pRb in the absence of prior phosphorylation by cdk4/cdk6-cyclin D and the complete phosphorylation

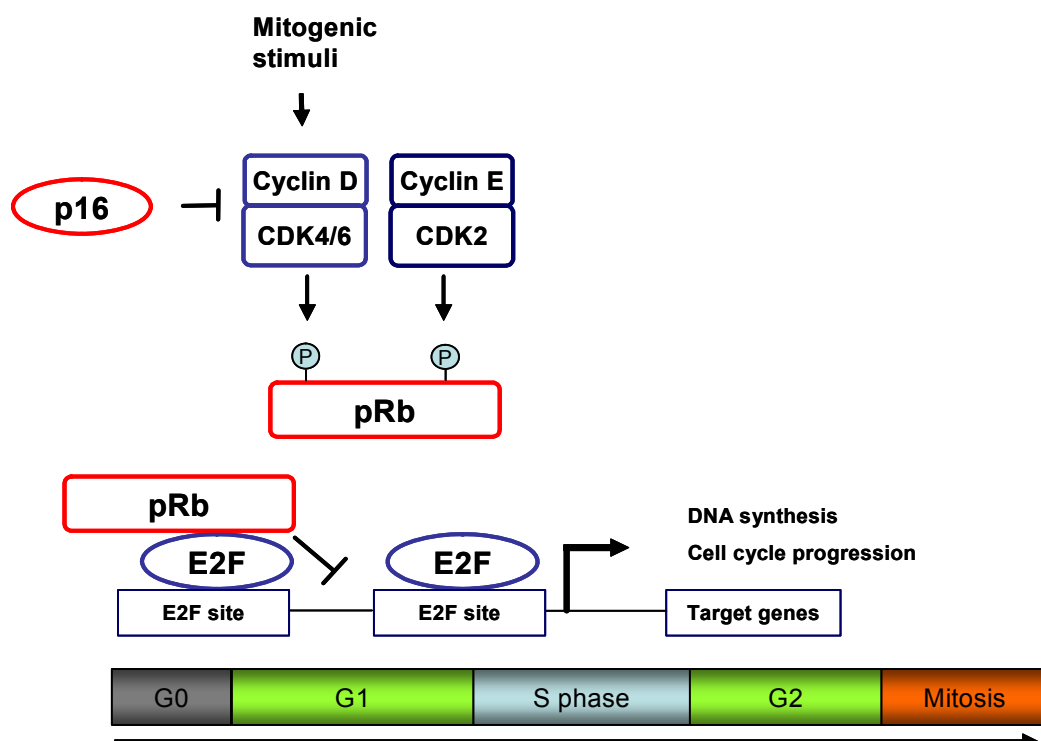


Figure 1-6 pRb binds to E2F1-3 in its unphosphorylated state and inhibits E2Fs to become transcriptionally active. In response to growth factor stimuli, activated cyclin-dependent kinase complexes phosphorylate pRb and cause it to become dissociated from E2Fs which can then activate transcription of genes involved cell proliferation.

of pRb requires both D- and E-type cdks (Hinds et al., 1992; Lundberg and Weinberg, 1998). This results in dissociation of pRb-E2F leading to activation of free E2F and transcription of E2F-responsive genes. E2F regulates several families of genes whose products are required for DNA synthesis, such as DHFR (dihydrofolate reductase) and DNA polymerase α , and for cell cycle progression, such as B-myb, cyclin A and cyclin E (DeGregori et al., 1995; Fry et al., 1997; Lam and Watson, 1993). Activation of these genes by E2F in late G₁ is sufficient to drive cells into S phase (DeGregori et al., 1995).

The transactivation domain of E2F1 is in the C-terminal region of the protein (residues 368-473). This region also holds an 18 amino-acid motif (409-426) essential for pRb binding (Helin et al., 1992). When pRb is unphosphorylated, it can repress E2F activity in two ways (Bell and Ryan, 2004). Unphosphorylated pRb binds E2F via the C-terminal pRb binding domain and inhibits E2F's ability to recruit to the basic transcriptional machinery (Dyson et al., 1993; Flemington et al., 1993). In this state E2F can occupy E2F DNA-binding sites, but is unable to activate gene expression and is therefore considered a 'passive' repressor. The other mechanism is by pRb-mediated recruitment of various chromatin modifiers such as histone deacetylases (HDACs), DNA and histone methyl transferases and polycomb group proteins to the pRb-E2F complex. These proteins are able to inhibit transcription and contribute to E2Fs 'active' repression (Ferreira et al., 2001; Robertson et al., 2000; Trimarchi et al., 2001; Trouche et al., 1997).

E2F target genes are bound by different E2F-pocket protein complexes through the cell cycle. Repressor E2F-pocket protein complexes occupy promoters in G₀ and G₁. E2F4 and p130 are the predominant complex found on promoters in quiescent cells which correlates with low levels of histone acetylation. Expression of many genes in G₁ is believed to occur through alleviation of this repression (de-repression). In late G₁, E2F4 is replaced largely by E2F1 and E2F3, which associates with histones H3 and H4 acetylation and gene activation. The activating E2Fs are most prevalent in early S phase as E2F target genes are highly expressed (Takahashi et al., 2000). However, some promoters are bound by E2F complexes both in late G₁ and during mid-S phase which contradicts current model. It has also been shown that several different E2F-pocket protein complexes can bind to the same DNA site simultaneously suggesting that cell

cycle regulation by E2F is a complex process and not yet completely understood (Wells et al., 2000).

In order to use a simpler system, the dE2F/dDP/RBF pathway present in *Drosophila melanogaster* has been utilized. *Drosophila* contains two E2F genes, one DP gene and two RB family members. These function in a similar way as their homologues in mammals. dE2F1 is a potent activator of transcription whereas dE2F2 is a repressor and both dimerize with dDP. Like pRb, RBF1 binds both E2Fs, whereas RBP2 interacts only with the repressor, dE2F2. Conversely, dE2F2/RBF1 or dE2F2/RBF2 complexes have been found to repress gene activation in *Drosophila melanogaster* in actively proliferating cells. These genes, not expressed in a cell cycle dependent manner, proved to be genes involved in cell differentiation (Dimova et al., 2003). Further analysis using this system may be able to clarify some of the complexities experienced in mammalian E2F mediated cell cycle regulation.

1.5 E2F1 regulation

E2F1 is both the founding member of the E2F1 family and is also the best studied. It is comprised of 473 residues and several defined domains that have high homology to the other family members. E2F1 has a cyclin A binding domain mapped to residues 67-108 and a nuclear localization signal (NLS) that is located directly after the cyclin A domain. The domain responsible for DNA binding runs from residue 120-191. The DNA binding domain is followed by a homologous hydrophobic repeat which is involved in homo- and hetero-dimerization called the leucine zipper. The transactivation domain is contained within residues 380-437. The pocket protein binding domain lies within the transactivation domain and maps to residues 409-426. The ARF binding domain is also found within the transactivation domain and is located on the far C-terminal end of the E2F1 poly-peptide (Mundle and Saberwal, 2003).

Several proteins involved in the cell cycle regulation are actively degraded at defined points during the cell cycle. In some cases, their degradation is essential to ensure accurate progression through the cycle. Cyclins, cyclin dependent kinases (cdks) and cdk inhibitors are degraded actively by the ubiquitin-proteasome pathway (Ciechanover, 1994; Hochstrasser, 1995).

Ubiquitin molecules are bound to lysine residues of the target protein.

Ubiquitination of proteins are performed by three different enzymes, an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and an ubiquitin-protein ligase (E3) (Scheffner et al., 1995). Poly-ubiquitinated proteins are then recognized by the 26S protease complex, the proteasome, which degrades the targeted protein.

E2F1 protein levels are also regulated by the ubiquitin-proteasome-dependent degradation pathway. Targeting of E2F1 for ubiquitination can occur through the C-terminal activation domain which also holds the pRb binding domain. The physical association of pRb blocks E2F1 ubiquitination and stabilizes E2F1 by protecting it from degradation (Campanero and Flemington, 1997; Hateboer et al., 1996b; Hofmann et al., 1996).

Events that affect pRb binding to E2F can therefore regulate E2F1's stability and activity during normal cell cycle. Phosphorylation of pRb in its C-terminal region by cdk4/cdk6-cyclin D releases HDAC activity from pRb and ablates the repressive action of pRb. This does not affect its E2F1 binding but promotes the phosphorylation of pRb by Cdk2-cyclin E resulting in complete dissociation from E2F1 (Harbour et al., 1999). Phosphorylation of E2F1 itself may also be important in the regulation of its activity during the cell cycle. E2F1 is phosphorylated at serine 332 and 337 residues by cdk4-cyclin D which prevents its interaction with pRb (Fagan et al., 1994).

E2F1 is also phosphorylated by cdk2-cyclin A. Whereas phosphorylation by cdk2-cyclin E alleviates pRb repression, phosphorylation by cdk2-cyclin A is able to reverse E2F1 transactivation. During S-phase as cyclin A concentrations increases, cdk2-cyclin A complex is able to bind a specific domain (residues 67-108) that is N-terminal to its DNA binding domain. After forming a stable complex with E2F1, the cdk2-cyclin A complex phosphorylates serine 375 on E2F1 and reduces the DNA binding ability of E2F1/DP1 heterodimer (Dynlacht et al., 1994; Krek et al., 1995; Xu et al., 1994). The cdk2-cyclin A complex can also phosphorylate DP1 leading to loss of E2F1 DNA binding ability (Guida and Zhu, 1999). This is believed to be a way to suppress activation of free E2F1 at the end of the cell cycle. Disruption of the cyclin A binding domain of E2F1

increases its stability and can lead to apoptosis, emphasizing on the importance of tight regulation of E2F1 by cdk2-cyclin A (Krek et al., 1995).

Another protein involved in mediating the activity of the transactivation domain of E2F1 is Mdm2. Mdm2 makes a functional contact with the transactivation domain of E2F1 and increases its activation function (Martin et al., 1995). The mechanism of Mdm2-mediated activation is unclear. It is unlikely that pRb displacement from E2F1 is the mechanism since the binding sites for Mdm2 and pRb are not overlapping.

The deacetylase activity bound to pRb appears to maintain E2F1 in deacetylated inactive form. E2F1 binds CBP co-activator via a small region in the activation domain without affecting its binding with pRb or Mdm2. CBP binding to E2F1 stimulates E2F1 activity (Trouche et al., 1996). Following dissociation of phosphorylated pRb, E2F1 is open to acetylation of lysine residues at 117, 120 and 125, positioned in or just outside the DNA binding domain toward the N-terminus. Acetylation on these residues enhances E2F1's stability as well as DNA binding activity. The acetyl transferase enzyme complex CBP/pCAF appears to be responsible for this acetylation, but whether CBP binding to the C-terminus of E2F1 is essential for its acetylation is not clear (Martinez-Balbas et al., 2000).

A class of E3 ubiquitin-protein ligases, referred to as SCF complexes (SKP1-CDC53 (cullin)-F-box protein complexes) have been identified which use F-box-proteins to recruit specific targets for ubiquitination by a relevant E2 enzyme. The F-box-containing protein p45^{SKP2} is a cell-cycle-regulated component of the ubiquitin protein ligase SCF^{SKP2} and recognizes substrates for this ligase. The N-terminal of E2F1 interacts with p45^{SKP2} leading to ubiquitination of E2F1. Disruption of the interaction between E2F-1 and p45^{SKP2} results in a reduction in ubiquitination of E2F1 and the stabilization and accumulation of transcriptionally active E2F-1 protein (Marti et al., 1999). E2F1 accumulates in late G1 phase but is rapidly degraded in S/G2 phase and SCF^{SKP2} mediated degradation of E2F1 may be one mechanism through which E2F is downregulated at the end of the cell cycle.

The alternative reading frame (ARF) protein, encoded from the INK4a locus, has been shown to promote growth inhibition, which in some cases can be overcome by ectopic E2F1 expression. ARF interacts with E2F and targets it for protein

degradation (Martelli et al., 2001). The mechanism for ARF-mediated E2F1 degradation is not clear. Whether Mdm2 is involved in the ARF-E2F1 effect is not known. The possibility that ARF binding to E2F1 leads to SCF^{SKP2} mediated degradation has also not been confirmed.

Nevertheless, Mdm2 has been shown to promote E2F1 stability by displacing SCF^{SKP2} from E2F1 and therefore inhibit its ubiquitination. Direct binding of E2F1 and Mdm2 is necessary for the negative effects of Mdm2 on E2F1 ubiquitination, but E2F1 downregulation is not due to either pRb or ARF (Zhang et al., 2005).

It has been noted that subcellular localization can also provide E2F regulation. The activating E2Fs have basic nuclear localization signal (NLS) in their N-terminal domain that is sufficient to mediate their nuclear localization. E2F1 accumulates in the nucleus at times which correlates with its activity. By contrast, the repressive E2Fs lack NLS and have to depend on either DP1 or pocket protein for their localization. The repressive E2Fs have a hydrophobic nuclear export signal (NES) and are mostly detected in the cytoplasm (Magaie et al., 1996; Muller et al., 1997; Verona et al., 1997).

1.5.1 E2F1 is stabilized following DNA damage

The regulation of E2F1 activity and stability during the cell cycle has been well documented. In addition, it has recently been shown that E2F1 levels increase following DNA damage. The first indication came in 1997 when Huang et al. showed that ionizing radiation increased the expression of E2F1 (Huang et al., 1997). Since then many reports have described E2F1 stabilization following various DNA damaging agents and chemotherapeutic drugs. E2F1 is upregulated in response to DNA damage caused by irradiation with X-rays or UVC, or following treatment with the DNA damaging agents actinomycin D, adriamycin and etoposide in a manner analogous to that of p53 (Blattner et al., 1999; Hofferer et al., 1999; Meng et al., 1999; O'Connor et al., 1995). This induction is specific to E2F1 rather than other members of the family and exhibits kinetics that closely resembles the induction of p53 (Blattner et al., 1999).

Recent studies have shed some clarity on the modifications that occur and the signalling pathways involved in E2F1 stability. They have also connected E2F1 induction with apoptosis in response to DNA damage. E2F1 is a target of DNA

damage-responsive protein kinases. E2F1 is phosphorylated at serine 31 by ATM/ATR kinase and by Cck2 kinase at serine 364 following DNA damage (Lin et al., 2001; Stevens et al., 2003). Both phosphorylation events lead to increased E2F1 protein stability and the induction of apoptosis. The ATM/ATR site at serine 31 is within the Skp2 binding domain and it has been suggested that phosphorylation at this site might alter the degradation of E2F1 by inhibiting Skp2 binding and therefore prevent the normal turnover of E2F1. Induction of both apoptosis and p53 phosphorylation by E2F1 is abolished by caffeine, which inhibits ATM/ATR kinases, this supports the notion that E2F1 uses the ATM signalling pathway to induce p53 and apoptosis (Powers et al., 2004; Sarkaria et al., 1999).

E2F1 is able to interact with Mdm2 via the E2F1 transactivation domain (residues 390-406) and this stimulates the activation capacity of E2F1/DP1 (Martin et al., 1995). Mdm2 expression can also lead to E2F1 stabilization through the E2F1 ubiquitination pathway (Zhang et al., 2005). Mdm2 inhibition can lead to an increase in expression of E2F1, similar to the way in which p53 is induced following Mdm2 removal (Blattner et al., 1999). Mdm2 expression has also been shown to promote degradation and antagonize the apoptotic properties of E2F1 (Loughran et al., 2000). Despite the controversy, given that E2F1 and p53 are similarly regulated by ATM/ATR and Chk2 kinases, it has been suggested that disruption of the E2F1-Mdm2 interaction might also play a role in the E2F1 induction following cellular stress. The Chk2 phosphorylation site at serine 364 is positioned close to the Mdm2 binding domain, but whether phosphorylation at this site affects Mdm2 binding is not clear.

It has been suggested that E2F1 phosphorylation may lead to stabilization through a member of the 14-3-3 family proteins. 14-3-3 proteins are a family of dimeric phosphoserine/phosphothreonine-binding proteins and are involved in a wide spectrum of biological processes (Fu et al., 2000). 14-3-3 τ interacts with ATM-phosphorylated (at serine 31) E2F1 during DNA damage and inhibits E2F1 ubiquitination. 14-3-3 τ is also required for the expression and induction of several E2F1 apoptotic target genes as well as apoptosis following DNA damage (Wang et al., 2004). Possible mechanism for this function includes 14-3-3 τ inhibition of E3 ligase binding, similar to the manner in which 14-3-3 σ inhibits Mdm2-mediated degradation of p53 following adriamycin treatment (Yang et al.,

2003). Another member of the family, 14-3-3 ϵ is able to bind DP2 via a region in the NLS of DP2. This interaction alters the cell cycle and apoptotic properties of E2F. In contrast with the effect of 14-3-3 τ on E2F1, etoposide treatment causes a decrease in the 14-3-3 ϵ -DP2 interaction and a subsequent induction in DP2 itself (Milton et al., 2006b).

Phosphorylation of p53 following DNA damage has been shown to promote the subsequent acetylation of lysine residues within its C-terminal region, leading to enhanced p53 DNA binding and translational activity (Sakaguchi et al., 1998). Whether phosphorylation of E2F1 promotes acetylation is not known. Nevertheless, E2F1 is acetylated by both p300/CBP and P/CAF at sites adjacent to the DNA binding domain (K117, K120, K125) which leads to increased stability, DNA binding and activation of E2F1 (Martinez-Balbas et al., 2000; Marzio et al., 2000). E2F1 acetylation occurs following DNA damage which requires P/CAF acetyltransferase activity and this leads to activation of E2F1 target genes involved in apoptosis rather than genes involved in cell cycle progression (Ianari et al., 2004; Pediconi et al., 2003). Proteosomal degradation requires that proteins are first targeted for destruction by ubiquitination of lysine residues. Therefore, it is possible that the increase in E2F1 stability observed is a result of a phosphorylation-acetylation cascade that prevents E2F1 turnover.

E2F1 is regulated by the co-activator and acetyltransferase p300 after DNA damage by a mechanism that links acetylation and ubiquitination. An acetylated form of E2F1 accumulates in cells following DNA damage, which can further be increased by p300 expression. Accumulation of ubiquitinated E2F1 is also induced by the same DNA damaging agents, which is in contrast to what could be expected since ubiquitination usually leads to rapid turnover of proteins. The increase in ubiquitinated E2F1 is again increased by p300 and requires acetylation of the three known acetylation sites (K117, K120, K125) (Galbiati et al., 2005). p300-induced E2F1 ubiquitination does not depend on the p45^{SKP2} E3 ligase since ubiquitination can occur with an E2F1 mutant devoid of the p45^{SKP2}-binding domain and is not influenced by pRb.

Another level of regulation of E2F1 activity following DNA damage is its ability to interact with p53 via the cyclin A binding site of E2F1. Cyclin A binds to E2F1

and prevents E2F1 from binding and cooperating with p53 to induce apoptosis. However, following DNA damage, cyclin A levels decrease with a parallel increase in E2F1-p53 formation, suggesting that E2F1 can directly stimulate apoptotic function of p53 following cellular stress (Hsieh et al., 2002). The induction of E2F1 was shown to be independent of transcriptional activity and Mdm2 binding.

1.6 E2F's role in cancer

Following the observation that showed E2F to be deregulated by a transforming virus it was speculated that E2F might be associated with cancer. pRb was subsequently found to be targeted by other viral oncoproteins, including SV40 large T antigen and E7 proteins from 'high-risk' human papilloma viruses (Chellappan et al., 1992). It is now thought, E2F is deregulated in most human cancers. This can occur by loss or mutation of Rb, viral infection, upregulation of the cdk/cyclin complexes that phosphorylate pRb or through loss of the cdk inhibitor p16 (Hall and Peters, 1996; Harbour et al., 1988).

1.6.1 Oncogene or tumour suppressor

Studies on E2F1 indicate that it may have a unique role compared to other E2Fs, showing characteristics of both an oncogene and a tumour suppressor (Pierce et al., 1999). A number of studies suggest that E2F1 functions as a oncogene by promoting the proliferation of cells beyond their normal constraints (Cress et al., 1993). Many genes that are involved in regulation of the cell cycle are direct targets of E2F1, such as Cdc2, cdc25a and cyclin E and genes which play a functional role in DNA synthesis, including thymidine kinase and DNA polymerase α (DeGregori et al., 1995; Dyson, 1998; Ohtani et al., 1995; Vigo et al., 1999). In addition, it has been shown that overexpression of E2F1 in quiescent cells is sufficient to induce entry into DNA synthesis, and E2F1 can function as an oncogene in transforming assays (Johnson et al., 1994; Lukas et al., 1996).

Additional support for E2F1's oncogenic potential comes from studies in mice carrying a germ-line mutation or deletion of the Rb gene. Embryos homozygous for mutation in Rb die between days 14 and 15 of gestation with defects in erythropoiesis, cell cycle control and apoptosis while Rb heterozygous mice

display pituitary tumours (Hu et al., 1994; Jacks et al., 1992). This phenotype can be modified by deletion of E2F1. Loss of E2F1 reduces the frequency of pituitary and thyroid tumours that occur in $Rb^{+/-}$, and greatly lengthens the lifespan if $Rb^{+/-}$; $E2F1^{-/-}$ animals (Yamasaki et al., 1998). In addition, $Rb/E2F1$ double mutants show significant suppression of apoptosis and S phase entry in certain tissues compared to Rb mutants, suggesting E2F1 is a critical mediator of these effects (Tsai et al., 1998). E2F3 has also been shown to make a contribution to the effect resulting from pRb loss. E2F3 mutation completely suppresses both the inappropriate proliferation and apoptosis arising in the Rb mutant embryos (Ziebold et al., 2001).

On the other hand, targeted deletion of the E2F1 gene in mice resulted in animals that developed and reproduced normally. However, $E2F1^{-/-}$ mice have excess mature T cells due to a defect in thymocyte apoptosis. As these mice age they exhibit a second phenotype marked by abnormal cell proliferation (Field et al., 1996). This suggests that in addition to promoting cell division, E2F1 has a role in regulating apoptosis and can suppress cell proliferation in some instances. The confirmation of E2F1's tumour suppressor function came when Yamasaki et al showed that mice lacking E2F1 develop broad spectrum of tumours in a number of tissues (Yamasaki et al., 1996).

Mouse models have also been used to explore the affect of increased or deregulated E2F1 activity. Increases in E2F1 activity can promote tumourigenesis by co-operating with v-Ha-ras transgene to induce skin tumours indicating that deregulated E2F activity can contribute to tumour development (Pierce et al., 1998a). Increased E2F1 activity can also promote tumourigenesis by co-operating with p53 deficiency to induce spontaneous skin carcinomas, verifying that increased E2F1 expression can contribute to tumour development. This suggests that p53 plays an important role in eliminating cells with deregulated E2F1 activity (Pierce et al., 1998b).

However, Pierce et al also showed that E2F1 can have tumour-suppressive properties in a transgenic model. As transgenic mice age they are predisposed to develop spontaneous tumours in a variety of tissues, but on the other hand, they are found to be resistant to skin tumour development following carcinogenic promotion (Pierce et al., 1999). These results demonstrate that

increased E2F1 activity can either promote or inhibit tumourigenesis in mice models, depending upon the experimental context.

The role of E2F1 in UV-induced apoptosis in vivo has also been assessed. Contrary to expectations, E2F1^{-/-} mice demonstrate enhanced keratinocyte apoptosis after UVB exposure, whereas apoptosis is suppressed by epidermis-specific overexpression of human E2F1. Apoptosis induced by γ -radiation was also repressed by E2F1, which implies that E2F1 can function as a suppressor of an apoptosis pathway that is initiated by DNA damage (Wikonkal et al., 2003). The reason for this function of E2F1 might lie in its role in DNA repair since inhibition of UV-induced apoptosis by E2F1 correlates with a stimulation of DNA repair. Mice lacking E2F1 are impaired in their ability to remove DNA photoproduct, while E2F1 transgenic mice repair UV-induced DNA damage at an accelerated rate compared to wild type mice (Berton et al., 2005).

1.7 E2F1 induced apoptosis

E2F1 is the only family member that is induced following DNA damage and most studies show that induction of apoptosis is a unique property of E2F1 (DeGregori et al., 1997; Kowalik et al., 1998; Leone et al., 2001; Lissy et al., 2000).

However, some research has shown that the other activating members, E2F2 and E2F3 also have this capacity (Vigo et al., 1999; Ziebold et al., 2001). However, recent work has shown that apoptosis induced by E2F3 is associated with accumulation of E2F1 and that E2F3-induced apoptosis is dependent on E2F1 (Dench and Helin, 2005). This suggests that accumulation of crucial levels of E2F1 activity, but not total E2F1 activity, is essential for the induction of apoptosis.

We have gained more understanding of the pathways involved in E2F1-induced apoptosis in recent years. Both overexpression experiments and mutant mouse models of E2F1 have shown that apoptosis can occur by mechanisms either dependent or independent of the tumour suppressor p53 (Fig. 1.7) (Field et al., 1996; Kowalik et al., 1995; Macleod et al., 1996; Pan et al., 1998; Qin et al., 1994; Shan and Lee, 1994; Vigo et al., 1999; Wu and Levine, 1994).

1.7.1 p53 dependent E2F1-induced apoptosis

Some studies show that the ability of E2F1 to promote apoptosis is at least partially dependent on p53. p53 mutant cells infected with an E2F1 adenoviral vector display attenuated apoptosis activity whereas augmentation of wild type p53 expression enhances cell death (DeGregori et al., 1995; Qin et al., 1994; Wu and Levine, 1994). p53 levels rise during E2F1 mediated apoptosis which corresponds with an increase in ARF expression (Bates et al., 1998; Guida and Zhu, 1999; Hiebert et al., 1995; Kowalik et al., 1998). The ARF protein binds to MDM2, and by doing so it blocks the ability of MDM2 to inactivate and ubiquitinate p53, which normally leads to p53 degradation (Bates et al., 1998; Honda and Yasuda, 1999; Kamijo et al., 1998; Pomerantz et al., 1998; Zhang et

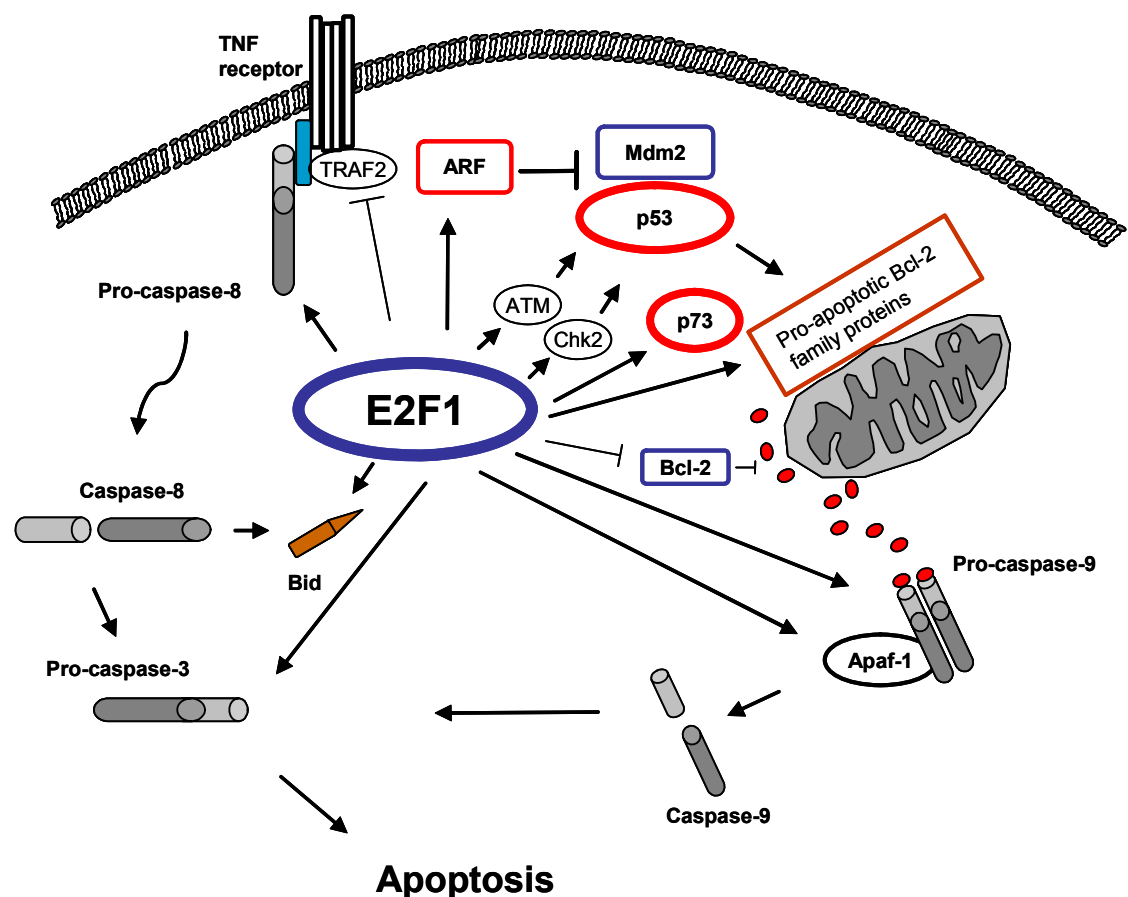


Figure 1-7 E2F1 can induce apoptosis in cells with and without functional p53 protein. E2F1 transcriptionally activates genes such as ARF, ATM and Chk2 leading to activation of p53 and apoptosis. But E2F1 has also been shown to activate genes leading to apoptosis in a p53-independent manner. Caspases 3, 7, 8 and 9 have all been shown to be upregulated by E2F1 activation as well as Apaf1, some pro-apoptotic Bcl-2 proteins and the p53 family member p73. Activation of these proteins all lead to increased apoptosis. E2F1 can also induce apoptosis by downregulating anti-apoptotic genes such as TRAF2 and Bcl-2.

al., 1998). E2F1 directly transactivates the ARF gene thereby promoting p53 stability (Bates et al., 1998). Indeed, the loss of ARF, as well as the overexpression of MDM2, impedes the ability of E2F to promote p53-dependent apoptosis (de Stanchina et al., 1998; Kowalik et al., 1998).

E2F1 can also induce apoptosis in a p53 dependent manner in mice and cells which lack ARF (Rogoff et al., 2002; Russell et al., 2002; Tolbert et al., 2002; Tsai et al., 2002). Several kinases phosphorylate and thus activate p53 upon DNA damage, including ATM/ATR, Chk1 and Chk2 (Banin et al., 1998; Canman et al., 1998; Shieh et al., 2000). E2F1 can directly activate transcription of both ATM and Chk2 genes, which phosphorylated p53 and alleviate its Mdm2-mediated degradation (Berkovich and Ginsberg, 2003). Therefore, ATM and Chk2 are most likely responsible for mediating the E2F1-driven phosphorylation of p53.

E2F1 also activates pro-apoptotic co-factors of p53. E2F1 (and E1A expression) upregulates the expression of ASPP1, ASPP2, JMY and TP53INP1 through a direct transcriptional mechanism (Chen et al., 2005; Fogal et al., 2005; Hershko et al., 2005). In addition, these factors then enhance the ability of p53 to induce cell death. It was also shown that activation of E2F1 leads to phosphorylation of p53 on serine 46 and this modification is important for E2F1-p53 cooperation in apoptosis.

1.7.2 p53 independent E2F1-induced apoptosis

In cell types lacking p53, for example, Saos-2 osteosarcoma cells, E2F1 has still been shown to induce death in absence of p53. E2F1 overexpression caused atrophy in testicles and sterility through a process involving increased apoptosis in mice which is independent of functional p53, since p53-nullizygous transgenic mice overexpressing E2F1 also suffer testicular atrophy (Holmberg et al., 1998).

In the absence of p53, the p53 family member, p73, may contribute to apoptosis. Expression of E2F1 in p53 nullizygous mice result in the induction of p73 expression along with transcription of p73 targets such as p21 and MDM2 (Irwin et al., 2000). Furthermore, inhibition of p73 function partially protects cells from E2F1-induced apoptosis (Stiewe and Putzer, 2000; Zaika et al., 2001).

E2F1 may also cause cellular death by directly targeting genes that regulate the apoptotic pathway. Deregulation of E2F1/DP1 in p53^{-/-} MEF or enforced E2F1 expression have been shown to give rise to an increase in expression of caspases 3, 7, 8 and 9 (Budd, 2001; Hitchens and Robbins, 2003; Nahle et al., 2002).

Additional members of the intrinsic apoptotic pathway are targets of E2F1. E2F1 and p53 both upregulate Apaf1 expression, but E2F1 also induces Apaf1 in the absence of p53 demonstrating that the E2F1 can directly control Apaf1 transcription (Furukawa et al., 2002; Moroni et al., 2001). E2F1 may also modulate the expression of the pro-apoptotic Bcl-2 family members Bad, Bak and Bid, which are responsible for governing the release of mitochondrial proteins such as cytochrome C (Stanella et al., 2002). E2F1 can also up-regulate the expression of the pro-apoptotic BH3-only proteins PUMA, Noxa and Bim, through a direct transcriptional mechanism (Biswas et al., 2005; Cao et al., 2004).

In addition to direct activation of pro-apoptotic genes, a second mechanism by which E2F1 sensitizes cells to apoptosis is inhibition of anti-apoptotic genes. E2F1 overexpression suppresses Bcl-2 protein and RNA levels, and restoration of Bcl-2 protein effectively blocks the accelerated apoptosis that occurs when E2F1 is overexpressed (Eischen et al., 2001).

E2F1's ability to inhibit survival factors to induce apoptosis is also supported by a study showing inhibition of necrosis factor κ B (NF- κ B). NF- κ B is stimulated via TNF receptor-associated factor 2 (TRAF2) following activation of TNF receptor, which contributes to inhibition of cell death (Chen and Goeddel, 2002). E2F1 induces apoptosis by a death receptor-dependent mechanism, by downregulating TRAF2 protein levels thereby inhibiting anti-apoptotic NF- κ B signaling (Phillips et al., 1999).

A number of reports have indicated that mutants of E2F1 which lack the transactivation domain are also able to induce death (Hsieh et al., 1997; Liu and Greene, 2001; Phillips et al., 1997). This suggests that induction of apoptosis by E2F1 may be partially mediated through alleviation of E2F-dependent transcriptional repression. Furthermore, only 75 amino acids from within the DNA binding domain of E2F1 is sufficient for cell death (Bell et al., 2006). This

domain of E2F1 does not bind DNA and is consequently unable to transactivate, repress or de-repress E2F target genes.

Overall, it is now clear that E2F1 can induce apoptosis by various mechanisms. E2F1-induced apoptosis can signal through both the extrinsic- and intrinsic- apoptotic pathways and be both caspase-dependent and caspase-independent. E2F1 can also inhibit anti-apoptotic pathways to induce apoptosis. In addition, the ability to both induce p53 expression and activity as well as promote expression of pro-apoptotic proteins downstream of p53 establishes the importance of E2F1 in apoptosis.

1.8 E2F1 pathway as a therapeutic target

Many anticancer studies set out either to boost cell death or to impede anti-apoptotic and proliferative pathways. For example, the restoration of the apoptotic pathway by reintroduction of p53 or activation of Apaf-1 increases the sensitivity of tumour cells to DNA damaging agents (Roemer and Friedmann, 1994; Soengas et al., 2001). However, mutant p53 in tumour cells can in some conditions weaken the function of wild type p53, and result in ineffective p53-based gene therapy (Stanelle and Putzer, 2006). Therefore, targets that compensate for or bypass cell death defects, regardless of p53 status, can be particularly useful.

E2F1 can induce apoptosis independently of p53 status, and the stabilization of E2F1 in response to DNA damage implies a role for E2F1 as a stress response gene that may play a role in enhancing chemosensitivity. Preclinical experiments using E2F1 as an anti-cancer therapy have been initiated. Induction of E2F1 via adenoviral mediated gene transfer results in tumour cell apoptosis *in vivo* with relative sparing of normal tissue (Kaelin, 2003). Moreover, E2F1 can sensitize cells to chemotherapy. E2F1 increases the effectiveness of chemotherapeutic agents that are most active in S-phase cells and/or require the presence of a particular cell-cycle dependent target for the induction of cell death (for example, topoisomerase). E2F1 protein levels increase in various human cancer cell lines following treatment with the topoisomerase inhibitors adriamycin or etoposide. This occurs independently of Rb or p53 status. Induction of E2F1 in tumour cells correlates with their sensitivity to adriamycin

or to etoposide, and fibroblasts from E2F1 knockout mice are more resistant to DNA damage than cells from normal mice. Overexpression of wild type E2F1 protein in various tumour cell lines (following transfection or infection with an adenovirus encoding wild-type E2F1) leads to enhanced cytotoxicity following exposure to DNA damaging agents, which results from enhanced apoptosis (Banerjee et al., 1998; Hofland et al., 2000; Meng et al., 1999; Nip et al., 1997).

Another approach, bearing in mind that the pRb pathway is deregulated in most tumours leading to increased E2F1 activity, is to use molecules to further increase E2F1 activity throughout the cell cycle. Cdk2/cyclin A can bind and neutralize E2F1 during S phase. Inhibition of the cdk2/cyclin A binding to E2F1 causes induction in apoptosis in tumour cells containing inactive pRb but not in normal cells (Chen et al., 1999).

It has been shown that Nutlins, small molecular inhibitors of Mdm2, inhibit Mdm2-p53 interaction and activate p53 signalling in cells with wild type p53 but not mutant p53. However, recent studies showed that Nutlin-3a increased cytotoxicity of genotoxic agents in human tumour cell lines with mutant p53. Nutlin-3a inhibited the binding of E2F1 to Mdm2 and induced stabilization and transcriptional activation of E2F1 following drug induced DNA damage, indicating that antagonism of Mdm2 by Nutlin-3a in cells with mutant p53 can enhance chemosensitivity in a E2F1 dependent manner (Ambrosini et al., 2007).

E2F1's downstream target, p73 is a valuable candidate for cancer therapy in tumour cells (Bell et al., 2007). Similar to E2F1, p73 efficiently induces apoptosis and enhances chemosensitivity of cells that are primarily resistant to apoptosis mediated by wild type p53 (Rodicker and Putzer, 2003). Moreover, therapeutic efficacy of E2F1 in pancreatic cancer correlates with p73 induction (Rodicker et al., 2001).

In addition to activating pro-apoptotic E2F1 target genes, molecules that target anti-apoptotic E2F1 regulated genes have been used in clinical studies in an attempt to hinder tumour growth. For example, anti-apoptotic Bcl-2 has been targeted by antisense oligonucleotides approaches that interfere with Bcl-2 activity, resulting in less Bcl-2 expression and in induction of apoptosis (Jansen et al., 2000; Klasa et al., 2002).

Overall, these results show that DNA damage can cooperate to selectively induce p53-independent apoptosis in cells that have deregulated E2F1 activity resulting from mutations in the pRb pathway. This implicates an important role for E2F1 in p53-independent sensitization to chemotherapy and provides a basis for pharmacologically increasing E2F1 protein levels for therapeutic gain.

1.9 Overview

Apoptosis is a complex process that regulates the destruction of a damaged cell. Therefore, it is thought that targeting the reactivation of apoptosis in malignant cells may be an effective method of cancer therapy. Treatment with existing chemotherapeutic drugs leads to changes in networks of proliferation, survival and apoptotic genes, some of which are differentially expressed in normal and malignant cells, leading to initiation of apoptosis.

The activation of the cell death pathway is influenced by the activity of many cellular genes. The transcription factor E2F1 has been shown to play an important role in apoptosis. E2F1 has the ability to induce both cell cycle progression and programmed cell death, leading potentially to both tumour-promoting and tumour-suppressive effects (DeGregori et al., 1997). The pathway to cell cycle progression is well known while the pathway to apoptosis is less well defined and more complex

In normal cells apoptosis can occur in response to DNA damage. Activation of oncogenes also sensitizes cells to apoptosis following treatment with chemotherapeutic drugs that cause DNA damage. E2F1 has been shown to respond to DNA damage and oncogene activation and may therefore play a vital role in tumour suppression and during cellular responses to chemotherapy. Therefore, investigating the response of E2F1 to DNA damage and oncogene activation should contribute to the understanding of E2F1's role in chemosensitivity.

Chapter 2: Materials and methods

2 Chapter 2: Materials and methods

Table 2-1 Composition of routinely used solutions and media

Substance	Composition
Phosphate Buffered Saline (PBS)	170 mM NaCl, 3.3 mM KCl, 1.8 mM Na ₂ HPO ₄ , 10.6 mM KH ₂ PO ₄
Tris-Buffered Saline (TBS)	25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 5 mM KCl
TBST	TBS + 0.1% Tween-20
L-Broth (LB)	1% Bacto-tryptone, 86 mM NaCl, 0.5% yeast extract
LB Agar	LB + 1.5% agar
Tris-EDTA (TE)	10 mM Tris-HCl, pH 8.0, 1 mM EDTA
Tris-acetate-EDTA (TAE)	40 mM Tris, 0.1% glacial acetic acid, 1 mM EDTA
2 x Western Sample Buffer	100 mM Tris, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 15% glycerol, bromophenol blue
SDS Running Buffer	0.1% SDS, 192 mM glycine, 25 mM Tris pH8.3
Transfer Buffer	192 mM glycine, 25 mM Tris, 20% methanol, 0.01% SDS
Tris Borate-EDTA (TBE)	45 mM Tris, 45 mM Boric acid, 0.625 mM EDTA pH 8.0
5% BSA (in TBST)	5% Albumin, bovine serum (Sigma), 0.01% Sodium Azide, TBST
5 x Western Sample Buffer	60 mM Tris-HCl, pH 6.8, 2% SDS, 14.4 mM β-mercaptoethanol, 25% glycerol, 0.1% bromophenol blue

2.1 Cell culture and treatments

2.1.1 Cell culture

Mouse Embryo Fibroblast (MEF) cells and Retinal Pigment Epithelial (RPE) cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, (FCS, Autogenbioclear), 2 mM L-glutamine, 60 µg/ml penicillin and 100 µg/ml streptomycin. Cells were routinely grown in a humidified atmosphere containing 5% CO₂ at 37°C. Cell culture was

performed in a Class II hood, using aseptic technique and sterile equipment and reagents.

Cells were passaged when sub-confluent; approximately every 2-3 days. After media was aspirated from the flask, 5ml of PBS was added to the cells then aspirated immediately. 4 ml TE + 0.25% trypsin was added and left for approximately 2 minutes at 37°C. Following trypsinisation fresh media was immediately added to the dissociated cells in order to neutralise the trypsin. Cells were centrifuged at 1000g for 5 minutes at room temperature and the media removed. The pelleted cells were then resuspended in fresh media at a ratio of 1:3 to 1:4.

Cryo-freezing was used for storage of all cell lines. Cells were trypsinised as described and following pelleting by centrifugation, cells were resuspended in a solution of 90% FCS, 10% dimethylsulphoxide (DMSO). Cells were aliquotted into cryo-tubes and frozen at 80°C overnight and then transferred to liquid Nitrogen. Thawing of cells was performed rapidly by placing cryo-tubes in a water bath at 37°C until just thawed. Cells were then mixed with fresh media, centrifuged at 1000g for 5 minutes and the supernatant aspirated off to ensure removal of DMSO prior to resuspension in 10% FCS DMEM

2.1.2 Transient transfection of plasmid DNA

Cells were seeded onto 100 mm tissue culture plates the day prior to transfection. A total of 15 µg plasmid DNA in 440 µl distilled H₂O (Invitrogen) was mixed with 500 µl 2 x HBS (50 mM HEPES, 250 mM NaCl, 1.5 mM NaHPO₄ pH 7.12) and 60 µl 2 M CaCl₂ dropwise with vigorous mixing. DNA calcium phosphate precipitate was allowed to form for 30 minutes at 37°C then added dropwise to tissue culture plates. The precipitate was removed after 16 hours and the cells washed twice in DMEM before 10% FCS DMEM added.

2.1.3 Retroviral infections

Retroviral infections were carried out using Phoenix-Ampho or Phoenix-Eco retroviral packaging cells which were maintained as described above. 2x10⁶ phoenix cells were plated in a 100 mm dish for each retroviral infection. The following day cells were transfected with 15 µg retroviral vector DNA for 16hours

as described above, then three harvests of infectious supernatant were collected in DMEM containing 20% FBS at 12 hour intervals. MEFs and RPEs were seeded at 0.75×10^6 / 100mm dish 24 hours before infection. Retroviral supernatants were purified through 0.45 μ m filter then added to the MEFs or RPEs cultures together with polybrene (hexadimethrine bromide; Sigma) at a final concentration of 5 μ g/ml. Three rounds of infection were undertaken at 12 hour intervals with retroviral pLPC vector alone or pLPC with insert of interest. After a 24 hour recovery period in 10% FCS DMEM, MEF cells were selected for four days in 2.5 μ g/ml puromycin (sigma) and RPE cells in 5.0 μ g/ml puromycin for 6 days.

2.1.4 Stable cell lines created using retroviral infection

Cell lines stably expressing scrambled short hairpin or shE2F1 were made using Phoenix-Ampho as a packaging cell and RPE and RPE E1A ER cells as a target cell line. Following retroviral infection, as described above, and 24 hour recovery period in 10% FCS DMEM, cells were selected in 5.0 μ g/ml puromycin for 6 days.

The following E2F1 knockdown cell lines were created.

Table 2-2 RPE cell lines with E2F1 knockdown

Cell line	Vector used
RPE shScr	pSuper Retro scramble short hairpin
RPE shE2F1	pSuper Retro E2F1 short hairpin
RPE E1A ER shScr	pSuper Retro scramble short hairpin
RPE E1A ER shE2F1	pSuper Retro E2F1 short hairpin

Cell lines stably expressing wild type E1A, wild type E2F1 or deletion mutants of E1A were made using Phoenix-Ampho as a packaging cell and PRE cells as a target cell line. Following retroviral infection, as described above, and 24 hour recovery period in 10% FCS DMEM, cells were selected in 5.0 μ g/ml puromycin for 6 days.

The following RPE cell lines were created.

Table 2-3 RPE cell lines expressing E1A truncations

Cell line	Vector used
RPE pLPC	pLPC
RPE E1A	pLPC wt E1A 12S
RPE E2F1	pLPC E2F1
RPE E1A Δ CR2	pLPC E1A Δ CR2
RPE E1A R2G	pLPC E1A R2G
RPE E1A Δ 2-11	pLPC E1A Δ 2-11
RPE E1A Δ 2-24	pLPC E1A Δ 2-24
RPE E1A Δ 2-36	pLPC E1A Δ 2-36
RPE E1A Δ 26-35	pLPC E1A Δ 26-35
RPE E1A Δ 48-60	pLPC E1A Δ 48-60
RPE E1A 143	pLPC E1A 143

Cell lines stably expressing wild type E2F1, truncated and/or mutated forms of E2F1 or E1A-ER fusion protein were made using Phoenix-Eco as a packaging cell and wild type MEFs, E2F1^{-/-}, pRb^{-/-} or pRB family triple knockout (pRb^{-/-}, 107^{-/-} and 130^{-/-}) MEFs as a target cell line. Following retroviral infection, as described above, and 24 hour recovery period in 10% FCS DMEM, MEF cells were selected for four days in 2.5 μ g/ml puromycin.

The following MEF cell lines were created.

Table 2-4 MEF cell lines expressing E1A-ER, full length or truncations/mutants of E2F1

Cell line	Vector used
MEF FL E2F1	pLPC FL E2F1
MEF E2F1 Tr. 1 Δ 374	pLPC E2F1 Truncation 1 GFP
MEF E2F1 Tr. 2 Δ 374, 132E	pLPC E2F1 Truncation 2 GFP
MEF E2F1 Tr. 3 Δ 284	pLPC E2F1 Truncation 3 GFP
MEF E2F1 Tr. 4 Δ 245	pLPC E2F1 Truncation 4 GFP
MEF E2F1 Tr. 5 Δ 191	pLPC E2F1 Truncation 5 GFP
MEF E2F1 Tr. 6 Δ 120	pLPC E2F1 Truncation 6 GFP
MEF E2F1 Tr. 7 ¹²⁰⁻³⁷⁴	pLPC E2F1 Truncation 7 GFP

MEF E2F1 Tr. 8 ²⁴⁵⁻³⁷⁴	pLPC E2F1 Truncation 8 GFP
MEF E2F1 Tr. 9 ³¹⁷⁻³⁷⁴	pLPC E2F1 Truncation 9 GFP
MEF E2F1 Tr. 10 ²⁴⁵⁻³¹⁷	pLPC E2F1 Truncation 10 GFP
MEF E2F1 Tr. 11 ¹²⁰⁻¹⁹¹	pLPC E2F1 Truncation 11 GFP
MEF GFP	pLPC GFP
MEF E2F1 S31A	pLPC E2F1 S31A
MEF E2F1 S364A	pLPC E2F1 S364A
MEF E2F1 D390A/F391A	pLPC E2F1 D390A/F390A
MEF E2F1 S31A/S364A	pLPC E2F1 S31A/ S364A
MEF E2F1 S31A/S364A/ D390A/F391A	pLPC E2F1 S31A/ S364A/D390A/F391A
MEF E2F1 K117R/K120R/K125R	pLPC E2F1 K117R/K120R/K125R
MEF E2F1 Tr. 8 S31A	pLPC E2F1 Truncation 8 S31A GFP
MEF E2F1 Tr. 8 ²⁴⁵⁻³⁷⁴ S31A K117R/K120R	pLPC E2F1 Truncation 8 S31A K117R GFP
MEF E2F1 Tr. 8 ²⁴⁵⁻³⁷⁴ S31A K117R/K120R T49A	pLPC E2F1 Truncation 8 S31A K117R T49A GFP
MEF E2F1 Tr. 8 ²⁴⁵⁻³⁷⁴ S31A K117R/K120R T49D	pLPC E2F1 Truncation 8 S31A K117R T49D GFP
MEF E2F1 Tr. 8 ²⁴⁵⁻³⁷⁴ S31A K117R/K120R T75A	pLPC E2F1 Truncation 8 S31A K117R T75A GFP
MEF E2F1 Tr. 8 ²⁴⁵⁻³⁷⁴ S31A K117R/K120R T75D	pLPC E2F1 Truncation 8 S31A K117R T75D GFP
MEF E2F1 Tr. 8 ²⁴⁵⁻³⁷⁴ S31A K117R/K120R Y100F	pLPC E2F1 Truncation 8 S31A K117R Y100F GFP
MEF E2F1 Tr. 8 ²⁴⁵⁻³⁷⁴ S31A K117R/K120R Y100D	pLPC E2F1 Truncation 8 S31A K117R Y100D GFP
MEF E2F1 Tr. 8 S31A K117R/K120R S104A	pLPC E2F1 Truncation 8 S31A K117R S104A GFP
MEF E2F1 Tr. 8 ²⁴⁵⁻³⁷⁴ S31A K117R/K120R S104D	pLPC E2F1 Truncation 8 S31A K117R S104D GFP
MEF E2F1 Tr. 6 ^{Δ120} S121A	pLPC E2F1 DNA Binding Domain S121A GFP
MEF E2F1 Tr. 6 ^{Δ120} S121D	pLPC E2F1 DNA Binding Domain S121D

MEF E2F1 Tr. 6 Δ^{120} S126A	pLPC E2F1 DNA Binding Domain S126A GFP
MEF E2F1 Tr. 6 Δ^{120} S126D	pLPC E2F1 DNA Binding Domain S126D GFP
MEF E2F1 Tr. 6 Δ^{120} Y128F	pLPC E2F1 DNA Binding Domain Y128F GFP
MEF E2F1 Tr. 6 Δ^{120} Y128D	pLPC E2F1 DNA Binding Domain Y128D GFP
MEF E2F1 Tr. 6 Δ^{120} T130A	pLPC E2F1 DNA Binding Domain T130A GFP
MEF E2F1 Tr. 6 Δ^{120} T130D	pLPC E2F1 DNA Binding Domain T130D GFP
MEF E2F1 Tr. 6 Δ^{120} T135A/T136A	pLPC E2F1 DNA Binding Domain T135A/T136A GFP
MEF E2F1 Tr. 6 Δ^{120} T135D/T135D	pLPC E2F1 DNA Binding Domain T135D/T135D GFP
MEF E2F1 Tr. 6 Δ^{120} T168A	pLPC E2F1 DNA Binding Domain T168A GFP
MEF E2F1 Tr. 6 Δ^{120} T168D	pLPC E2F1 DNA Binding Domain T168D GFP
MEF E2F1 GFP	pLPC E2F1 E2F1 GFP
WT MEF E1A-ER	pWZL blast E1A-ER
E2F1 $^{-/-}$ MEF E1A-ER	pWZL blast E1A-ER
pRB $^{-/-}$ MEF E1A-ER	pWZL blast E1A-ER
TKO $^{-/-}$ MEF E1A-ER	pWZL blast E1A-ER

2.1.5 siRNA Transfections

The following siRNA duplexes from Ambion were used:

Table 2-5 Oligos used in siRNA transfections

Gene	Accession No.	Catalogue No.	Sense and Antisense sequence
Human p400	NM 015409	AM167708A (131906)	GCCACCAAUAAGGCACUAU
			AUAGUGCCUUAUUGGUGGC
		AM167708A (131907)	GGAUACGCUGACAGAACAA
			UUGUUCUGUCAGCGUAUCC
		AM167708A (131908)	GCUUACACCAAUUGAAAAA
			UUUUUCAAUUGGUGUAAGC

All oligonucleotides were obtained as annealed and desalted duplexes from Ambion, having been designed with proprietary algorithms purported to increase the likelihood of silencing the desired target. 21-mer constituent oligonucleotides were synthesised as shown below, having complementary target-specific (N)₁₉ sequences on each strand and 3' diuridine or dideoxythymidine overhangs. RPE cells were plated in 35 mm wells 24 hours before transfection at a density of 1.5×10^5 cells per well. 1.0 μ l of 20 μ M stock siRNA oligo was added to Optimem (Invitrogen) to a final volume of 185 μ l. In another tube 4 μ l oligofectamine (Invitrogen) was added to 11 μ l Optimem and incubated for 5-10 minutes before being mixed, vortexed briefly and incubated at room temperature for 15-20 minutes. Meanwhile cells were washed twice with Optimem then 800 μ l of Optimem was added to each well. 200 μ l of the lipid complexes were added dropwise to each well, (to a final concentration of 20 nM), and incubated for 5-6 hours at 37°C then Optimem replaced with 2 ml 10% FCS DMEM in each well. Cells were treated and harvested at the times indicated for each assay.

2.2 Molecular cloning

2.2.1 Restriction digests

Restriction Digests were performed with enzymes from New England Biolabs (NEB). Five to ten-fold unit excess of enzyme was incubated with 2-10 μ g DNA for 1 hour at the appropriate temperature. For sequential digests DNA was purified using Qiagen PCR purification kit and resuspended in the appropriate

buffer for the sequential digest. After digestion, cleaved vector DNA was incubated for a further 30 minutes at 37°C with 5U Calf Intestine Alkaline Phosphatase (NEB) then DNA was purified using Qiagen PCR purification kit and resuspended in TE.

2.2.2 Ligation

Restriction fragments for ligation were purified by gel electrophoresis followed by excision using a Qiagen Gel Band Purification Kit. Ligations were carried out using Rapid DNA Ligation Kit (Roche). An approximate 2 fold molar excess of the insert fragment over the vector fragment was combined in a final volume of 10 µl DNA Dilution Buffer. 10 µl T4 DNA Ligation buffer was then added along with 5U T4 DNA ligase. This was incubated at room temperature for 5 minutes.

2.2.3 Transformation of competent cells

E. Coli DH5α supercompetent cells (Stratagene) were transformed for plasmid storage and preparation. Cells, which were stored at -80°C and highly temperature sensitive, were thawed on ice to prevent loss of transformation ability. Typically 10-20 ng of plasmid DNA was gently mixed into 50 µl of supercompetent cells. The cells were incubated for 30 minutes on ice before being heat shocked at 42°C for exactly 30 seconds and then transferred to ice for a further 2 minutes. 250 µl of 37°C SOC medium (LB broth, 0.04% glucose, 10 mM MgSO₄, 10 mM MgCl₂) was added before incubation for 1 hour on an orbital shaker (225-250 rpm). Typically 150 µl of the transformation mixture was then plated on LB agar plates containing 50 µg/ml ampicillin or kanamycin and the plates were incubated at 37°C overnight to allow growth and colony-formation of the transformed cells.

2.2.4 Screening of transformants

Colonies obtained from transformation were grown in overnight cultures of LB with 50 µg ampicillin/kanamycin, and plasmid DNA miniprepmed using QIAprep Spin Miniprep Kits (Qiagen #27104). Analytical restriction digests were performed and resolved upon agarose gels to validate successful cloning. Insert-

vector boundaries were always sequence analysed. If the insert was PCR derived, the entire insert was also sequence analysed.

2.2.5 Preparation of plasmid DNA

For large scale plasmid DNA preparation, a single isolated bacterial colony was selected from a freshly streaked plate and used to inoculate 4 ml LB medium containing 50 µg/ml ampicillin/kanamycin. This was allowed to incubate with vigorous shaking at 37°C for about 6 hours to form a mini culture and was subsequently used to inoculate 100 ml LB containing 50 µg/ml ampicillin/kanamycin. Following an overnight incubation at 37°C on an orbital shaker (300 rpm), cells were harvested by centrifugation at 3000g for 20 minutes at 4°C and plasmid DNA retrieved using the Qiagen Plasmid Maxi Kit.

The bacterial pellet was resuspended in 10 ml of Buffer P1 (50 mM Tris pH 8.0, 10 mM EDTA, 100 µg/ml RNase A) and then gently but thoroughly mixed with 10 ml Buffer P2 (200 mM NaOH, 1% SDS) to initiate an alkaline lysis reaction. This reaction was allowed to proceed for 5 minutes at room temperature before neutralising the lysate by the addition of 10 ml chilled buffer P3 (3M potassium acetate, pH 5.5) which subsequently resulted in formation of a precipitate of dodecyl sulphate. The SDS-denatured proteins and chromosomal DNA were co-precipitated with the detergent whilst the plasmid DNA remained in solution due to a lack of close protein associations. Precipitation was enhanced by 20 minute incubation on ice and the precipitate was then pelleted by centrifugation at 4000g for 30 minutes at 4°C. The supernatant containing plasmid DNA was promptly removed and applied to a QIAGEN-tip 500 pre-equilibrated with 10 ml of Buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15% Isopropanol, 0.15% Triton X-100). Gravity flow allowed the supernatant to pass through the anion-exchange resin to which plasmid DNA is able to tightly bind. The resin was then washed twice with 30 ml of buffer QC (1 M NaCl, 50 mM MOPS pH 7.0, 15% Isopropanol) and the purified plasmid DNA was subsequently eluted with 15 ml Buffer QF (1.25 M NaCl, 50 mM Tris pH 8.5, 15% isopropanol) and precipitated with 10.5 ml (0.7 volume) of room temperature isopropanol. This was immediately followed with a 4000g centrifugation at 4°C for 30 minutes. The plasmid DNA pellet was then washed with 70% ethanol, dried at room

temperature for 5-10 minutes and resuspended in an appropriate volume of sterile TE buffer (pH 8.0 10 mM Tris, 1 mM EDTA).

2.2.6 Agarose gel electrophoresis

Agarose (Sigma) was melted by heating in TAE, and then Ethidium Bromide was added to 0.5 µg/ml, prior to solidification. Agarose was allowed to solidify in gel trays for at least 1 hour. Samples for electrophoresis were diluted with 5 x gel loading buffer (30% glycerol, bromophenol blue) and electrophoresed at 100V in TAE gel running buffer. Bands were visualised using a UV transilluminator.

2.3 RNA/cDNA techniques

2.3.1 Preparation of total cellular RNA

Total cellular RNA was isolated from cells when approximately 80% confluent using TRI reagent (Sigma), in accordance with the manufacturers instructions. Media was aspirated off cells grown in 6-well plates or 100 mm plates. Cells from each well were harvested by scraping in 500 µl of TRI reagent per well or 1 ml per 100 mm plate and transferred to a sterile eppendorf tube. Cells were left to stand for 5 minutes to ensure complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform per 1 ml of TRI reagent used was then added to each tube and the samples vortexed for 15 seconds. The samples were then allowed to stand for a further 15 minutes at room temperature prior to being centrifuged at 13,000g for 15 minutes at 4°C. This resulted in separation of the samples into three phases: A lower red organic phase containing protein, a middle white interphase containing precipitated DNA and an upper colourless aqueous phase containing RNA. The upper phases were carefully removed, ensuring no contamination from the remaining phases and transferred to fresh eppendorf tubes. 500 µl Isopropanol per 1 ml TRI reagent used was added to each of the tubes containing the aqueous RNA and thoroughly mixed by repeated inverting. Following 5-10 minutes incubation at room temperature to allow maximal precipitation of RNA, samples were centrifuged at 13,000g for 10 minutes at 4°C. The supernatant was then removed and the remaining RNA pellet was washed with 1 ml of 75% ethanol made up with diethylpyrocarbonate (DEPC)-treated dH₂O (Ambion). The samples were vortexed briefly,

microcentrifuged at 7500g for 5 minutes at 4 °C and the supernatant aspirated off. Appropriate volumes of DEPC-H₂O, in the range of 5-30 µl were added to resuspend the RNA pellet. RNA concentration was determined by UV spectrophotometry. A ratio of absorbance at 260 nm to 280 nm in the range of 1.8-2.0 indicated the RNA samples were relatively free from contamination from DNA or protein. RNA was stored at 70 °C.

2.3.2 Preparation of cDNAs

cDNAs were prepared using the GeneAmp RNA PCR kit (Applied Biosystems) as per the manufacturers instructions using 1 µg RNA on Peltier Thermal Cycler (MJ Research). Primer annealing was carried out in a final volume of 20 µl including 4 µl 25 mM MgCl₂, 2 µl 10 x PCR Buffer II (500 mM KCl, 100 mM Tris-HCl, pH 8.3), 8 µl 10 mM dNTPs, 20U Rnase inhibitor, 50U MuLV Reverse Transcriptase, 1 µl Random Hexamers (50 µM) for 10 minutes before reverse transcription was allowed to proceed at 42°C for 30 minutes. The reaction was stopped by heating at 70°C for 15 minutes and cDNAs were then stored at -20°C.

2.3.3 Reverse transcriptase – Polymerase chain reaction (RT-PCR)

PCRs were carried out using a Peltier Thermal Cycler (MJ research, Helena Bioscience). 2 µl of cDNA was amplified in a final volume of 20 µl containing 1 nM of each primer, 0.5U AmpliTaq DNA polymerase, 2 µl 10 x PCR Buffer II, 1.2 µl 25mM MgCl₂ and 20 µM dNTP.

The following forward and reverse primers were used:

Table 2-6 RT-PCR primers

Primer	Sequence
E2F1 F	5'-GCCACCATAGTGTCACCACC-3'
E2F1 R	5'-GGTGAGGTCCCCAAAGTCAC-3'
GAPDH F	5'-TCCACCACCCTGTTGCTG-3'
GAPDH R	5'-ACCACAGTCCATGCCATCAC-3'

PCR reaction were carried out under the following cycling parameters: 95 °C for 3.00 minutes, 20 cycles for GAPDH, 25 cycles for E2F1 of 95 °C for 30 seconds, 66 °C for 40 seconds 72 °C for 1.00 minute and then cooled down to 4 °C.

Reaction products were resolved using Agarose Gel Electrophoresis and bands visualised using a UV transilluminator as described above.

2.3.4 Site-directed mutagenesis

Site-Directed Mutagenesis was carried out using QuickChange Site-Directed Mutagenesis Kit (Stratagene) with 5 µl of 10x reaction buffer, 50 ng of dsDNA template, 125 ng forward primer, 125 ng reverse primer, 1 µl dNTP mix, 2.5 µl DMSO in the final volume of 50 µl. Then 1 µl of Pfu Turbo DNA polymerase (2.5 U/µl) was added to the reaction. The PCR reactions were carried out under the following cycling parameters: 95 °C for 30 seconds, 18 cycles of 95 °C for 30 seconds, 55 °C for 5.30 minutes, and then cooled down to 4 °C, using on a Peltier Thermal Cycler (MJ Research). Then 1 µl of the Dpn I restriction enzyme (10 U/µl) was added to each amplification reaction and incubated at 37 °C for 1 hour to digest the parental (i.e. the nonmutated) supercoiled dsDNA. 1 µl of the digested reaction was used to transform *E. Coli* DH5α supercompetent cells (Stratagene) as described above. The day after, 10 colonies were picked for each reaction, grown over night, and plasmid DNA miniprep. Analytical restriction digests were performed and resolved upon agarose gels to validate successful amplification and then entire inserts sequence to verify successful mutagenesis.

The following primers were used in Site-Directed Mutagenesis

Table 2-7 Primers used in Site-Directed Mutagenesis

Primer	Sequence
E2F1 S31A F	CGGCTGCTCGACTCCTCGCAGATCGTCATCATCTCCGC
E2F1 S31A R	CGGCTGCTCGACTCCGCGCAGATCGTCATCATCTCCGC
E2F1 S364A F	GCTGTTGTCCCGGATGGGCAGCCTGCGGGCTCCCGTGGAC

E2F1 S364A R	GCTGTTGTCCCGGATGGGCGCCCTGCGGGCTCCCGTGGAC
E2F1 D390A/F391A F	CCTGGAGCATGTGCGGGAGGCCGCTCCGGCCTCCTCCCT GAGG
E2F1 D390A/F391A R	CCTCAGGGAGGAGGCCGGAGGCGGCCTCCCGCACATGCTC CAGG
E2F K117R/K120R/K125R F	GCCATCCAGGAGAAGGTGTGAGATCCCCGGGGGAGAGGT CACGCTATGA
E2F K117R/K120R/K125R R	TCATAGCGTGACCTCTCCCCGGGGATCTCACACCTTCTCC TGGATGGC
E2F1 Tr. 8 K117/120R F	CGCCATCCAGGAGAAGGTGTGAGACCGGATCCACC
E2F1 Tr. 8 K117R/K120R R	GGTGGATCCGGTCTCACACCTTCTCCTGGATGGCG
E2F1 Tr. 8 T49A F	CCGCCGGCTCCCGCCGGCCCCGCGG
E2F1 Tr. 8 T49A R	CCGCGGGGCGCGGGAGCCGGCGG
E2F1 Tr. 8 T49D F	CCGCCGGCTCCCGACGGCCCCGCGG
E2F1 Tr. 8 T49D R	CCGCGGGGCGGTCGGGAGCCGGCGG
E2F1 Tr. 8 T75A_F	CGCCCCGGCCCGCACCCAGTGCGCC
E2F1 Tr. 8 T75A R	GGCGCACTGGGTGCGGGCCGGGGCG
E2F1 Tr. 8 T75D F	CGCCCCGGCCCGACCCAGTGCGCC
E2F1 Tr. 8 T75D R	GGCGCACTGGGGTCGGGCCGGGGCG
E2F1 Tr. 8 Y100D F	GACCATCAGGACCTGGCCGAGAGC
E2F1 Tr. 8 Y100D R	GCTCTCGGCCAGGTCCTGATGGTC
E2F1 Tr. 8 Y100F F	GACCATCAGTTCCTGGCCGAGAGC
E2F1 Tr. 8 Y100F R	GCTCTCGGCCAGGAACTGATGGTC
E2F1 Tr. 8 S104A F	CCTGGCCGAGGCCAGTGGGCCAGC
E2F1 Tr. 8 S104A R	GCTGGCCCACTGGCCTCGGCCAGG
E2F1 Tr. 8 S104D F	CCTGGCCGAGGACAGTGGGCCAGC
E2F1 Tr. 8 S104D R	GCTGGCCCACTGTCCTCGGCCAGG
E2F1 Tr. 15 S121A F	CAGGAAAAGGTGTGAAAGCCCCGGGGGAGAAGTCACG
E2F1 Tr. 15 S121A R	CGTGACTTCTCCCCGGGGCTTTCACACCTTTTCCTG
E2F1 Tr. 15 S121D F	CAGGAAAAGGTGTGAAAGACCCGGGGGAGAAGTCACG
E2F1 Tr. 15 S121D R	CGTGACTTCTCCCCGGGTCTTTCACACCTTTTCCTG
E2F1 Tr. 15 S126A F	TGAAATCCCCGGGGGAGAAGGCACGCTATGAGACCTCACT
E2F1 Tr. 15 S126A R	AGTGAGGTCTCATAGCGTGCCTTCTCCCCGGGGATTTC

E2F1 Tr. 15 S126D F	TGAAATCCCCGGGGGAGAAGGACCGCTATGAGACCTCACT
E2F1 Tr. 15 S126D R	AGTGAGGTCTCATAGCGGTCTTCTCCCCGGGGATTTC
E2F1 Tr. 15 T130A F	GGAGAAGTCACGCTATGAGGCCTCACTGAATCTGACCAC
E2F1 Tr. 15 T130A R	GTGGTCAGATTCAGTGAGGCCTCATAGCGTGACTTCTCC
E2F1 Tr. 15 T130D F	GGAGAAGTCACGCTATGAGGACTCACTGAATCTGACCAC
E2F1 Tr. 15 T130D R	GTGGTCAGATTCAGTGAGTCCTCATAGCGTGACTTCTCC
E2F1 Tr. 15 Y128D F	CCCGGGGGAGAAGTCACGCGATGAGACCTCACTGAATCT
E2F1 Tr. 15 Y128D R	AGATTCAGTGAGGTCTCATCGCGTGACTTCTCCCCGGG
E2F1 Tr. 15 Y128F F	CCCGGGGGAGAAGTCACGCTTTGAGACCTCACTGAATCT
E2F1 Tr. 15 Y128F R	AGATTCAGTGAGGTCTCAAAGCGTGACTTCTCCCCGGG
E2F1 Tr. 15 T135A/T136A F	CACTGAATCTGGCCGCCAAGCGCTTCC
E2F1 Tr. 15 T135A/T136A R	GGAAGCGCTTGGCGGCCAGATTCAGTG
E2F1 Tr. 15 T135D/T136D F	CACTGAATCTGGACGACAAGCGCTTCC
E2F1 Tr. 15 T135D/T136D R	GGAAGCGCTTGTCGTCCAGATTCAGTG
E2F1 S144D/S146D F	GAGCTGCTGGACCACGACGCTGACGGTG
E2F1 S144D/S146D R	CACCGTCAGCGTCGTGGTCCAGCAGCTC
E2F1 Tr. 15 T168A F	GCGGCGCATCTTTGACATCACC
E2F1 Tr. 15 T168A R	GGTGATGTCAAAGATGCGCCGC
E2F1 Tr. 15 T168D F	GCGGCGCATCGATGACATCACC
E2F1 Tr. 15 T168D R	GGTGATGTCATCGATGCGCCGC

2.3.5 Quantitative real time polymerase chain reaction (qRT-PCR)

cDNA synthesis and qRT-PCR was carried out using the DyNAmo SYBR Green 2-step qRTPCR Kit with 1 µg RNA, 10 µl RT buffer (including dNTP mix and 10 mM MgCl₂), 1 µl Random Hexamers (300 ng/µl) and 2 µl M-MuLV Rnase H⁺ reverse transcriptase in a final volume of 20 µl per sample. cDNA synthesis was carried out under the following parameters: Primer Extension - 25°C for 10 minutes, cDNA Synthesis - 37°C for 30 minutes, Reaction Termination - 85°C for 5 minutes, on a Peltier Thermal Cycler (MJ Research).

Prior to analysis, a large preparation of serially diluted RPE total RNA was made and frozen in multiple aliquots at -70°C in order to provide material to construct reproducible standard curves. qRT-PCR was carried out with 2 µl of cDNA, 10 µl 2x qPCR master mix, (contains modified hot start *Tbr* DNA polymerase, SYBR Green I, optimized PCR Buffer, 5 mM MgCl₂ and dNTP mix including dUTP), 5.5 µl H₂O and 2.5 µl designed primers as indicated (QuantiTect Primer Assays - Qiagen) for gene of interest. 18S rRNA control primers were also used for each tested cDNA and used to standardise data. Three repeats were carried out for each sample. For analysis the control sample was normalised to 1 and all other samples were compared relative to this value.

Reactions were pipetted into 96 well optical plate (BioRad) minimizing exposure of the qPCR master mix to light and sealed using optically clear flat cap strips (BioRad). qPCR was carried out under the following cycling parameters using MJ Opticon Monitor Analysis Software version 3.1, on a Peltier Thermal Cycler (MJ Research) with a Chromo 4 continuous fluorescence detector.

Initial Denaturation - 95°C for 15 minutes, 40 cycles of Denaturation - 94°C for 10 seconds, Annealing - 55°C for 30 seconds, Extension - 72°C for 30 seconds, plate read (data acquisition), followed by Final Extension - 72°C for 10 minutes, Melting Curve 70-90°C read 0.3C hold 1 second and Reannealing - 72°C for 10 minutes. Data analysed using MJ Opticon Monitor Analysis Software version 3.1

The following Primers were used

Table 2-8 Primers used in qRT-PCR

Gene	Catalogue No.
Hs E2F1	QT00016163
Hs p400	QT00079807

2.4 Protein immunoblotting

2.4.1 Separation of proteins by polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were resolved on denaturing polyacrylamide gels according to molecular weight by electrophoresis on Amersham Biosciences SE400 standard format vertical unit tanks. Typically, cells were lysed in a 2x western sample buffer. Equal numbers of cells were used per plate and equal volumes of sample were boiled for 2 minutes prior to loading then electrophoresed on SDS-polyacrylamide gels in 1 x SDS running buffer at 55V overnight until the dye front had reached the bottom of the gel. SDS-PAGE gels consisted of a separating gel overlaid by a stacking gel. Depending on the molecular weight of the product to be visualised, the separating gel was between 6% and 15% acrylamide (from 30% stock solution (37.5:1 acrylamide:bis acrylamide) Severn Biotech Ltd) and otherwise constituted 375mM Tris-HCl, pH8.8, 0.1% SDS, polymerised with 0.05% ammonium persulphate, 0.1% TEMED (Sigma). Stacking gel was 4% acrylamide and otherwise constituted 125mM Tris-HCl, pH 6.8 , 0.1% SDS, polymerised with 0.05% ammonium persulphate, 0.1% TEMED.

2.4.2 Western blotting

Electrophoretic transfer of proteins resolved by SDS-PAGE to nitrocellulose membrane was achieved using Hoefer TE 42 Protein Transfer tanks. SDS Gels were blotted in a sandwich with sponge and 2 sheets Whatman 3MM paper, all equilibrated in Transfer Buffer, either side of the gel and a matching piece of nitrocellulose membrane (Amersham Biosciences). Transfer was carried out in 1 x Transfer Buffer at 0.5 Amps for 4 hours. After transfer to nitrocellulose membranes, ponceau-S (Sigma) staining was undertaken as a further assessment of loading and to check the fidelity of transfer.

Table 2-9 Antibodies

Antibody	Type	Company	Dilution
Anti-Actin A-4700	Monoclonal	Sigma	1:2000
Anti-Adenovirus (M58 + M73)	Monoclonal	Abcam	1:500
Anti- Adenovirus type 5 E1A	Monoclonal	BD Pharmingen	1:500
Anti-Adenovirus 2/5 E1A	Polyclonal	Santa Cruz	1:500
Anti-E2F1 C-20	Polyclonal	Santa Cruz	1:1000
Anti-E2F1 KH95	Monoclonal	Santa Cruz	1:1000
Anti-E2F1 KH95/KH20	Monoclonal	Upstate Biotechnology	1:1000
Anti-GFP	Monoclonal	Roche	1:1000
Anti-GFP	Monoclonal	Covance	1:3000
Anti-p38	Polyclonal	Cell Signalling	1:1000
Anti-p53 (DO-1)	Monoclonal	BD Pharmingen	1:5000
Anti-PARP	Monoclonal	BD Pharmingen	1:1000
Anti-pRb C-15	Polyclonal	Santa Cruz	1:500
Anti mouse IgG HRP linked		Cell Signalling	1:3000
Anti rabbit IgG HRP linked		Cell Signalling	1:3000

2.4.3 Probing

Following transfer, the membrane was blocked in TBST milk buffer (TBST + 5% skimmed milk powder (Marvel) for 1 hour at room temperature. Membranes were incubated with primary antibodies (typically a 1:1000 dilution in milk buffer or 5% BSA in TBST for 2 hour at room temperature or over night at 4°C. Excess primary antibody was removed by washing the blot 3 times in TBST for 10 minutes before incubating for 1 hour at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody (Cell Signalling) at a 1:3000 dilution. To ensure the removal of excess secondary antibody, the blot was washed 3 times for 15 minutes in TBST. The blot was developed using the enhanced chemiluminescence method, (ECL Amersham), as directed by the manufacturers and X-ray film (Fuji Medical X-ray film).

2.4.4 Immunoprecipitation

Protein-G sepharose beads (SIGMA) were washed twice with 1ml LSAB (20mM HEPES, 150mM NaCl, 0.5% Triton and complete mini protease inhibitor tablet (Roche). Beads were resuspended at 50:50 ratio of beads to LSAB buffer

Cells with wild type E1A or E1A Δ CR2 as indicated were washed with PBS then scraped in 1ml LSAB and transferred to a sterile eppendorf tube. Samples were spun at 13000g for 10 seconds and the supernatant was transferred to a fresh sterile eppendorf tube (60 μ l kept for SDS page analysis of Input). Samples were pre-incubated with 20 μ l protein-G sepharose beads at 4°C for 1 hour on an orbital shaker. The beads were carefully pelleted by pulse microcentrifugation and the supernatants carefully removed and transferred to a fresh eppendorf. 6 μ l of anti-E1A mouse antibody and 50 μ l of bead:buffer mix was added to each sample and incubated overnight at 4°C on an orbital shaker. The beads were carefully pelleted by pulse microcentrifugation and the supernatants carefully removed. The beads were then washed three times with cold LSAB before the bound material was released by addition of an equal volume of 2 x western sample buffer and analysed by SDS PAGE and western blotting.

2.5 Cell death assays

After the treatments and times indicated, total populations of cells, including floating and adherent cells, were processed for flow cytometric analysis (FACScan, Becton Dickinson). Each sample was harvested by collection of media in 15ml falcon tube. The cells were then washed in 2 ml PBS which was also collected into the 15 ml falcon tube and 2 mls of TE + 0.25% trypsin was added to remove adherent cells, and subsequently transferred to the falcon tube. The samples were then centrifuged for 5 minutes at 1000g and the supernatants removed. Cells were resuspended in 500 μ l PBS followed by 5 ml ice cold methanol while vortexing the samples to avoid clumping. The cells were then left to fix at 4°C for a minimum of 3 hours.

Directly before FACS analysis the cells were collected by centrifugation and resuspended in 400 μ l PBS, 20 μ l 1 mg/ml Propidium iodide (Sigma) and 0.2 μ l RNase (100 mg/ml) and incubated for 30 minutes at room temperature then

sorted for and analysed for DNA content. The percentage of cells with a sub-G1 DNA content was taken as a measure of the apoptotic rate of the cell population.

2.6 Plasmid sources and construction

pLPC

Retroviral expression vector deriving transcription from a CMV promoter. Obtained from Scott Lowe.

pLPC E2F1 WT

Retroviral expression vector for WT E2F1. Obtained from Kevin Ryan, Beatson Institute for Cancer Research.

pLPC E2F1 WT (GFP)

Retroviral expression vector for WT E2F1 fused to GFP. Obtained from Kevin Ryan, Beatson Institute for Cancer Research

pLPC GFP

Retroviral expression vector for GFP. Obtained from Kevin Ryan, Beatson Institute for Cancer Research.

pLPC Tr. 1 GFP

Retroviral expression vector for Tr. 1 GFP. Obtained from Kevin Ryan, Beatson Institute for Cancer Research.

pLPC Tr. 2 GFP

Retroviral expression vector for Tr. 2 GFP. Obtained from Kevin Ryan, Beatson Institute for Cancer Research.

pLPC Tr. 3 GFP

Retroviral expression vector for Tr. 3 GFP. Obtained from Kevin Ryan, Beatson Institute for Cancer Research.

pLPC Tr. 4 GFP

Retroviral expression vector for Tr. 4 GFP. Obtained from Kevin Ryan, Beatson Institute for Cancer Research.

pLPC Tr. 5 GFP

Retroviral expression vector for Tr. 5 GFP. Obtained from Kevin Ryan, Beatson Institute for Cancer Research.

pLPC Tr. 6 GFP

Retroviral expression vector for Tr. 6 GFP. Obtained from Kevin Ryan, Beatson Institute for Cancer Research.

pLPC Tr. 7 GFP

Retroviral expression vector for Tr. 7 GFP. Obtained from Kevin Ryan, Beatson Institute for Cancer Research.

pLPC Tr. 8 GFP

Retroviral expression vector for Tr. 8 GFP. Obtained from Kevin Ryan, Beatson Institute for Cancer Research.

pLPC Tr. 9 GFP

Retroviral expression vector for Tr. 9 GFP. Obtained from Kevin Ryan, Beatson Institute for Cancer Research.

pLPC Tr. 10GFP

Retroviral expression vector for Tr. 10 GFP. Obtained from Kevin Ryan, Beatson Institute for Cancer Research.

pLPC Tr. 11 GFP

Retroviral expression vector for Tr. 11 GFP. Obtained from Kevin Ryan, Beatson Institute for Cancer Research.

pLPC E1A WT

Retroviral expression vector for WT E1A 12S. Obtained from Scott Lowe.

pLPC E1A Δ CR2

Retroviral expression vector for E1A Δ CR2. Obtained from Scott Lowe.

pLPC E1A R2G

Retroviral expression vector for WT E1A R2G. Obtained from Scott Lowe.

pLPC E1A Δ 2-11

Retroviral expression vector for WT E1A Δ 2-11. Obtained from Scott Lowe.

pLPC E1A Δ 2-24

Retroviral expression vector for WT E1A Δ 2-24. Obtained from Scott Lowe.

pLPC E1A Δ 2-36

Retroviral expression vector for WT E1A Δ 2-36. Obtained from Scott Lowe.

pLPC E1A Δ 26-35

Retroviral expression vector for WT E1A Δ 26-35. Obtained from Scott Lowe.

pLPC E1A Δ 48-60

Retroviral expression vector for WT E1A Δ 48-60. Obtained from Scott Lowe.

pLPC E1A 143

Retroviral expression vector for WT E1A 143. Obtained from Scott Lowe.

pSuperRetro Scr

Retroviral expression vector for scrambled hairpin. Obtained from Doron Gingsberg

pSuperRetro E2F1

Retroviral expression vector for E2F1 hairpin. Obtained from Doron Gingsberg

pWZL blast E1A-ER

Retroviral expression vector for E1A-ER fusion protein. Obtained from Andy Phillips

Chapter 3: Results

3 Chapter 3: Results

The essential role of E2F1 in apoptosis is well documented. The pathways leading to programmed cell death that are directed by E2F1 are complex and overlapping (Field et al., 1996; Kowalik et al., 1998; Macleod et al., 1996; Qin et al., 1994; Shan and Lee, 1994; Wu and Levine, 1994). E2F1 has the ability to induce both cell cycle progression and programmed cell death, leading potentially to both tumour-promoting and tumour-suppressive effects. The effect of E2F1 overexpression on tumour growth has been evaluated in several types of human cancers and E2F1 has been shown to sensitize tumour cells to chemotherapy (Kaelin, 2003; Stanelle and Putzer, 2006).

Oncogenes have been shown to deregulate E2F1 through inhibition of pRB and oncogenic stress can induce E2F1's apoptotic activity. E2F1 has also been shown to respond to DNA damage. However, how cancer cells evade E2F1-induced apoptosis is not clear. Investigating the mechanism(s) leading to the DNA damage response and defining the role of E2F1 in DNA-damage induced apoptosis should contribute to the understanding of E2F1's role in chemosensitivity. That may ultimately help to provide novel targets in the E2F1 pathway for therapeutic intervention.

The aims of this project are to investigate E2F1's response to DNA damage and oncogenic stress.

Chapter 3-1: E2F1's involvement in chemotherapeutic response

3.1 E2F1's involvement in chemotherapeutic response

In mammalian cells, DNA damage induces robust changes in gene expression and these changes contribute to the execution of cellular responses to mutations and DNA damage, including DNA repair, cell cycle arrest and apoptosis. DNA damage, caused by radiation or genotoxic drugs is detected by DNA damage-sensing proteins. They then generate the signals leading to the responses, which represent defences against genomic instability and tumorigenesis resulting from unrepaired damage. The response depends on the severity of the damage and the type of cell affected. The tumour suppressor p53 is well studied as one of the main player in DNA damage checkpoint regulation. It is stabilized and activated as a transcriptional factor in response to genomic DNA damage (Latonen and Laiho, 2005; Vousden and Lu, 2002). In 1997, Huang et al. showed that ionizing radiation increased the expression of E2F1 (Huang et al., 1997). Later E2F1 was shown to be upregulated in response to DNA damage caused by irradiation with X-rays or UVC, or following treatment with the DNA-damaging agents actinomycin D, adriamycin and etoposide in a manner analogous to that of p53 (Blattner et al., 1999; Hofferer et al., 1999; Meng et al., 1999). Since then several groups have studied E2F1's response to various DNA damaging agents. Some discoveries have now been published suggesting the post-translational modifications of E2F1 that follow DNA damage and lead to E2F1 stabilization. Some controversy has risen about the physiological importance of these modifications and specially their applications in E2F1's ability to induce apoptosis.

Several genes involved in the activation or execution of apoptosis have been shown to be unregulated at the transcriptional level by E2F1 overexpression. But how E2F1's response to DNA damage translates into the activation of this specific subset of E2F1 target genes and the importance of E2F1 in drug induced apoptosis is not as well studied. However, some groups have managed to bridge the DNA damage response of E2F1 to increased E2F1 activity. In 2000, Martinez-Balbas et al. showed that E2F1 is acetylated by the p300/CBP-associated factor P/CAF, and the consequences of this acetylation is increased DNA-binding ability, activation potential and protein half-life (Martinez-Balbas et al., 2000).

In 2003, E2F1 was shown to switch from cell cycle progression to apoptotic E2F1 target genes in response to adriamycin, and p73 was an important E2F1 target gene in the DNA damage response (Pediconi et al., 2003). These results indicate that E2F1 and downstream targets of E2F1 may prove to be valuable candidates for cancer therapy in tumour cells.

The aim of the studies in this chapter is to further investigate E2F1's response to chemotherapeutic drugs and the importance of E2F1 in drug-induced apoptosis.

3.1.1 E2F1 is induced in response to various chemotherapeutic drugs

In order to investigate E2F1's response to DNA damage, three chemotherapeutic drugs used in the clinics; actinomycin D, adriamycin and etoposide were used. Actinomycin D binds DNA at the transcription initiation complex and prevents elongation by RNA polymerase, and therefore interferes with DNA replication. It is one of the older chemotherapy drugs and has been used in therapy for many years. Adriamycin (doxorubicin) is also a DNA-interacting drug widely used in chemotherapy. It interacts with DNA by intercalation and inhibits the progression of the enzyme topoisomerase II, which unwinds DNA for transcription. Etoposide is also an inhibitor of topoisomerase II and is used as a form of chemotherapy for malignancies such as lung, ovarian and testicular cancer.

In order to determine E2F1's response to these chemotherapeutic drugs, Mouse Embryonic Fibroblast (MEF) cells were treated with 100 ng/ml actinomycin D, 2.0 µg/ml adriamycin and 20 µM etoposide. The cells were then lysed and endogenous E2F1 protein levels measured by Western blotting using an antibody specific for E2F1. This revealed a detectable induction of E2F1 protein levels only 6 hours following treatment with all three drugs, compared with untreated cells, and the induction continues up to 12 hours of treatment (Fig. 3-1). The MAP kinase p38 showed no induction following treatment and was used to ensure equal loading of proteins, suggesting that the increase in E2F1 protein levels in response to DNA damage is specific.

In order to confirm E2F1's response to DNA damage in primary human cells, TERT-immortalized Retinal Pigment Epithelial (RPE) cells were treated for the same time period as the MEFs and with the same concentration of adriamycin and endogenous E2F1 levels measured by Western blotting. Similar results were obtained as in the MEFs, with detectable E2F1 induction after 6 hours following treatment and the induction continuing up to 12 hours (Fig. 3-2a). In order to investigate if the increase in E2F1 protein levels observed were post-transcriptional or a result of enhanced transcription of the E2F1 gene itself, a real-time quantitative PCR (qPCR) was carried out. RNA was isolated from RPE cells following same treatment as in figure 3-2a, cDNA prepared and the inferred

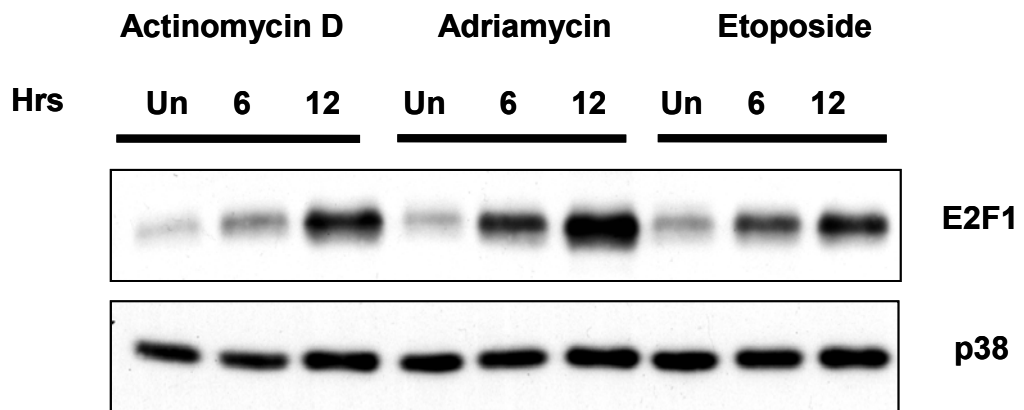


Figure 3-1 E2F1 is induced following treatment with actinomycin D, adriamycin and etoposide.

WT MEFs were treated with 100 ng/ml actinomycin D, 2.0 μ g/ml adriamycin, or 20 μ M etoposide. Cells were lysed in SDS lysis buffer 6 and 12 hours following treatment. Extracts were then resolved by SDS-PAGE and endogenous E2F1 levels measured by Western blotting using E2F1 specific antibody (C-20). Blots were re-probed with a p38 antibody to ensure equal loading.

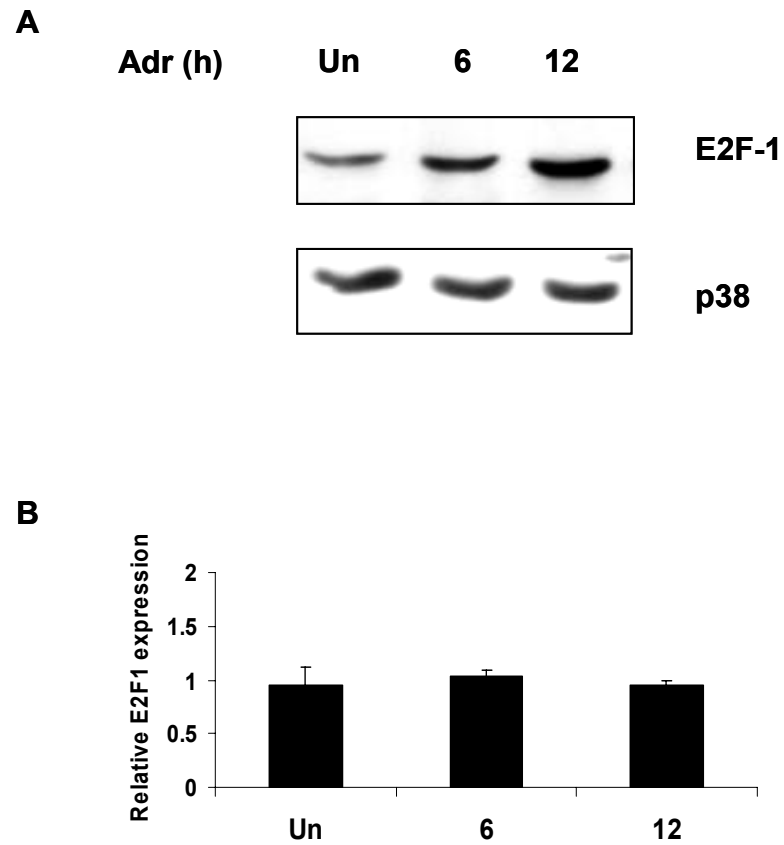


Figure 3-2 E2F1 is post-transcriptionally induced following treatment with adriamycin in RPE cells.

A + B) RPE cells were treated with 2.0 $\mu\text{g/ml}$ adriamycin for 6 and 12 hours. **A)** Following treatment cells were lysed in SDS lysis buffer. Extracts were then resolved by SDS-PAGE and endogenous E2F1 levels measured by Western blotting using E2F1 specific antibody (C-20). Blots were re-probed with a p38 antibody to ensure equal loading. **B)** RNA was extracted and cDNA made. Relative expression of E2F1 was determined by real-time qPCR.

E2F1 mRNA levels measured using E2F1 specific primers. No changes in the mRNA levels following adriamycin treatment were observed (Fig. 3-2b), indicating that the changes in E2F1 protein levels are not due to increased transcription, but are post transcriptional. Whether the post transcriptional induction of E2F1 is a result of increased translation of E2F1 mRNA or increased protein stability, or both, remains to be determined.

3.1.2 E2F1 is involved in drug induced apoptosis

Given the pro-apoptotic activity of E2F1 and E2F1's induction following DNA damage which can also lead to apoptosis, E2F1's involvement in drug induced apoptosis was tested. Full length E2F1 was infected into E2F1^{-/-} MEFs (E2F1^{-/-} + FL E2F1) and drug induced apoptosis compared with wild type MEFs and E2F1^{-/-} MEFs. Following treatment with 10 μ M Etoposide for 48 hours, both wild type and E2F1^{-/-} + FL E2F1 MEFs showed over 40 % apoptosis measured by flow cytometry whereas E2F1 knockout MEFs showed only 12 % apoptosis (PI staining and sub-G1 content used as an indicator of apoptotic cells) (Fig. 3-3). This indicates that E2F1 is involved in drug induced apoptosis.

To verify E2F1's involvement in drug induced apoptosis, RPE cells stably expressing a shRNA targeting E2F1 and a scrambled hairpin as a control were generated. Significant knockdown of E2F1 protein levels was obtained compared with a cell line stably expressing a scrambled hairpin with no effect seen on p38 in either line (Fig. 3-4a).

Following treatment with adriamycin both cell lines showed induction of E2F1 (Fig. 3-4b). However, after 12 hours of treatment, the levels of E2F1 protein in the cells expressing the E2F1 shRNA only reached levels that were comparable to the basal level of E2F1 in the control shRNA expressing cells (compare lines 1 and 6). p53 was also induced following the treatment. In untreated cells p53 levels were very low and hardly detectable by immunoblotting, but following 6 hours of treatment, p53 levels were easily seen with similar protein accumulation following 12 hours of treatment. E2F1 knockdown did not have an effect on basal levels of p53 or the accumulation of p53 following treatment (Fig. 3-4b).

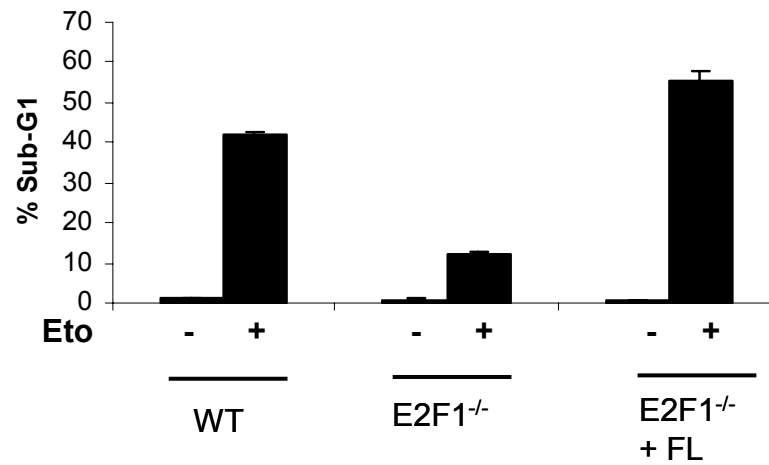


Figure 3-3 E2F1 knockout cells show reduced drug-induced apoptosis

Wild type MEFs (WT), E2F1 knockout MEFs (E2F1^{-/-}) and E2F1 knockout MEFs expressing full length human E2F1 (E2F1^{-/-} + FL) were treated with 10 μ M etoposide (Eto) for 48 hours. Following treatment the DNA content of cells was assessed by flow cytometry. The percentage of cells with a sub-G1 DNA content was taken as the apoptotic population.

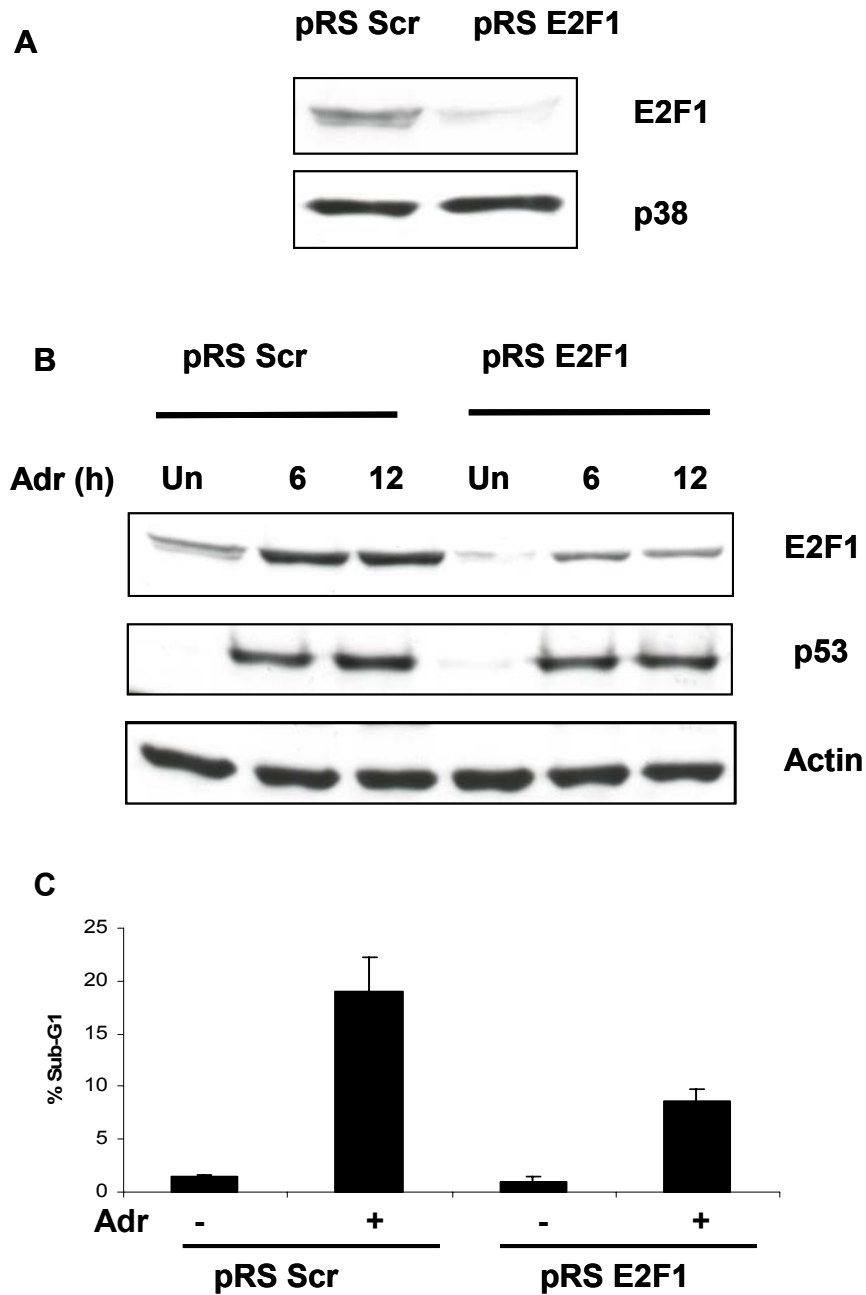


Figure 3-4 Knockdown of E2F1 reduces drug induced apoptosis

RPE cells were retrovirally infected with scrambled or E2F1 specific shRNA (pRS Scr and pRS E2F1). **A**) Cells were lysed in SDS lysis buffer, extracts resolved by SDS-PAGE and endogenous E2F1 levels measured by Western blotting using E2F1 specific antibody (C-20). Blots were re-probed with a p38 antibody to ensure equal loading. **B**) pRS Scr and pRS E2F1 cells were treated with 2.0 µg/ml adriamycin and lysed in SDS lysis buffer 6 and 12 hours following treatment. Extracts were then resolved by SDS-PAGE. Endogenous E2F1 and p53 levels were measured by Western blotting. Blots were re-probed with a p38 antibody to ensure equal loading. **C**) pRS Scr and pRS E2F1 cells were treated with 0.5 µg/ml adriamycin (Adr) for 48 hours. Following treatment DNA content of cells was assessed by flow cytometry. The percentage of cells with a sub-G1 DNA content was taken as the apoptotic population.

To test the affect of E2F1 knockdown on drug induced apoptosis, these cell lines were treated with adriamycin for 72 hours and apoptosis measured by flow cytometry. Whereas adriamycin induced extensive apoptosis in the cell line expressing scrambled hairpin, apoptosis was robustly inhibited by E2F1 knockdown (Fig. 3-4c).

These results clearly demonstrate a critical role for E2F1 in drug induced apoptosis and establish the importance of further investigation of E2F1's induction following DNA damage and its role in chemosensitivity of tumour cells following drug treatment.

3.1.3 Summary

DNA damaging agents that are regularly used to treat cancer were used to investigate the affect of E2F1 protein levels. These drugs cause DNA damage and therefore activate DNA damage responsive pathways within cells. E2F1 was shown to be upregulated by a post-transcriptional mechanism in response to adriamycin, actinomycin D and etoposide in a time dependent manner. These results are in line with previous studies which also have examined E2F1's response to various DNA damaging agents (Blattner et al., 1999; Meng et al., 1999). Our data also support the notion that E2F1 plays a role in drug induced apoptosis in a p53-independent manner. This connection between DNA damage and E2F1-depentent apoptosis following drug treatment is of particular significance. The frequent deregulation of E2F1 in human tumours, in addition with its apoptotic potential and its stabilization following DNA damage suggests that E2F1 plays a role in the enhanced sensitivity of tumour cells to DNA damage induced cell death. Further studies investigating the mechanism leading to E2F1 accumulation following DNA damage may therefore provide a way to increase chemosensitivity in tumours.

Chapter 3.2: Mechanisms of E2F1 induction following DNA damage

3.2 Mechanisms of E2F1 induction following DNA damage

Recent studies have shown that the induction of E2F1 following DNA damage are mediated by both phosphorylation and acetylation. E2F1 was shown to be phosphorylated by the damage-responsive protein kinase ATM following DNA damage and the phosphorylation site was mapped at Serine 31 (Lin et al., 2001). This site was shown to be required for ATM-mediated stabilization as E2F1 with this site mutated to alanine showed no accumulation following treatment with the DNA damaging agent neocarzinostatin (NCS). In a different study E2F1 was showed to be phosphorylated by the checkpoint kinase 2 (Chk2) in response to etoposide (Stevens et al., 2003). The phosphorylation occurred within a Chk2 consensus phosphorylation site at serine 364 and resulted in protein stabilization and increased half-life. Mutation of this site to alanine resulted in approximately four times less protein accumulation compared with wild type. E2F1 is acetylated by P/CAF leading to increased stability of E2F1 (Martinez-Balbas et al., 2000). Later it was shown that this occurs following treatment with adriamycin (Pediconi et al., 2003). These acetylation sites were mapped to the N-terminus, adjacent to the DNA binding domain (K117, K120 and K125) of E2F1 (Fig. 3-5).

During a normal cell cycle E2F1 protein levels are regulated by ubiquitin-proteasome-dependent degradation (Campanero and Flemington, 1997). The physical association of pRb can stabilize E2F1 by protecting it from degradation (Hateboer et al., 1996a; Hofmann et al., 1996). Furthermore, it has been suggested that E2F1 modifications following DNA damage also inhibit E2F1's ubiquitination and degradation. However, the way DNA damage affects E2F1 ubiquitination or degradation still remains unknown.

There is also some evidence that E2F1 stabilization is affected by direct binding to other cellular proteins. E2F1 was shown to physically interact with MDM2, which targets p53 for rapid degradation by the ubiquitin proteasome pathway (Haupt et al., 1997; Martin et al., 1995). Conversely MDM2 was shown to stimulate the activation capacity of E2F1, but the effect on E2F1 stability or accumulation following DNA damage was not tested.

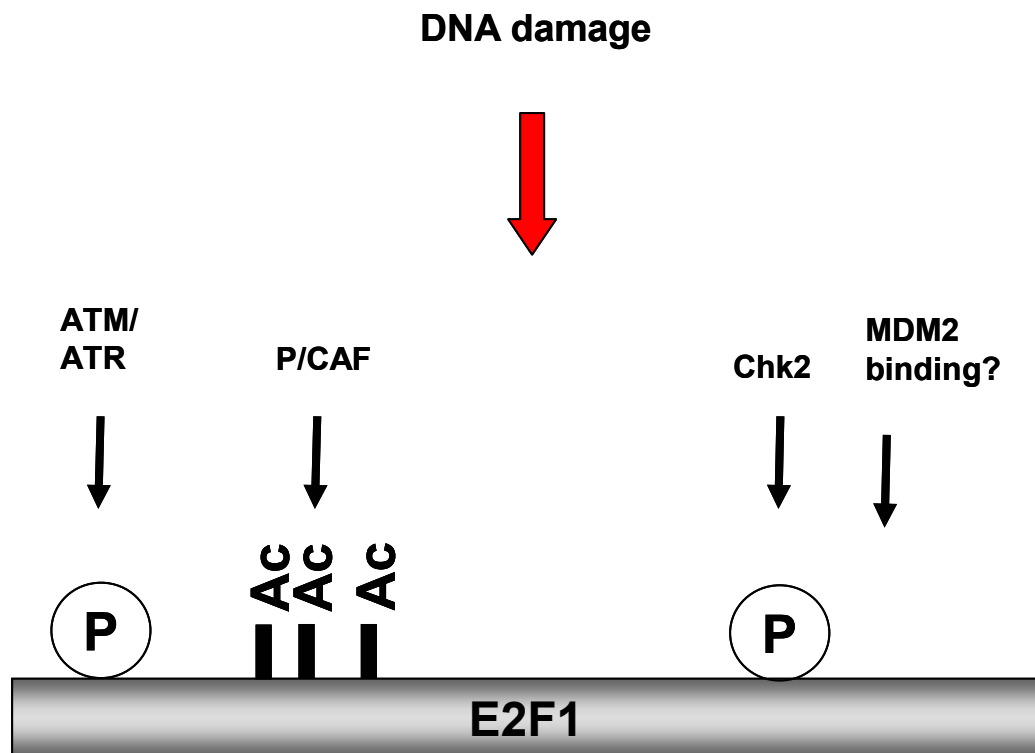


Figure 3-5 Known mechanisms of E2F1 induction following DNA damage

E2F1 has been shown to be phosphorylated at serine 31 by ATM kinase and at serine 364 by Chk2 kinase in response to DNA damage. It has also been shown to be acetylated by P/CAF following DNA damage. Physical interaction between C-terminal end of E2F1 and MDM2 activates E2F1 but whether it leads to increased expression following DNA damage is not known.

The contribution of each of the known mechanisms on E2F1 stability following DNA damage, and in which context they play a role in, is not known in detail. Further investigation is therefore needed to fully understand the post-transcriptional modifications that occur on E2F1 following cellular stress and DNA damage and the consequences of these changes.

The aim of this chapter is to investigate the importance of previously characterized mechanisms of E2F1 induction following treatment with chemotherapeutic drugs that cause DNA damage. In addition, it was considered if unidentified mechanism(s) could also account for E2F1 stabilization.

3.2.1 Contribution of known mechanisms to E2F1 induction following DNA damage

In order to further understand E2F1's induction following DNA damage and analyse the importance of previously known mechanism, E2F1 knockout MEFs were used and stably infected by full length or mutated forms of E2F1. The advantage of using E2F1^{-/-} MEFs is that there is no endogenous E2F1 that can have an effect on the response of the exogenous protein. In addition, as most of previous studies have been carried out in cancer cells, which might already have lost some of the mechanism leading to E2F1 accumulation following cellular stress and DNA damage, it was envisaged that MEFs might prove to be a better cell line to investigate the mechanisms leading to E2F1 induction.

Firstly, in order to test exogenous E2F1's response to DNA damage a retrovirus expressing full length E2F1 was infected into E2F1^{-/-} MEFs and examined. In the untreated cells this exogenously expressed E2F1 was undetectable by Western blotting (Fig. 3-6a lane 1). However, after 6 hours of treatment with 2.0 µg/ml adriamycin, E2F1 could be detected with a more robust level of E2F1 being detected after 12 hours. This pattern of response was very similar to what was observed in wild type MEFs (Fig. 3-1). The MAP kinase p38 showed no induction following treatment and was used to ensure equal loading of proteins, suggesting that the increase in E2F1 protein levels in response to DNA damage is specific. In order to investigate if the increase in E2F1 protein levels observed here is a result of enhanced transcription from the inserted E2F1 gene itself or if the changes are post-transcriptional, RNA from the same samples was prepared and mRNA levels measured by RT PCR using E2F1 specific primers and GAPDH primers as control. This revealed no changes in the mRNA levels following adriamycin treatment (Fig. 3-6b) indicating that the changes in exogenous E2F1 protein levels are post-transcriptional and not due to increased transcription.

In order to test if E2F1 in this system is degraded by the proteasome, cells were treated with the proteasome inhibitor MG132. E2F1 protein levels were rapidly induced following MG132 treatment for 4 hours (Fig 3-7a). The induction following MG132 treatment was similar to E2F1 induction following 10 hours

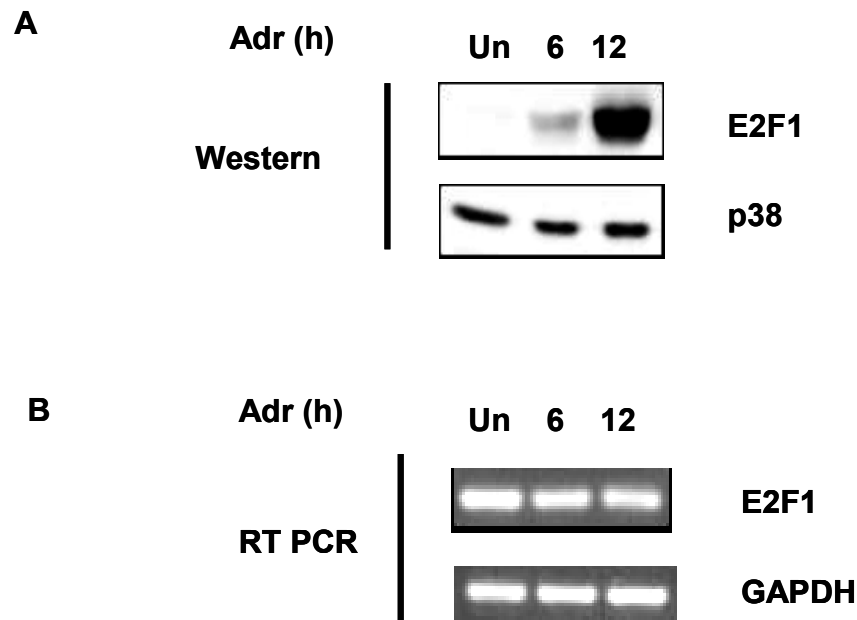


Figure 3-6 Exogenous E2F1 is post-transcriptionally induced following treatment with adriamycin in E2F1^{-/-} cells

A + B) E2F1^{-/-} MEFs were retrovirally infected with full length E2F1 in order to make cell line stably expressing E2F1 (MEF E2F1^{-/-} + FL). Cells were then treated with 2.0 µg/ml adriamycin for 6 and 12 hours. **A)** Cells were lysed in SDS lysis buffer following treatment. Extracts were resolved by SDS-PAGE and E2F1 levels measured by Western blotting using E2F1 specific antibody (C-20). Blots were re-probed with a p38 antibody to ensure equal loading. **B)** RNA was extracted and cDNA prepared. Relative expression of E2F1 following adriamycin treatment was determined by RT-PCR.

adriamycin treatment. However, small differences were observed, with E2F1 levels higher following adriamycin treatment, which is possibly due difference in timing of treatment (10 hours adriamycin treatment compared with 4 hours MG132 treatment). When cells were treated with both MG132 and adriamycin, a similar level of induction was observed. That indicates that E2F1 induction following DNA damage is likely due to inhibition of degradation by the proteasome.

The effect of acetylation on E2F1 stability was also tested. Cells were treated with the histone de-acetylase (HDAC) inhibitor trichostatin A (TSA) for 24 hours (100 μ M final concentration). Following TSA treatment, E2F1 levels increased rapidly, indicating that E2F1 can be acetylated leading to protein induction (Fig 3-7b).

Next it was tested whether previously described mechanisms for E2F1 accumulation were being utilised in this system. First it was investigated whether phosphorylation on serines 31 and 364 were playing a role. To do this, constructs were made with these sites mutated leading to alanine substitutions (alanine cannot be phosphorylated) and stably expressed in E2F1^{-/-} MEFs. These cell lines were then treated with adriamycin for 6 hours and E2F1 protein levels measured by Western blotting. The protein levels in these cell lines were marginally higher than in the cell line expressing the FL E2F1 and interestingly both of these cell lines showed robust E2F1 induction following adriamycin treatment (Fig. 3-8). The MAP kinase p38 showed no induction following treatment and was used to ensure equal loading of proteins.

This indicates that either phosphorylation on these sites is not required for the response or combination of mutations would be required to inhibit the induction following DNA damage. In order to test if both of these phosphorylation events contribute to the induction of E2F1, but one would be enough to stabilize the protein to the same level as full length, a double mutant carrying serine alanine substitutions on both amino acid sites 31 and 364 (S31A/S364A), was created and stably expressed in E2F1^{-/-} MEFs. This cell line still showed E2F1 accumulation following adriamycin treatment for 6 hours, with

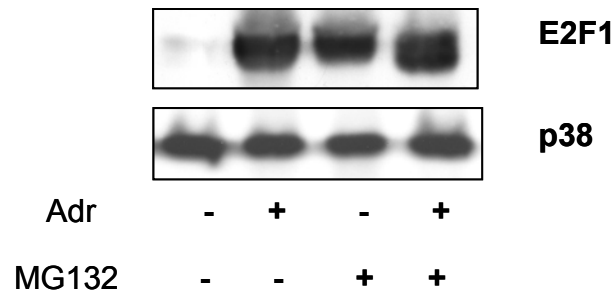
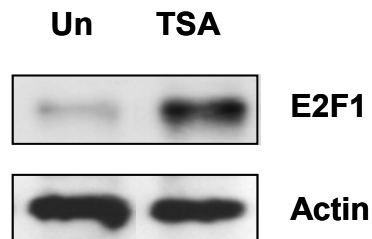
A**B**

Figure 3-7 DNA damage inhibits proteosomal degradation of E2F1

A) MEF E2F1^{-/-} + FL were treated with 2.0 µg/ml adriamycin for 10 hours and/or the proteosomal inhibitor MG132 (10 µM final concentration) for 4 hours. Cells were then lysed in SDS lysis buffer and extracts resolved by SDS-PAGE. E2F1 levels were measured by Western blotting using E2F1 specific antibody (C-20). Blots were re-probed with a p38 antibody to ensure equal loading. **B)** MEF E2F1^{-/-} + FL were treated with the HDAC inhibitor trichostatin A (TSA) (100 µM final concentration). Cells were then lysed in SDS lysis buffer and extracts resolved by SDS-PAGE. E2F1 levels were measured by Western blotting using E2F1 specific antibody (C-20). Blots were re-probed with an actin antibody to ensure equal loading.

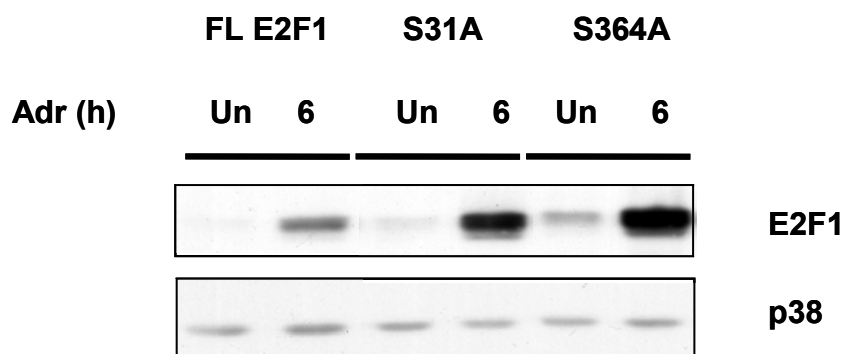


Figure 3-8 E2F1 with S31A or S364A substitution is induced following treatment with adriamycin

E2F1^{-/-} MEFs expressing FL or mutated E2F1 (S31A or S364A) were treated with 2.0 µg/ml adriamycin and lysed in SDS lysis buffer 6 hours following treatment. Extracts were then resolved by SDS-PAGE and E2F1 levels measured by Western blotting using E2F1 specific antibody (C-20). Blots were re-probed with a p38 antibody to ensure equal loading.

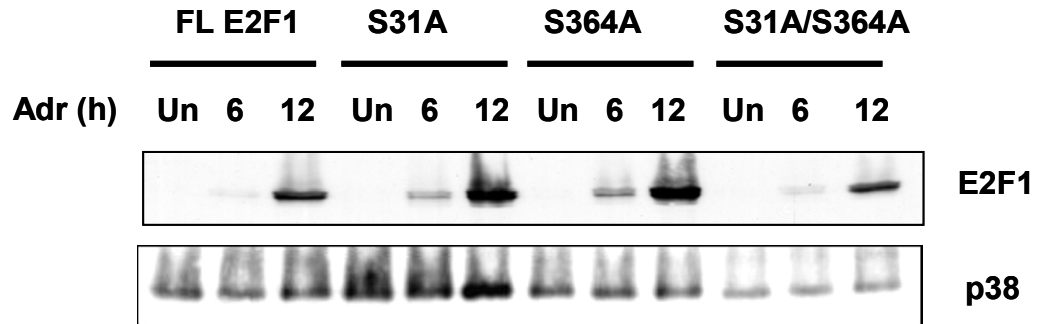


Figure 3-9 A 'double mutant' of E2F1 with S31A and S364A substitutions is induced following treatment with adriamycin

E2F1^{-/-} MEFs expressing FL or mutated E2F1 (S31A, S364A or S31A/S364A) were treated with 2.0 µg/ml adriamycin and lysed in SDS lysis buffer 6 and 12 hours following treatment. Extracts were then resolved by SDS-PAGE and E2F1 levels measured by Western blotting using E2F1 specific antibody (C-20). Blots were re-probed with a p38 antibody to ensure equal loading.

more induction after 12 hours treatment (Fig. 3-9). This indicates that neither of the two known DNA damage responsive phosphorylation events are required for the induction seen on full length E2F1 following DNA damage.

This raised the question if, in combination or independently, other mechanisms played a role in the induction. In order to investigate if MDM2 binding to the C-terminal activation domain of E2F1 was responsible for the E2F1 induction seen on the S31A/S364A double mutant, a MDM2 binding deficient mutant with the aspartic acid at site 390 and phenylalanine at 391 substituted with alanine (D390A/F391A) was created in addition to the double S31A/S364A mutant. This mutant, with both known DNA damage phosphorylation sites and deficient in binding to MDM2, was expressed in E2F1^{-/-} MEFs. Basal E2F1 levels of triple mutant (S32A, S364A and D390A/F391A) were detectable compared to undetectable levels of the previously made full length E2F1 cell line (Fig 3-10 compare lane 1 and 4). Following adriamycin treatment the levels of triple mutant E2F1 increased, almost to the same levels as seen on the full length protein, with detectable induction after 6 hours following treatment and robust induction after 12 hours.

As previously discussed E2F1 has also been shown to be a target for acetylation on lysine 117, 120 and 125 by P/CAF which leads to stabilization following adriamycin treatment (Martinez-Balbas et al., 2000; Pediconi et al., 2003). One possibility is that acetylation on these lysines plays a major part in the E2F1 induction seen on the triple mutant in our system. In order to test that, a mutant with all three lysine residues substituted to arginine (K117R/K120R/K125R) was created, either on full length E2F1 or on the triple mutant (S32A, S364A and D390A/F391A). These mutants were then stably expressed in E2F1^{-/-} MEFs and tested for E2F1 induction following adriamycin treatment. As seen in Fig. 3-11, arginine substitutions for these lysine residues on the triple mutant did not impair E2F1 induction following treatment with adriamycin indicating that these residues are not critical for E2F1 to accumulate following DNA damage (Fig 3-11a).



Figure 3-10 A 'triple mutant' of E2F1, with S31A and S364A substitutions and mutations impairing MDM2 binding is induced following treatment with adriamycin

E2F1^{-/-} MEFs expressing FL or mutated E2F1 (S31A/S364A/D390A/F391A) were treated with 2.0 µg/ml adriamycin and lysed in SDS lysis buffer 6 and 12 hours following treatment. Extracts were then resolved by SDS-PAGE and E2F1 levels measured by Western blotting using E2F1 specific antibody (C-20). Blots were re-probed with a p38 antibody to ensure equal loading.



Figure 3-11 Acetylation mutations in addition on the ‘triple mutant’ of E2F1, is induced following treatment with adriamycin

A) E2F1^{-/-} MEFs expressing the E2F1 ‘triple mutant’ with in addition, acetylation sites at residue 117, 120 and 125 mutated to arginine (S31A/S364A/D390A/F391AK117R/K120R/K125R) were treated with 2.0 µg/ml adriamycin and lysed in SDS lysis buffer 6 and 12 hours following treatment. Extracts were then resolved by SDS-PAGE and E2F1 levels measured by Western blotting using E2F1 specific antibody (C-20). Blots were re-probed with a p38 antibody to ensure equal loading. **B)** E2F1^{-/-} MEFs expressing E2F1 with acetylation sites at residue 117, 120 and 125 mutated to arginine (K117R/K120R/K125R) were treated with the HDAC inhibitor trichostatin A (TSA) (100 µM final concentration). Cells were then lysed in SDS lysis buffer and extracts resolved by SDS-PAGE. E2F1 levels were measured by Western blotting using E2F1 specific antibody (C-20). Blots were re-probed with an actin antibody to ensure equal loading.

In addition, the acetylation mutant retains its inducibility following TSA treatment. That indicates that additional acetylation sites might play a role in the stabilization of E2F1 following DNA damage (Fig. 3-11b).

These results indicate that there are still mechanism(s) to be found that lead to post-transcriptional E2F1 accumulation in response to the chemotherapeutic drug adriamycin. Further studies are therefore required to understand E2F1 induction under these circumstances.

3.2.2 Response of regions of E2F1 to DNA damage

3.2.2.1 Description of E2F1 truncations

In order to further understand E2F1's induction following DNA damage and in attempt to analyse what regions of E2F1 are responsible for the induction, previously made and described truncated forms of E2F1 were used (Bell et al., 2006). These series of N- and C-terminal truncations of E2F1 were fused at the C-terminus to Green Fluorescent Protein (GFP). That allowed easy detection by Western blotting of all of the E2F1 truncated forms when expressed in cells. The truncations had a series of E2F1 domains removed and could therefore be used to determine which components of E2F1 may be important for induction in an attempt to find a minimal region of E2F1 that responds to DNA damage (Fig. 3-12).

3.2.2.2 C-terminal truncations

The first truncation (tr. 1 Δ^{374}) used lacks the entire transactivation domain of E2F1 (amino acids 375-437) including the pRb binding domain and the region necessary for MDM2 binding. It has been shown previously that E2F1 is actively degraded by the ubiquitin-proteasome pathway and efficient degradation depends on the availability of selected sequences within the C-terminus of E2F1 and pRb binding (Hofmann et al., 1996). The importance of this region in DNA damage response has not been determined. Truncation 1 has previously been shown to be able to induce cell death in transactivation independent manner by

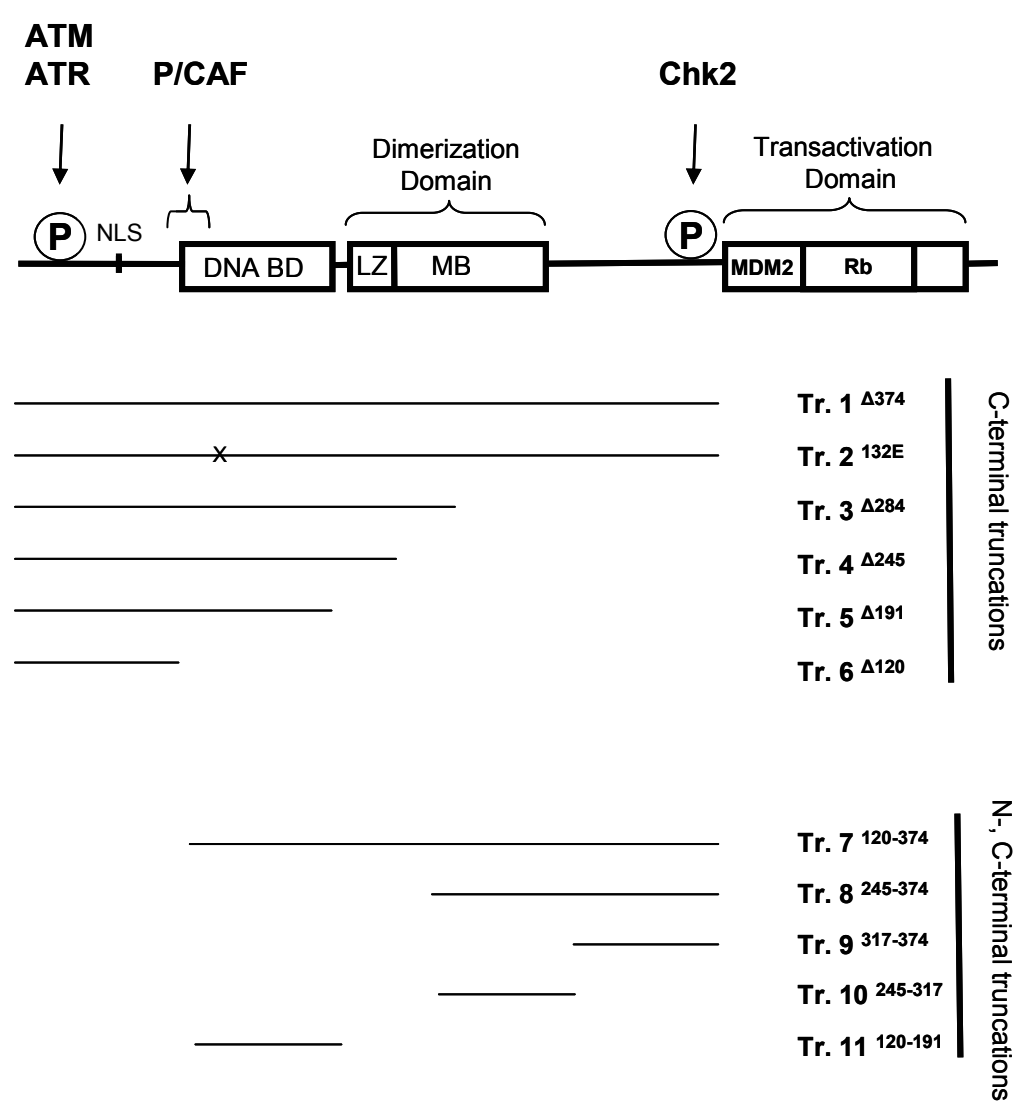


Figure 3-12 Description of E2F1 truncations

A series of C-terminal and N-, C-terminal truncations of E2F1 were used in order to find a minimal region of E2F1 that responds to DNA damage. Truncation 1 lacks the whole transactivation domain of E2F1. Truncation 2 has a mutation in the DNA binding domain. All other C-terminal truncations lack part of the C-terminus. All N-, C-terminal truncations lack part of the N-terminus in addition of lacking the transactivation domain. All truncations were fused to GFP at the C-terminus for detection by Western blotting.

a mechanism considered to be via de-repression of E2F1 target genes (Hsieh et al., 1997; Liu and Greene, 2001; Phillips et al., 1997). Truncation 2 (tr. 2^{Δ374, 132E}) has a glutamic acid substitution at amino acid 132 within the DNA-binding domain that abrogates DNA binding and also lacks the transactivation domain, like all the other remaining truncations made (Cress et al., 1993; Helin and Harlow, 1994). This truncation is impaired in activating E2F1 target genes and does not induce apoptosis when overexpressed (Bell et al., 2006; Hsieh et al., 1997; Phillips et al., 1997). Truncation 3 (tr. 3^{Δ284}) lacks amino acids 285 to 437 and therefore lacks both the entire transactivation domain, including the pRb binding domain, and the Chk2 phosphorylation site at S364. It also lacks a part of the marked box within the dimerization domain. Truncation 4 (tr. 4^{Δ245}) lacks amino acids 246 to 437 and therefore the whole marked box. Truncation 5 (tr. 5^{Δ191}) lacks the entire dimerization domain and is therefore unable to bind to DP family of proteins. Truncation 6 (tr. 6^{Δ120}), the smallest C-terminal truncation made, lacks amino acids 121-473 and therefore the entire DNA binding domain in addition to the transactivation domain.

3.2.2.3 N- and C-terminal truncations

Truncation 7 (tr. 7¹²⁰⁻³⁷⁴) is an N- and C-terminal domain truncation which lacks the first 120 amino acids plus the transactivation domain. This truncation therefore lacks the nuclear localisation signal (NLS) of E2F1 and is expressed throughout the entire cell when overexpressed compared with truncations that remains the NLS which are primarily, though not exclusively, localised in the nucleus, a pattern generally observed for WT E2F1 (L.A. Bell, Ph.D. thesis, University of Glasgow, 2006). Truncation 8 (tr. 8²⁴⁵⁻³⁷⁴) only consists of amino acids 245 to 374 so it includes the marked box and the Chk2 phosphorylation site at 364. Truncation 9 (tr. 9³¹⁷⁻³⁷⁴) consists of amino acids 317 to 374. Truncation 10 (tr. 10²⁴⁵⁻³¹⁷) consists of the marked box only (amino acids 245 to 317). Truncation 11 (tr. 11¹²⁰⁻¹⁹¹), the smallest truncation, consists only of the DNA binding domain of E2F1 (amino acids 120 to 191). This domain has been shown to induce apoptosis in a transactivation independent manner and does not bind DNA (Bell et al., 2006).

3.2.2.4 DNA damage response of GFP linked full length E2F1

Before infection with the truncations it was decided to infect full length E2F1 protein linked to GFP in to E2F1^{-/-} MEFs to certify that C-terminal GFP linked E2F1 remains it's induction following DNA damage as seen with full length unlinked E2F1 (Fig. 3-6a). Following infection of FL E2F1-GFP and GFP alone as control into E2F1^{-/-} MEFs, cells were treated with adriamycin for 6 and 12 hours. The E2F1 protein levels were measured by Western blotting following treatment using GFP specific antibody. This revealed a clear induction after 6 hours following treatment and with similar levels after 12 hours (Fig. 3-13). No induction was seen following adriamycin treatment in cells expressing GFP alone indicating that the induction seen on E2F1 is not due to GFP stabilization following drug treatment. It was noticed that the basal level of GFP alone was higher than GFP fused with E2F1 and could indicate that it lacks domains found in E2F1 that is responsible for active degradation and is therefore more stable in untreated cells. These results indicated that E2F1-GFP behaves in similar manner in terms of induction following DNA damage as wild type E2F1 (Fig 3-1 and 3-6a). Therefore, using the truncations of E2F1 may be a good model in an attempt to find minimal regions of E2F1 that are responsive to DNA damage.

3.2.2.5 DNA damage response of C-terminal truncations

In order to analyse the response of C-terminal truncations to DNA damage, tr. 1 Δ^{374} was first stably expressed in E2F1^{-/-} MEFs and analysed. This revealed low basal expression and barely detectable with the GFP specific antibody (Fig. 3-14a). When these cells were treated with adriamycin a detectable induction was seen after 6 hours with the accumulation increasing at 12 hours. This indicates that the transactivation domain, with the pRb binding domain and sequences necessary for MDM2 binding is not necessary for E2F1 induction following DNA damage. However, it can not be concluded that pRb or MDM2 do not contribute to the induction of the full length E2F1 under these conditions, since quantitative comparison is not possible between these different cell-lines, especially when the basal expression levels differ.

Next the question if DNA binding of E2F1 was necessary for its induction following DNA damage was asked. Following infection of tr. 2 $\Delta^{374, 132E}$ and treatment with adriamycin for 6 and 12 hours, E2F1 levels were measured by

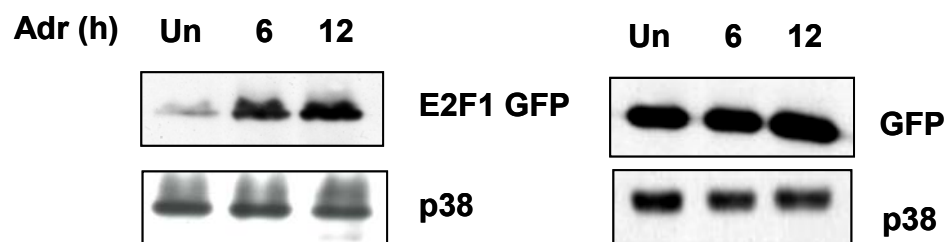


Figure 3-13 E2F1 fused to GFP is induced following treatment with adriamycin in E2F1^{-/-} MEFs

E2F1^{-/-} MEFs expressing E2F1 fused with GFP (E2F1 GFP) or GFP alone were treated with 2.0 µg/ml adriamycin and lysed in SDS lysis buffer 6 and 12 hours following treatment. Extracts were resolved by SDS-PAGE and E2F1 and GFP levels measured by Western blotting using GFP specific antibody. Blots were re-probed with a p38 antibody to ensure equal loading.

Western blotting. This revealed a modest induction after 6 hours following treatment and similar levels of accumulation after 12 hours (Fig. 3-14b). This indicates that DNA binding is not necessary for E2F1 to respond to DNA damage.

In order to further map the domain that could be responsible for induction seen on tr. 1^{Δ374} and tr. 2^{Δ374, 132E}, tr. 3^{Δ284} was infected into E2F1^{-/-} MEFs and analysed for protein accumulation following drug treatment. Tr. 3^{Δ284} responded to adriamycin treatment in a similar fashion as tr. 1^{Δ374} and tr. 2^{Δ374, 132E} indicating that the region responsible for induction seen on tr. 1^{Δ374} does not contain amino acids 284 to 374 and therefore does not require phosphorylation of serine 364 (Fig 3-14c). Similar results were obtained with tr. 4^{Δ245} and tr. 5^{Δ191}. Both truncations showed induction after 6 and 12 hours following adriamycin treatment when stably expressed in E2F1^{-/-} MEFs (Fig. 3-14 d and e). This ruled out the dimerization domain as a region responsible for the induction experienced on tr. 1^{Δ374} and indicated that the induction is not dependent on E2F1 binding to its DP binding partners.

The next question asked was whether induction seen by tr. 5^{Δ191} in response to DNA damage was dependent on the DNA binding domain. Tr. 6^{Δ120} was therefore expressed in E2F1^{-/-} MEFs and analysed for induction following DNA damage. This truncation showed higher basal expression compared with truncations 1-5, but whether that was due to higher viral infection efficiency or increased protein stability was not determined. When cells expressing tr. 6^{Δ120} were treated with adriamycin for 12 hours period, clear induction was seen after 6 hours and even more induction after 12 hours following treatment (Fig. 3-14f).

This indicates that the first 120 amino acids of E2F1 are able to respond to DNA damage with the possibility that some modifications may occur that lead to protein accumulation of this N-terminal truncation. The only previously described DNA damage responsive mechanisms within this region are the ATM phosphorylation site at serine 31 and the acetyl responsive lysines at 117 and 120. In order to investigate if tr. 6^{Δ120} is induced by modifications at these sites, mutagenesis on S31, K117 and K120 on this truncation was performed, leading to serine/alanine and lysine/arginine substitutions when expressed in cells. Firstly, cells were infected with virus expressing tr. 6^{Δ120} S31A to determine the importance of ATM phosphorylation on tr. 6^{Δ120}. As figure 3-15

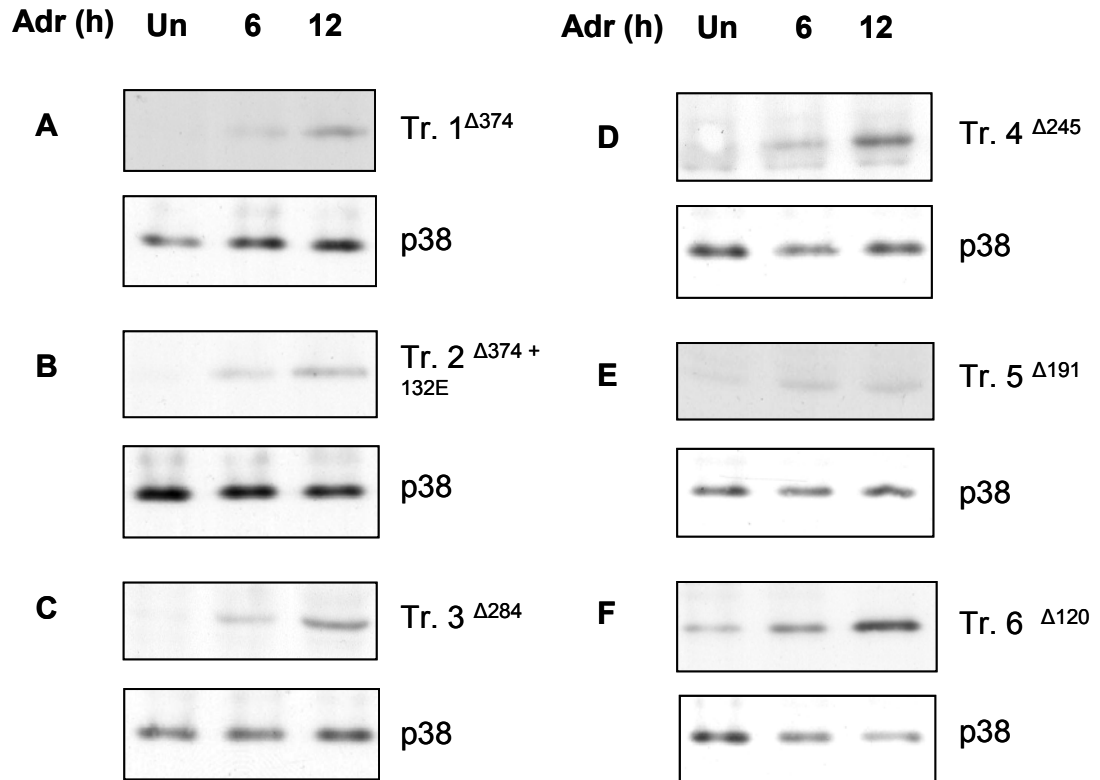


Figure 3-14 Response of C-terminal truncations of E2F1 to adriamycin treatment

E2F1^{-/-} MEFs expressing C-terminal truncations of E2F1 were treated with 2.0 μ g/ml adriamycin and lysed in SDS lysis buffer 6 and 12 hours following treatment. Extracts were then resolved by SDS-PAGE and E2F1 levels measured by Western blotting using GFP specific antibody. Blots were re-probed with a p38 antibody to ensure equal loading. The truncations are as follows **A)** Truncation 1 lacks the entire transactivation domain. **B)** Truncation 2 has glutamic acid substitution at amino acid 132 in addition with lacking the transactivation domain. **C)** Truncation 3 lacks amino acids 285-437. **D)** Truncation 4 lacks amino acids 246-437. **E)** Truncation 5 lacks amino acids 293-437. **F)** Truncation 6 lacks amino acids 121-473.

indicates, ATM phosphorylation at S31 is not responsible for this induction, since tr. 6^{Δ120} S31A showed similar induction after 6 and 12 hours following adriamycin treatment compared with unmutated tr. 6^{Δ120} (Fig. 3-15). To assess if acetylation on lysine 117 and/or 120 was responsible for the accumulation of tr. 6^{Δ120} in this assay, tr. 6^{Δ120} and tr. 6^{Δ120} S31A were expressed with the lysine arginine substitutions. This revealed that tr. 6^{Δ120} is induced by some previously unknown mechanism, since induction of tr. 6^{Δ120} S31A/K117A/K120A was similar to induction seen on tr. 6^{Δ120} (Fig. 3-16).

These data indicate that at least one mechanism of E2F1 induction following DNA damage is yet to be discovered and that mechanism is in the first 120 amino acids of E2F1. However, the significance of previously characterized mechanisms to E2F1 induction in this assay is difficult to ascertain. The basal expression levels of exogenous proteins can not be perfectly controlled and therefore vary between different cell lines. In addition, care should be taken when direct comparison of protein accumulation between different Western blot membranes is made because of differences in antibody detection and differences in exposure time. Nevertheless, these data prompted us to further examine E2F1 induction following adriamycin treatment in order to find other regions within full length E2F1 that are responsive to DNA damage and examine the importance of previously known mechanisms for induction of these regions.

3.2.2.6 DNA damage response of N-, C-terminal truncations

In order to analyse the response of N- and C-terminal truncations to DNA damage, tr. 7¹²⁰⁻³⁷⁴ was first stably expressed in E2F1^{-/-} MEFs and analysed. This revealed that the basal expression was low and barely detectable with the GFP specific antibody (Fig 3-17a). Following adriamycin treatment for 6 and 12 hours a clear induction was observed, with a detectable induction after 6 hours and further accumulation after 12 hours (Fig 3-17a). This pattern was very similar observed with the C-terminal truncations and reveal that even though some unknown mechanism exists in the first 120 amino acids of E2F1, they are not necessary for the DNA damage response.

In an attempt to find the minimal responsive region within the C-terminal end of E2F1, truncations 8-10 were stably expressed in E2F1^{-/-} MEFs. The largest truncations of these three, tr. 8²⁴⁵⁻³⁷⁴ consists of amino acids 245 to 374 and

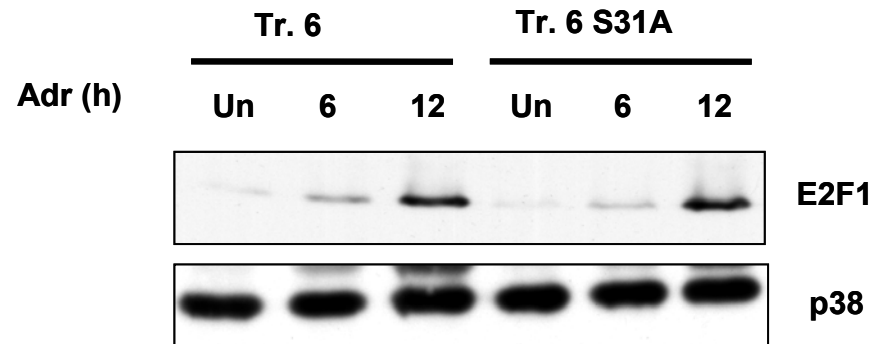


Figure 3-15 Truncation 6 with a serine to alanine substitution at amino acid 31 is induced following adriamycin treatment

E2F1^{-/-} MEFs expressing truncation 6 (Tr. 6) or truncations 6 with serine 31 substituted to alanine (Tr. 6 S31A) were treated with 2.0 µg/ml adriamycin and lysed in SDS lysis buffer 6 and 12 hours following treatment. Extracts were then resolved by SDS-PAGE and E2F1 levels measured by Western blotting using GFP specific antibody. Blots were re-probed with a p38 antibody to ensure equal loading.

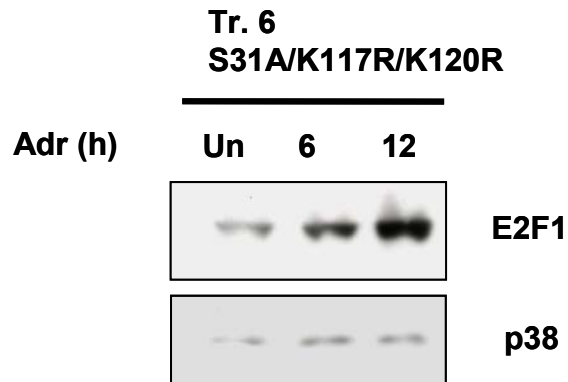


Figure 3-16 Truncation 6 with a serine to alanine substitution at amino acid 31 and with acetylation sites K117 and K120 mutated to arginine is induced following adriamycin treatment

E2F1^{-/-} MEFs expressing truncation 6 with lysine 117 and 120 substituted with arginine in addition to serine 31 substituted to alanine (Tr. 6 S31A/K117R/K120R) were treated with 2.0 µg/ml adriamycin and lysed in SDS lysis buffer 6 and 12 hours following treatment. Extracts were then resolved by SDS-PAGE and E2F1 levels measured by Western blotting using GFP specific antibody. Blots were re-probed with a p38 antibody to ensure equal loading.

includes the marked box and the Chk2 phosphorylation site at 364. Following adriamycin treatment protein levels were measured by Western blotting. This revealed no induction after 6 or 12 hours of treatment indicating that this truncation is unable to respond to DNA damage (Fig. 3-17b). Similar results were obtained with tr. 9³¹⁷⁻³⁷⁴ and tr. 10²⁴⁵⁻³¹⁷ with no visible induction under these conditions (Fig. 3-17 c and d). These results indicated that the mechanism responsible for induction seen on tr. 7¹²⁰⁻³⁷⁴ is either in the DNA binding domain of E2F1 or within amino acids 192 to 245, which consists primarily of the leucine zipper region of the dimerization domain.

In order to ensure that the induction seen on tr. 7¹²⁰⁻³⁷⁴ was not dependent on Chk2 phosphorylation on S364, a mutant version of 7¹²⁰⁻³⁷⁴ carrying serine to alanine substitution at site 364, was made and expressed in cells. Following adriamycin treatment tr. 7¹²⁰⁻³⁷⁴ S364A showed similar induction as seen on 7¹²⁰⁻³⁷⁴ (Fig. 3-17e). In order to further narrow down the region responsible, tr. 11¹²⁰⁻¹⁹¹ which only consists of the DNA binding domain, was expressed in E2F1^{-/-} MEFs and protein levels measured by Western blotting following treatment with adriamycin for 6 and 12 hours. This revealed a detectable induction after 6 hours of treatment with similar induction after 12 hours (Fig. 3-17f).

The DNA binding domain does not include the known ATM and Chk2 phosphorylation sites and must therefore use different mechanism for induction. This domain comprises two of the known DNA damage responsive acetylation sites (K120 and K125) that could be responsible for the induction seen. However, that was not considered to be a likely explanation since substitution of all three lysines on full length E2F1 did not alter its inducibility (Fig. 3-11) and neither did substitution of two of them on tr. 6^{Δ120} (Fig. 3-16). This data therefore indicates that the DNA binding domain of E2F1 can respond to DNA damage and be induced following adriamycin treatment by a mechanism(s) not previously described.

In summary, using the C-terminal and N-, C-terminal truncations of E2F1, two minimal regions of E2F1 were found to be responsive to DNA damage (Fig. 3-18). The data suggest that the mechanism for the induction seen on tr. 6^{Δ120} and tr. 11¹²⁰⁻¹⁹¹ are not by previously identified mechanisms. That prompted us to

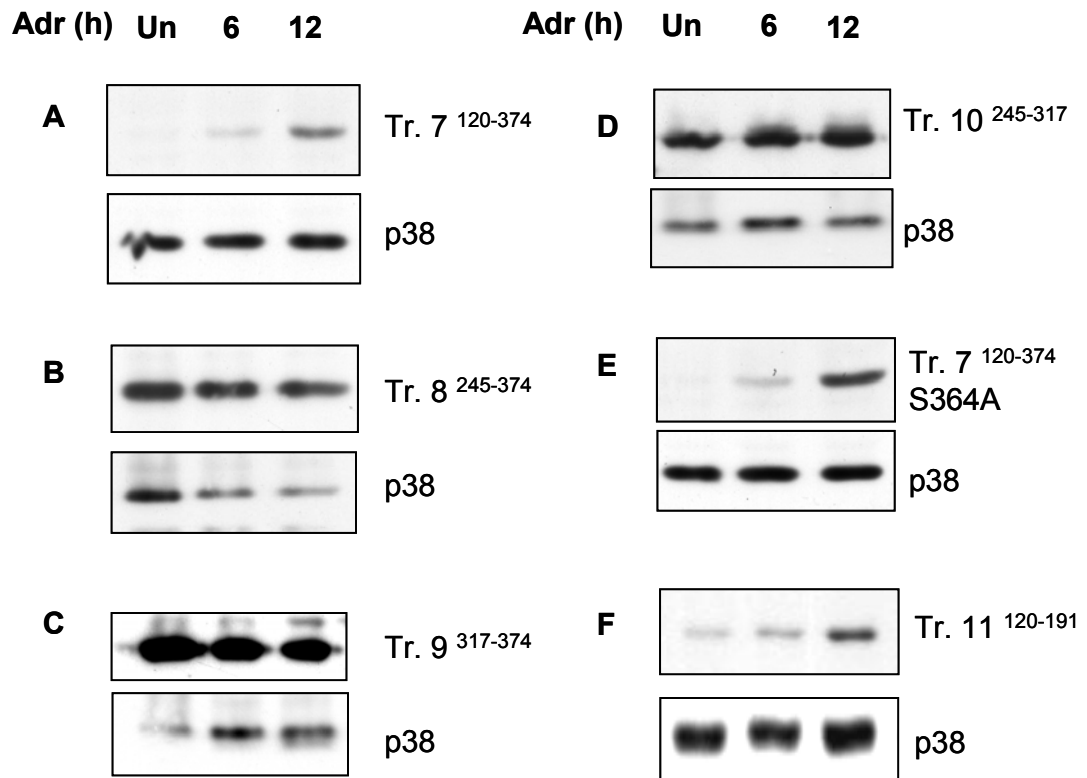


Figure 3-17 Response of N-, C-terminal truncations of E2F1 to adriamycin treatment

E2F1^{-/-} MEFs expressing N- and C-terminal truncations of E2F1 were treated with 2.0 µg/ml adriamycin and lysed in SDS lysis buffer 6 and 12 hours following treatment. Extracts were then resolved by SDS-PAGE and E2F1 levels measured by Western blotting using GFP specific antibody. Blots were re-probed with a p38 antibody to ensure equal loading. The truncations are as follows **A)** Truncation 7 lacks the first 120 amino acids in addition with the transactivation domain. **B)** Truncation 8 consists of amino acids 245-374. **C)** Truncation 9 consists of amino acids 317-374. **D)** Truncation 10 consists of amino acids 245-317. **E)** Truncation 7 with serine 31 to alanine substitution. **F)** Truncation 11 consists only of the DNA binding domain (amino acids 120-191).

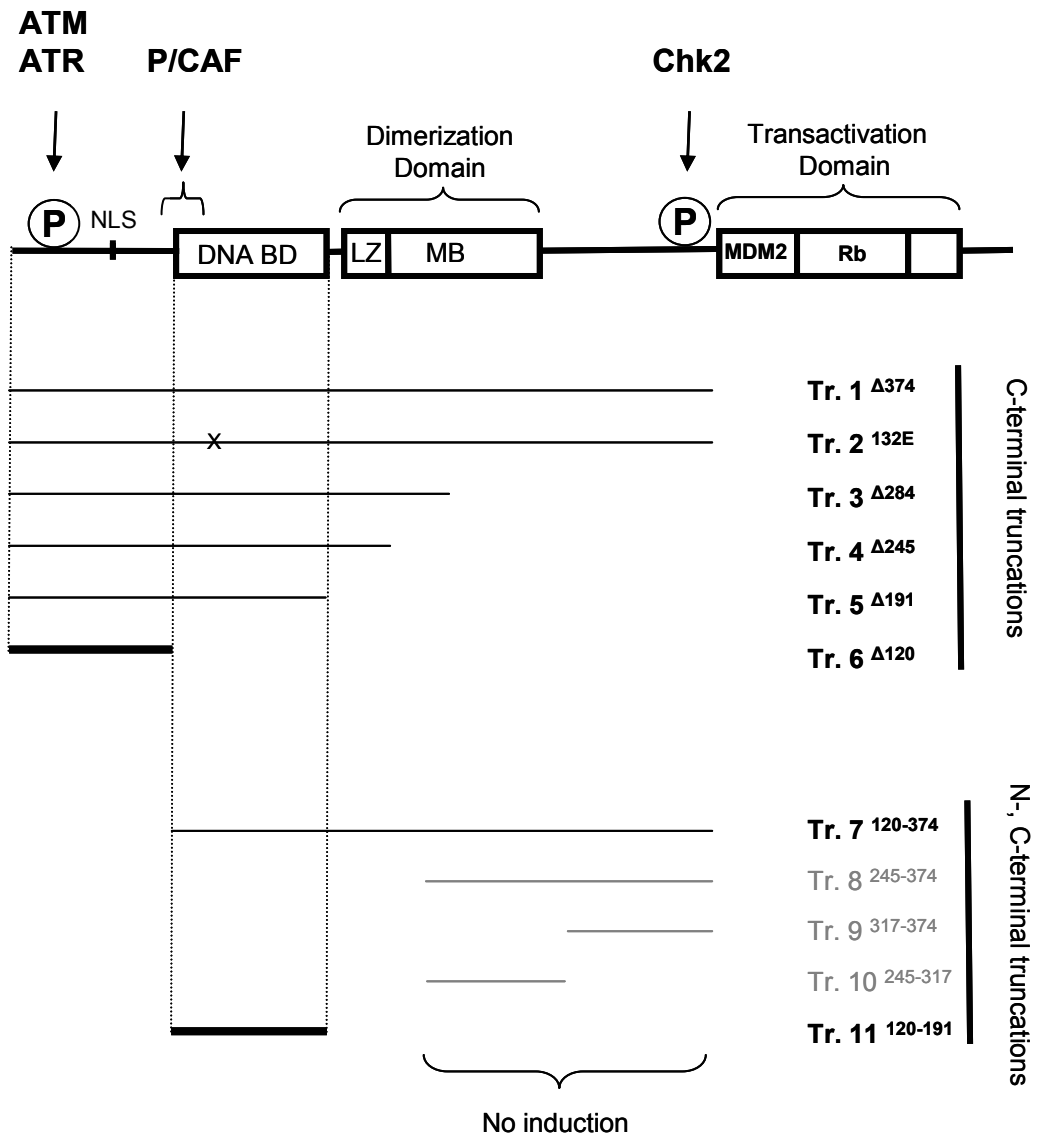


Figure 3-18 Summary of induction of E2F1 truncation following DNA damage

A series of C-terminal and N-, C-terminal truncations of E2F1 were expressed in E2F1^{-/-} MEFs in order to find a minimal region of E2F1 that responds to DNA damage. This revealed that two small regions of E2F1, truncation 6 (Tr. 6 $\Delta 120$) and truncation 11 (Tr. 11 120-191) are both induced following DNA damage. That indicates that E2F1 can be induced by mechanism(s) other than those that have been previously described. Truncations that showed induction following DNA damage are in bold. Two minimal regions, truncation 6 and truncation 11 are highlighted. C-terminal truncation 8, 9 and 10 showed no induction following DNA damage.

further examine the induction of both truncations to try to search for the potential mechanism(s) involved.

3.2.2.7 Identification of potential phosphorylation sites in tr. 6^{Δ120}

In order to further investigate the induction of tr. 6^{Δ120} and in an attempt to find the mechanism responsible for the response it was decided to search for potential phosphorylation sites within this region. Protein phosphorylation affects a multitude of cellular signalling processes and two phosphorylation sites within E2F1 that affect stabilization have been identified (Lin et al., 2001; Stevens et al., 2003). Human p53 has also been reported to responsive to DNA damage. Increased phosphorylation in the N-terminal domain of p53 has been demonstrated for at least nine phosphorylation sites in response to ionizing radiation or UV light (reviewed in (Appella and Anderson, 2001; Brooks and Gu, 2003; Latonen and Laiho, 2005). Therefore, in light of the data obtained with the E2F1 truncations, it was believed to be a strong possibility that unknown phosphorylation sites play a role in the induction observed on tr. 6^{Δ120}. Using NetPhos 2.0 (<http://www.cbs.dtu.dk/services/NetPhos/>), a neural network-based method for predicting potential phosphorylation sites at serine, threonine or tyrosine residues in protein sequences, several potential phosphorylation sites in tr. 6^{Δ120} were identified (Fig. 3-19) (Blom et al., 1999). These potential phosphorylation sites were threonine 49 and 75, tyrosine 100 and serine 104. This indicated that phosphorylation of any of these sites might, singularly or in combination, play a role in the induction of E2F1 following DNA damage. This prompted us to further analyse the importance of these potential phosphorylation sites.

3.2.2.8 Tr. 6^{Δ120} with potential phosphorylation sites mutated is induced following DNA damage

In order to investigate the role of potential phosphorylation sites in induction of tr. 6^{Δ120} mutant constructs of all potential phosphorylation sites that showed score similar or higher than serine 31, the only known site within the region, were made. These mutations were made on the tr. 6^{Δ120} S31A/K117A/K120A (tr. 6^{Δ120+3mut}) to make certain these residues did not play a role in the response.

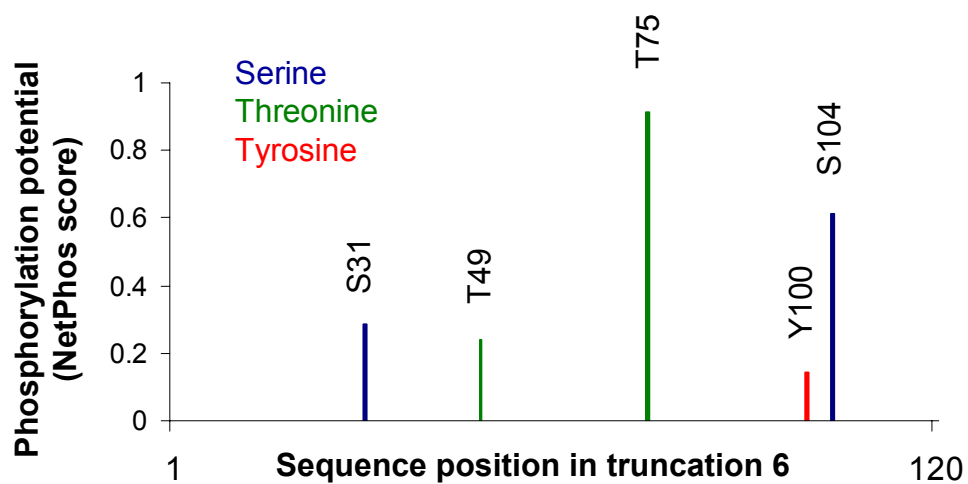


Figure 3-19 Predicted phosphorylation sited in truncation 6

Potential phosphorylation sites were found in truncation 6 using NetPhos 2.0. NetPhos score is the output score (from 0 or low potential to 1 or high potential) from the ensembles of neural networks trained on that acceptor residue type. Figure adapted from NetPhos 2.0 Server – prediction results.

To block potential phosphorylation, threonine 49 was mutated to alanine and, in order to mimic phosphorylation at this site, threonine 49 was substituted with aspartic acid. These constructs, tr. 6^{Δ120+3mut} T49A and T49D, were then expressed in E2F1^{-/-} MEFs and protein levels following adriamycin treatment analysed by Western blotting. This revealed that neither alanine nor aspartic acid substitution inhibited the inducibility of tr. 6^{Δ120+3mut} after 6 and 12 hours of treatment (Fig. 3-20a) and the induction was comparable with the induction seen on unmutated tr. 6^{Δ120+3mut} (Fig. 3-16).

Next the involvement of threonine 75 phosphorylation was assessed. Tr. 6^{Δ120+3mut} T75A and T75D constructs were made and expressed in E2F1^{-/-} MEFs. Following infections and adriamycin treatment, cells were analysed for protein expression. As seen on figure 3-20b, these substitutions did not completely inhibit the induction as clear protein accumulation was observed after 12 hours following treatment (Fig. 3-20b).

Constructs with tyrosine 100 substituted with phenylalanine or aspartic acid were next analysed. Detectable inductions were obtained after 12 hours following adriamycin treatment (Fig 3-20c), indicating that phosphorylation on this tyrosine residue was not completely responsible for the induction experienced on tr. 6^{Δ120+3mut}. Lastly, tr. 6^{Δ120+3mut} S104A and S104D constructs were generated and analysed. Detectable accumulation was obtained after 6 and 12 hours following treatment (Fig. 3-20d).

These data clearly indicate, since none of these mutations completely inhibited the response to DNA damage that either they are not involved in the induction of tr. 6^{Δ120} or combination of mutations would be required to inhibit the induction. However, other post transcriptional mechanism, such as acetylation might play a role and be responsible for the induction. Further studies are therefore required to fully understand the mechanism involved in the induction of tr. 6^{Δ120}, the smallest N-terminal truncations that responded to DNA damage in this study.

3.2.2.9 Identification of potential phosphorylation sites in tr. 11¹²⁰⁻¹⁹¹

In attempt to find the mechanism responsible for the induction seen on tr. 11¹²⁰⁻¹⁹¹ which consists of the DNA binding domain of E2F1, following DNA damage, it

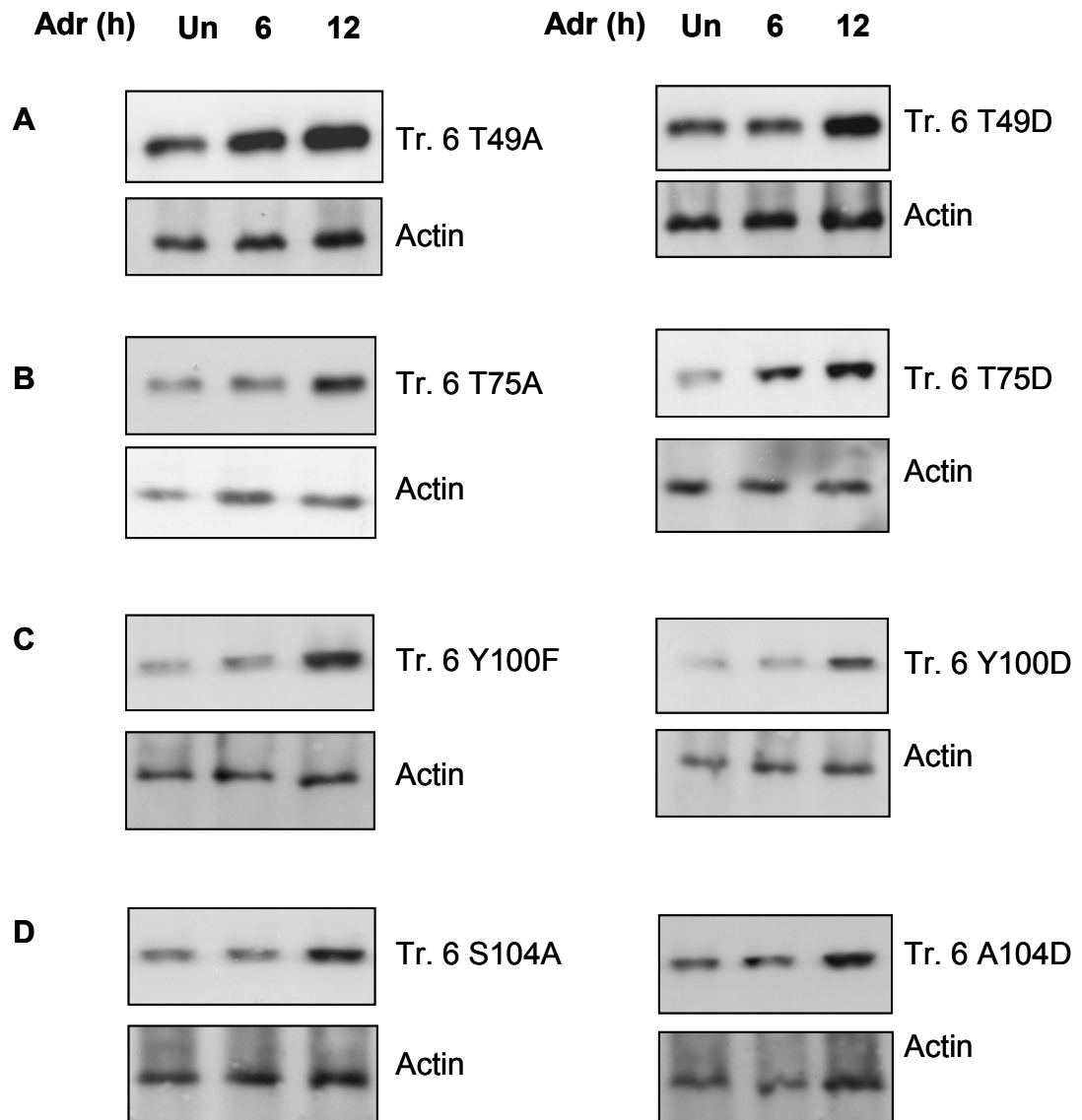


Figure 3-20 Response of truncation 6 with potential phosphorylation sites mutated to adriamycin treatment

E2F1^{-/-} MEFs expressing truncation 6 (tr. 6 $\Delta_{120+3mut}$) with potential phosphorylation sites mutated were treated with 2.0 μ g/ml adriamycin and lysed in SDS lysis buffer 6 and 12 hours following treatment. Extracts were then resolved by SDS-PAGE and E2F1 levels measured by Western blotting using GFP specific antibody. Blots were re-probed with an actin antibody to ensure equal loading. The truncations are as follows **A**) tr. 6 $\Delta_{120+3mut}$ T49A and T49D. **B**) tr. 6 $\Delta_{120+3mut}$ T75A and T75D. **C**) tr. 6 $\Delta_{120+3mut}$ Y100F and Y100D. **D**) tr. 6 $\Delta_{120+3mut}$ S104A and S104D.

was also decided to search for potential phosphorylation sites within this region. This was done by using the same network based method as used for tr. 6^{Δ120}, NetPhos 2.0 (<http://www.cbs.dtu.dk/services/NetPhos/>), which predicts potential phosphorylation sites at serine, threonine or tyrosine residues in protein sequences (Blom et al., 1999). Using this software it was possible to find several potential phosphorylation sites in tr. 11¹²⁰⁻¹⁹¹ (Fig. 3-21). These potential phosphorylation sites were serine 121 and 126, threonine 130, 135 and 136, and tyrosine 128 and 168. This indicated that phosphorylation of any of these sites might play a role in the induction of E2F1 following DNA damage, which prompted us to further analyse the importance of these potential phosphorylation sites.

3.2.2.10 Tr. 11¹²⁰⁻¹⁹¹ with potential phosphorylation sites mutated is induced following DNA damage

In order to investigate the importance of the potential phosphorylation sites within tr. 11¹²⁰⁻¹⁹¹ mutagenesis was used to substitute potential phosphorylation sites for amino acids that can not be phosphorylated (alanine or phenylalanine) or to a phospho-mimicking amino acid (glutamic acid). Firstly, E2F1^{-/-} MEFs expressing tr. 11¹²⁰⁻¹⁹¹ S121A or S121D were analysed for protein induction 6 and 12 hours following adriamycin treatment. This revealed a modest induction after 6 hours of treatment and more detectable induction after 12 hours (Fig. 3-22a). This indicated that phosphorylation on serine 121 was not completely responsible for the induction of tr. 11¹²⁰⁻¹⁹¹. Mutations were next made on serine 126. Following infection into E2F1^{-/-} MEFs, cells were analysed for protein accumulation following DNA damage. Similar to what seen for serine 121 substitution, tr. 11¹²⁰⁻¹⁹¹ S126A and S126D were insignificantly induced after 6 hours of treatment with more accumulation after 12 hours (Fig. 3-22b).

This indicated that phosphorylation on serine 126 did not play an essential role in truncation tr. 11¹²⁰⁻¹⁹¹ induction. To further analyse the role of the potential phosphorylation sites to DNA damage response of tr. 11¹²⁰⁻¹⁹¹, mutation were made on tyrosine 128 leading to phenylalanine and aspartic acid substitutions. Neither tr. 11¹²⁰⁻¹⁹¹ Y128F nor Y128D substitution showed complete inhibition of induction of tr. 11¹²⁰⁻¹⁹¹ following adriamycin treatment (Fig. 3-22c).

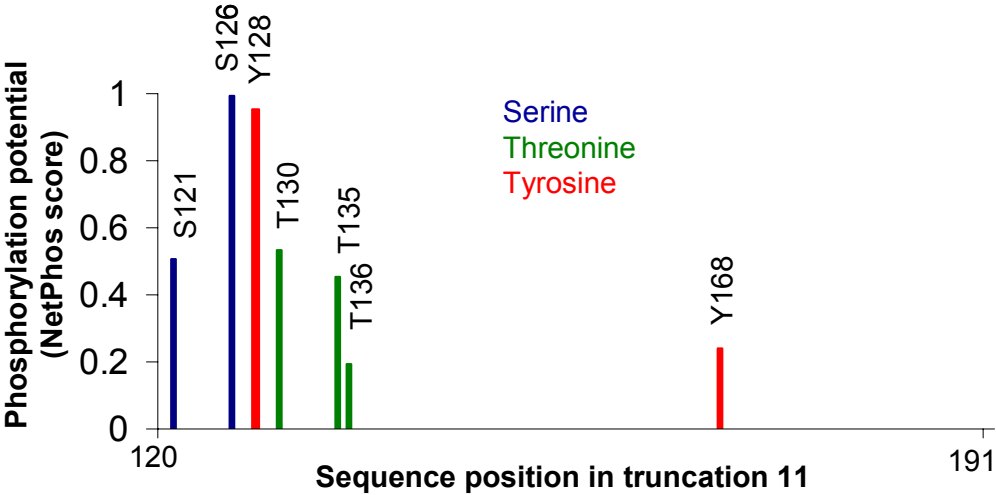


Figure 3-21 Predicted phosphorylation sited in truncation 11

Potential phosphorylation sites were found in truncation 11 using NetPhos 2.0. NetPhos score is the output score (from 0 or low potential to 1 or high potential) from the ensembles of neural networks trained on that acceptor residue type. Figure adapted from NetPhos 2.0 Server – prediction results.

Threonine 130 was next substituted with alanine and aspartic acid. Cells were made and subsequently treated with adriamycin for 6 and 12 hours. Both cell lines showed detectable induction after 12 hours of treatment (Fig. 3-22d).

The close proximity of threonines at position 135 and 136, which both showed to be potential phosphorylation sites by the NetPhos 2.0 program, allowed us to mutate them both using single oligo pair by PCR. Constructs, leading to both threonines to alanines and aspartic acids substitutions were made. Following infection into E2F1^{-/-} MEFs, cells were analysed for protein induction following DNA damage. This revealed detectable induction after 12 hours following treatment which indicated that neither of these threonines were essential in tr. 11¹²⁰⁻¹⁹¹ response to DNA damage (Fig. 21e).

The last potential phosphorylation site to be investigated was threonine at position 168. Constructs leading to T168A and T168D substitution were made on tr. 11¹²⁰⁻¹⁹¹. Cell lines expressing these constructs were treated with adriamycin for 6 and 12 hours and protein accumulation analysed. This revealed a detectable induction after 12 hours of treatment indicating that phosphorylation at threonine 168 could not be the sole explanation for the DNA damage response of tr. 11¹²⁰⁻¹⁹¹ (Fig. 3-22f).

These data clearly indicate that none of the phosphorylation sites tested were completely responsible for the inducibility of tr. 11¹²⁰⁻¹⁹¹. It is though a possibility that a combination of mutations would be required to inhibit the induction and therefore no effect seen when mutated one by one. Other post transcriptional mechanism that phosphorylation might also be responsible for the induction. The previously identified acetylation responsive lysines at position 120 and 125 could play a role or even some unidentified DNA damage responsive lysines within this domain. Therefore, in order to search for the mechanism responsible for this induction, further studies are required to fully understand the mechanism involved in the induction of the DNA binding domain of E2F1.

3.2.3 Chapter summary

It was demonstrated in the previous chapter that E2F1 is induced following treatment with adriamycin, a DNA damaging agent that is commonly used in treatment of various human cancers. Adriamycin was therefore used to

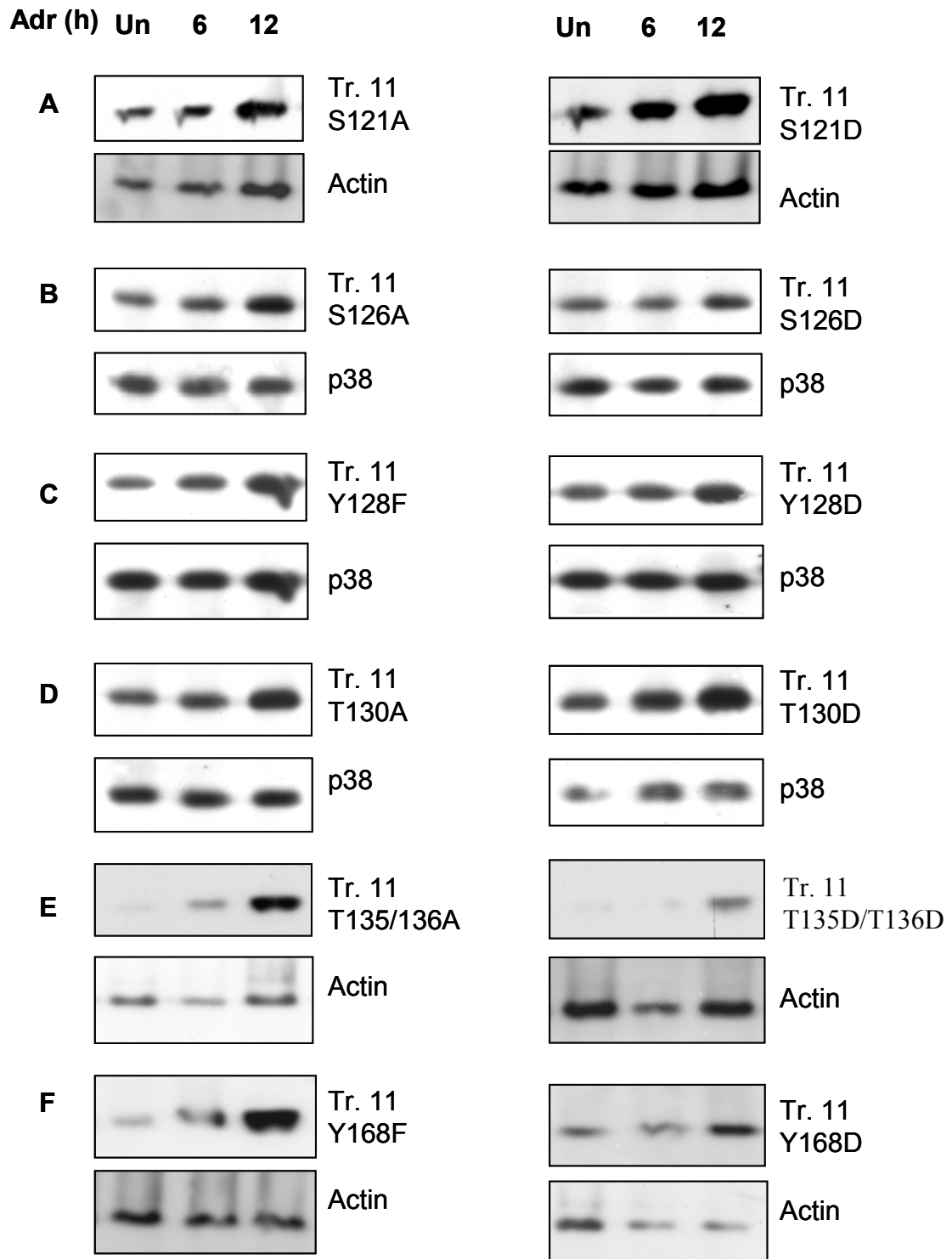


Figure 3-22 Response of truncation 11 with potential phosphorylation sited mutated to adriamycin treatment

E2F1^{-/-} MEFs expressing truncation 11 (tr. 11 120-191) with potential phosphorylation sited mutated alanine, phenylalanine or aspartic acid were treated with 2.0 µg/ml adriamycin and lysed in SDS lyses buffer 6 and 12 hours following treatment. Extracts were then resolved by SDS-PAGE and E2F1 levels measured by Western blotting using GFP specific antibody. Blots were re-probed with a p38 antibody to ensure equal loading. The truncations are as follows **A)** tr. 11 120-191 S121A and S121D. **B)** tr. 11 120-191 S126A and S126D. **C)** tr. 11 120-191 Y128F and Y128D. **D)** tr. 11 120-191 T130A and T130D. **E)** tr. 11 120-191 T135/136F and T135/136D. **F)** tr. 11 120-191 Y168F and Y168D

investigate the contribution of some of the previously characterized mechanisms leading to E2F1 activation and stabilization. This revealed that singularly or in combination they were not fully responsible for the E2F1 induction. It could not be concluded that these mechanisms were not activated and contributed to some degree to the induction, but it was apparent that they were dispensable, and therefore some other mechanism(s) must play a role in E2F1's response to DNA damage under these conditions.

In order to find a minimal region of E2F1 that is responsive to DNA damage, truncated forms of E2F1 were assessed for inducibility. These studies revealed that the first 120 amino acids (tr. 6^{Δ120}) of E2F1 respond to adriamycin treatment which could not be explained by serine 31 phosphorylation, and therefore indicate that at least one mechanism of E2F1 induction following DNA damage is yet to be discovered. Using a mutagenesis approach, the potential phosphorylation sites within this region were excluded as the sole sites essential for this induction. But whether other phosphorylation events or other modifications are responsible for the induction remains to be discovered.

In addition, the DNA binding domain (tr. 11¹²⁰⁻¹⁹¹) of E2F1 responded to DNA damage. This is a separate region from truncation 6 and indicates that another mechanism of E2F1 accumulation is yet to be discovered. Mutation of potential phosphorylation sites within this region did not completely inhibit the induction and indicates that either a combination of phosphorylation events or different modifications are responsible for this induction.

Together with previously identified pathways leading to E2F1 stabilization, these data indicate that E2F1's response to DNA damage is complex and perhaps overlapping and may involve numerous pathways and different modifications. Whether these mechanisms are inhibited in tumour development will need thorough investigation since, restoration of the apoptotic pathway of E2F1, for example by activating E2F1's sensitivity to continuous occurrence of mutations in tumours, could prove to be useful strategy to kill tumour cells. Further studies on E2F1 induction are therefore crucial in the coming years.

Chapter 3.3: E2F1's response to oncogene activation

3.3 E2F1's response to oncogene activation

Oncogenes have been shown to deregulate E2F1 through inhibition of pRb and oncogenic stress can induce E2F1's apoptotic activity (Chellappan et al., 1992; Harbour and Dean, 2000; Whyte et al., 1988). The adenoviral early region 1A (*E1A*) oncogene can deregulate E2F1 by interacting with pRb and other cellular components involved in cell cycle regulation. Therefore, E1A has been used as a tool to identify cellular regulatory pathways that modulate cell proliferation and, when altered, contribute to cancer formation. The gene encoding E1A has evolved to influence cellular decisions leading to cell division and differentiation, however, E1A also sensitises cells to the induction of apoptosis by diverse stimuli, including many anticancer agents (Lowe et al., 1993).

These E1A activities are mediated through binding the RB family proteins (pRb, p107 and p130) and via the E1A N-terminal domain that interacts with different cellular protein complexes including the histone acetyltransferases p300 and p400 (Frisch and Mymryk, 2002). E1A has been shown to lead to significant activation of the TAp73 promoter, with mutants lacking the p300-and/or pRb-binding sites showing reduced ability to activate the TAp73 promoter (Flinterman et al., 2005). The importance of p400 has also been evaluated. In 2001 Fuchs et al. concluded that the p400 complex is an essential E1A transformation target and p400 has also been shown to be required for E1A to promote apoptosis (Fuchs et al., 2001; Samuelson et al., 2005).

A more complete understanding of the role and activity of these complexes will provide further insight into the underlying platform and pathways involved in oncogene activation. It was therefore decided to further investigate the effect of E1A expression on E2F1 activity and the contribution of the E1A bound cellular complexes to the increased sensitivity of cells with deregulated E2F1 caused by pRb inhibition.

3.3.1 E2F1 is induced following E1A expression

Expression of adenovirus 5 early region 1 (Ad5 E1, which directs expression of both E1A and E1B proteins) has been shown to cause increase in E2F1 protein levels (Hateboer et al., 1996b). However, the effect of E1A expression alone on E2F1 protein levels has not been thoroughly investigated. To examine the effect of oncogene activation on E2F1 levels, wild type E1A was retrovirally infected into RPE cells and endogenous E2F1 protein levels examined by Western blotting. This revealed a noticeable induction of E2F1 protein levels following E1A expression when compared with empty vector (pLPC) infection (Fig. 3-23a). The E2F1 protein levels were similar to the protein levels seen when E2F1 was overexpressed in the same system. Blots were re-probed with an actin antibody to ensure equal loading.

In order to examine if the increase in E2F1 protein levels following E1A expression was a result of enhanced transcription of the E2F1 gene itself or if the changes were post transcriptional, real time PCR was carried out. RNA was isolated from the same RPE cell lines and the E2F1 mRNA levels measured using E2F1 specific primers. No changes were observed in the mRNA levels following E1A expression compared with vector control (Fig. 3-23b). RPE cells over-expressing E2F1 were used as positive control and resulted in around 30 fold mRNA increase compared to vector alone.

These data indicate that the changes in E2F1 protein levels are not due to increased transcription, but are a post-transcriptional effect.

In order to confirm these results it was decided to use a system where E1A activity can be induced by tamoxifen in RPE cells. In this system E1A is fused with a mutant version of the hormone-binding domain of the oestrogen receptor (ER). This version has affinity to the synthetic ligand tamoxifen. E1A-ER fusion is inactive in the absence of tamoxifen because it is complexed with intracellular polypeptides such as Hsp90. Tamoxifen binding releases the receptor from the inhibitory complexes leading to nuclear import and activation of E1A-ER fusion protein (Fig. 3-24).

Cells were treated with tamoxifen and lysed 24 hours following treatment. Endogenous E2F1 protein levels were measured by Western blotting which

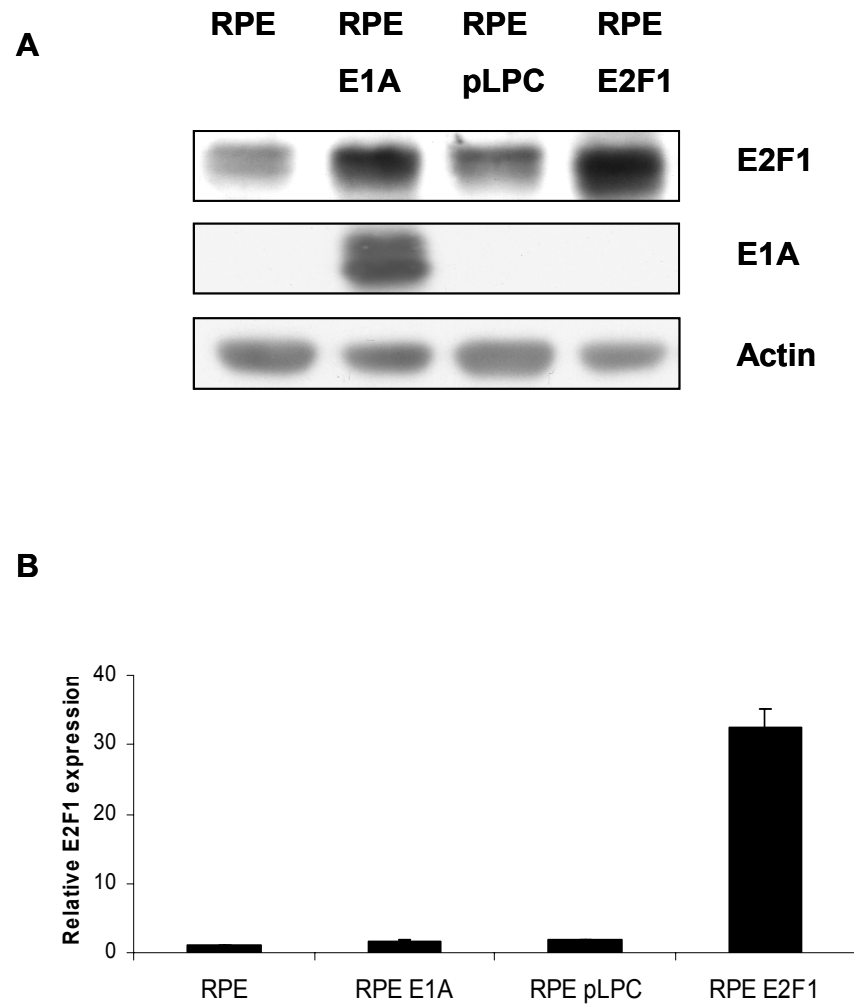


Figure 3-23 Endogenous E2F1 protein levels are induced in cells overexpressing wild type E1A

A + B) RPE cells were retrovirally infected with wild type E1A, empty vector (pLPC) and E2F1. **A)** Cells were harvested in SDS buffer and protein levels estimated by Western blot analysis. Extracts were probed with anti E2F1 antibody (C-20) to determine E2F1 expression and anti E1A mouse antibody (M58 + M73) to determine E1A expression. Blots were re-probed with an actin antibody to ensure equal loading. **B)** RNA was extracted and cDNA prepared. Relative expression of E2F1 was determined by real-time qPCR.

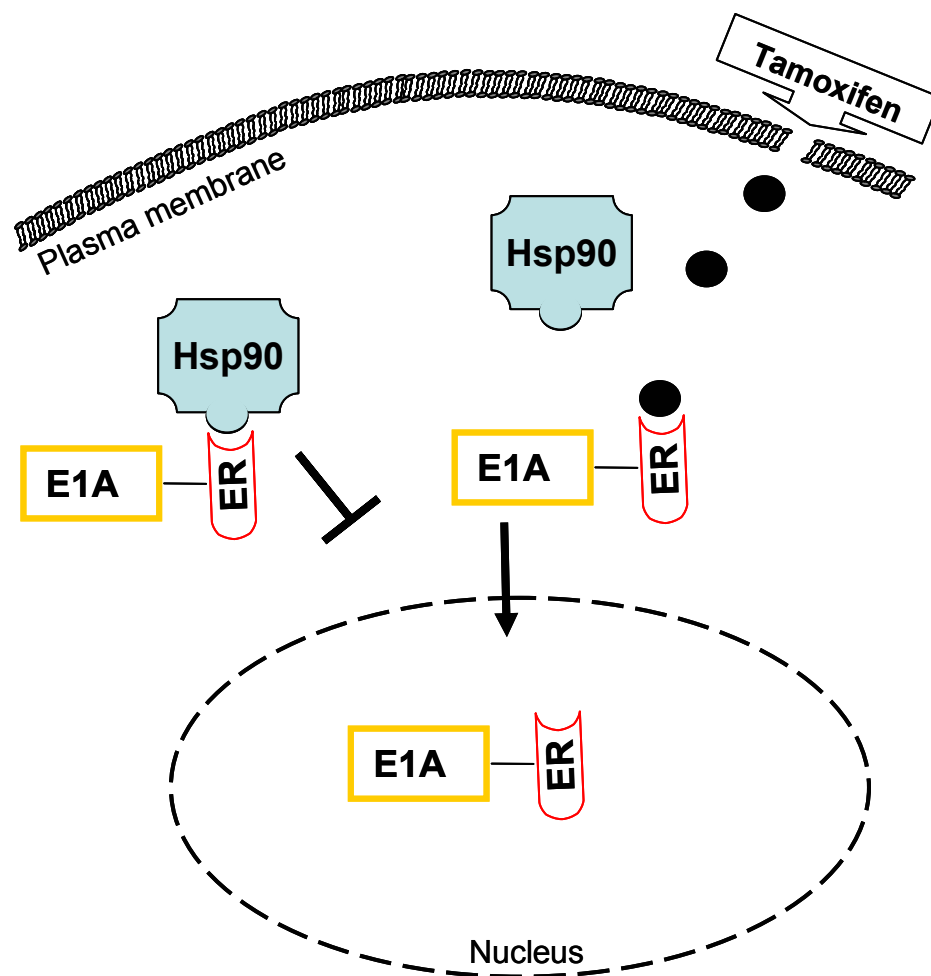


Figure 3-24 The E1A ER system

E1A is fused with a mutant version of the hormone-binding domain of the oestrogen receptor (ER) which has affinity for the synthetic ligand tamoxifen. Inhibitory proteins such as Hsp90 bind to ER and inhibit normal function of the E1A-ER fusion protein. Following tamoxifen treatment, Hsp90 binding is released from the ER leading to nuclear import and activation of the E1A-ER fusion protein

revealed noticeable induction in treated cells (Fig. 3-25). E1A levels were also induced following tamoxifen treatment. No induction was seen in actin levels which were used to ensure equal loading of proteins.

3.3.2 E1A-induced E2F1 is not further induced following DNA damage

E2F1 can be induced by DNA damage and oncogene activation. In order to examine if E2F1 induced by E1A expression can be further induced by DNA damage, RPE cells that express the E1A-ER fusion protein were treated with adriamycin for 6 and 12 hours in the absence or presence of tamoxifen. 24 hours prior to adriamycin treatment cells were treated with tamoxifen to activate E1A. E2F1 levels were then measured by Western blotting. Consistent with previous experiments, E2F1 was induced following adriamycin treatment and following E1A activation (Fig. 3-26, lane 1 and 4). However, when E2F1 levels were already induced by E1A activation, no further induction was seen following DNA damage (Fig. 3-26, lines 4, 5 and 6).

The tumour suppressor p53 was significantly induced following DNA damage and showed detectable induction following E1A activation. In contrast with E2F1, p53 levels further accumulated following DNA damage in the presence of E1A activation. These results indicate that, different to p53 the mechanism leading to E2F1 induction following E1A expression are the same or overlap the mechanisms involved in E2F1's DNA damage response. However, the possibility that E2F1 levels reach saturation point following E1A activation, and can therefore not be further induced following DNA damage, can not be excluded.

3.3.3 E1A expression sensitises cells to drug induced apoptosis

E1A can sensitise cells to the induction of apoptosis by diverse stimuli, including many anticancer agents (Lowe et al., 1993). In order to confirm this was the case in our system, E1A expressing cells were treated with 0.5 µg/ml adriamycin for 48 hours. Apoptosis was measured by flow cytometry (PI staining and sub-G1 content used as an indicator of apoptotic cells). Consistent with previous reports, E1A expressing cells become very sensitive to drug induced apoptosis

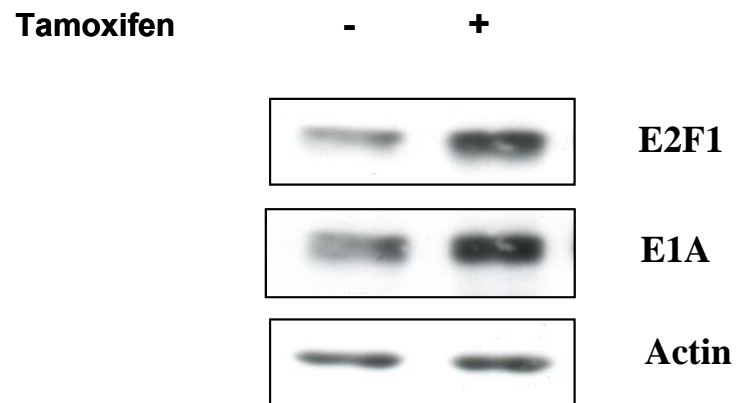


Figure 3-25 Endogenous E2F1 protein levels are induced following E1A activation in an E1A-ER system following treatment with tamoxifen

RPE cells expressing E1A-ER fusion protein were treated with tamoxifen. 24 hours following treatment, cells were harvested in SDS buffer and protein levels estimated by Western blot analysis. Extracts were probed with anti E2F1 antibody (C-20) to determine E2F1 and E1A specific antibody to determine E1A expression. Blots were re-probed with an actin antibody to ensure equal loading.

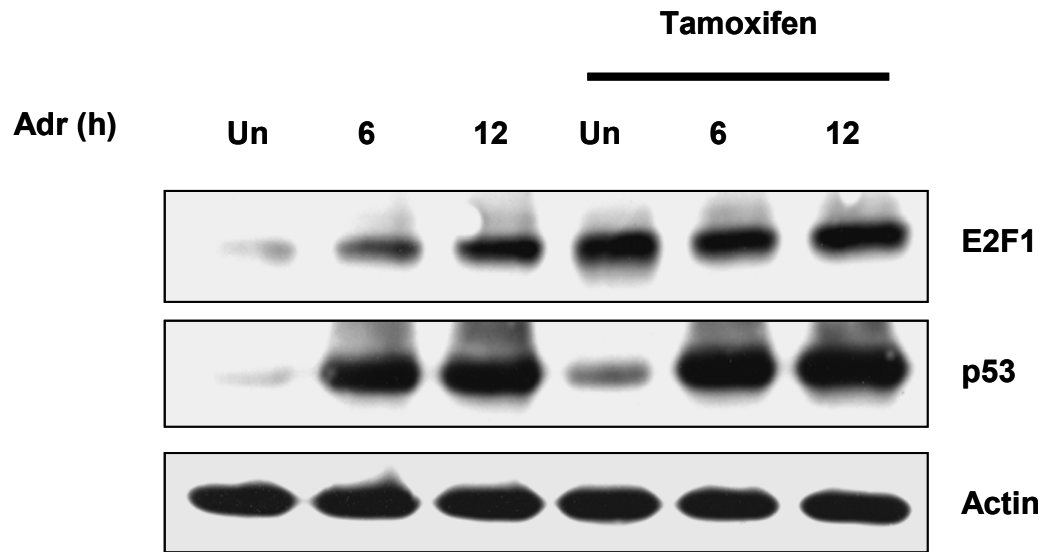


Figure 3-26 Endogenous E1A-induced E2F1 protein levels are not further induced following DNA damage

RPE cells expressing E1A-ER fusion protein (RPE E1A-ER) were treated with 2.0 $\mu\text{g/ml}$ adriamycin (Adr) for 6 and 12 hours (h). 24 hours prior to adriamycin treatment cells were treated with tamoxifen or control. Cells were then harvested in SDS buffer and protein levels estimated by Western blot analysis. Extracts were probed with anti E2F1 antibody (C-20) to determine E2F1 levels and anti p53 antibody (DO1) to determine p53 levels. Blots were re-probed with an actin antibody to ensure equal loading.

and showed about 30% accumulation in sub-G1 content compared to 1% in parental RPE cells (Fig. 3-27a).

Similar results were obtained using RPE cells expressing E1A-ER (RPE E1A-ER). Following E1A activation by tamoxifen cells were treated with adriamycin. 48 hours following treatment cells were harvested and apoptosis measured by FACS analysis. This resulted in 30% drug-induced cell death when E1A was active, compared with 3% cell death when no tamoxifen was added (Fig. 3-27b). Tamoxifen treatment alone had no effect on cell death.

3.3.4 Deregulated E2F1 is involved in drug induced apoptosis

E1A binds all the RB family proteins (pRb, p107 and p130) which regulate both the activating and repressive E2Fs (E2F1-3 and E2F4-5 respectively). However, E1A induced drug sensitivity is dependent on E1A binding to pRb, which binds the activating E2Fs (E2F1-3) (Samuelson and Lowe, 1997; Samuelson et al., 2005). E2F1 is the only family member that is induced following DNA damage and many studies show that E2F1 is the only member that can induce apoptosis (DeGregori et al., 1997; Kowalik et al., 1998; Leone et al., 2001; Lissy et al., 2000). It is therefore most likely that E2F1 induction following E1A expression plays a crucial role in the increased drug sensitivity.

The importance of high levels of deregulated E2F1 in drug induced apoptosis was tested. An RNAi approach was used to silence E2F1 levels in RPE E1A-ER cells. Cell line stably expressing a scrambled hairpin was created as a control (RPE E1A-ER pRS-Scr). Significant knockdown of E2F1 protein was obtained compared with a cell line stably expressing a scrambled hairpin with no effect seen on actin (Fig. 3-28a, compare lane 1 and lane 3). Both cell lanes showed E2F1 induction following tamoxifen treatment, however the levels of E2F1 were significantly lower in the pSR-E2F1 line compared to the cells expressing the scrambled control. E2F1 knockdown did not have an effect on basal levels of p53, which also showed marginal induction following E1A activation in both cell lines.

The effect of E2F1 knockdown on drug-induced apoptosis in E1A expressing cells was tested. The two cell lines were treated with tamoxifen and adriamycin for 48 hours and apoptosis measured by flow cytometry. There was a detectable

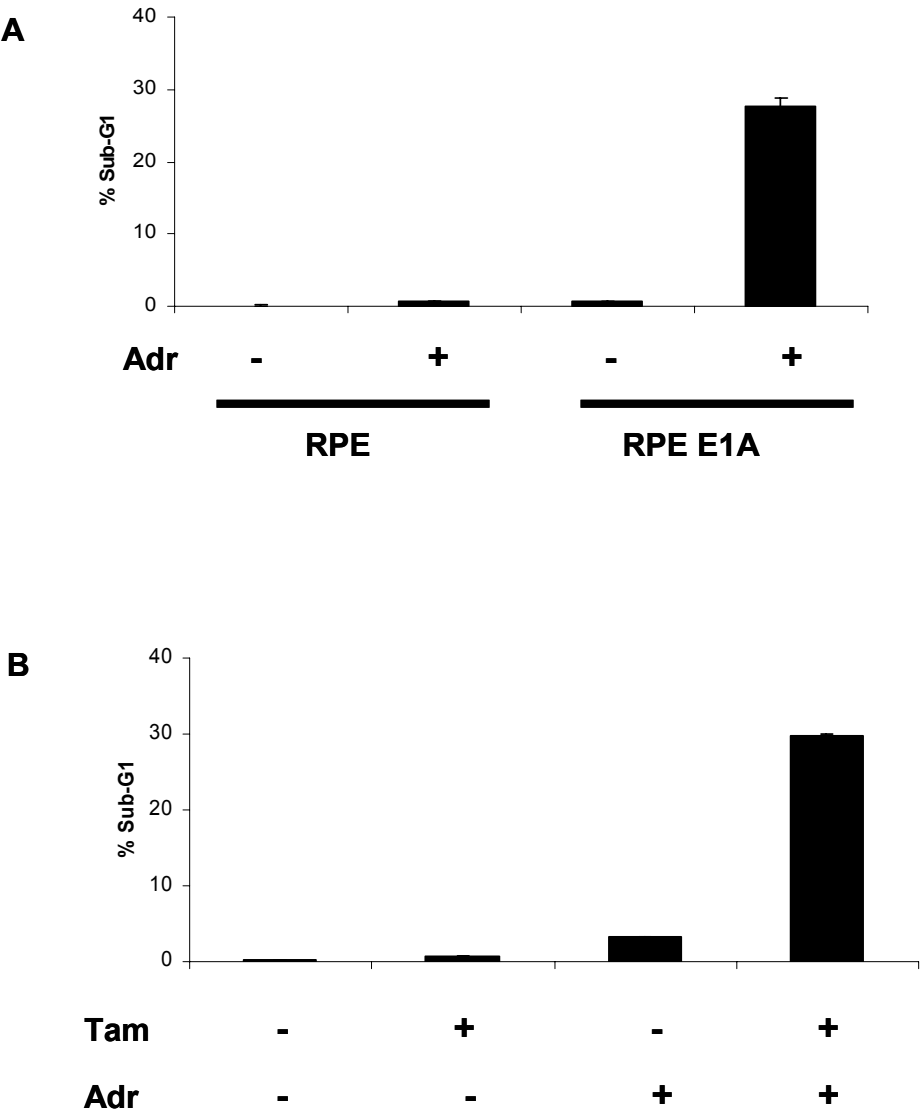


Figure 3-27 E1A expression sensitizes cells to drug induced apoptosis

A) RPE cells expressing wt E1A (RPE E1A) were treated with 0.5 μ g/ml adriamycin (Adr) for 48 hours. Following treatment DNA content of cells was assessed by flow cytometry. The percentage of cells with a sub-G1 DNA was taken as a measure of apoptotic rate. **B)** RPE cells expressing E1A-ER fusion protein (RPE E1A-ER) were treated with tamoxifen (Tam), 0.5 μ g/ml adriamycin (Adr) or tamoxifen plus adriamycin for 48 hours. Following treatment DNA content of cells was assessed by flow cytometry. The percentage of cells with a sub-G1 DNA was taken as a measure of apoptotic rate.

decrease in apoptosis in the cell line with E2F1 knockdown, compared with cells expressing the scrambled hairpin (Fig. 3-28b).

These results demonstrate that induced E2F1 protein levels contribute to increased drug sensitivity in circumstances when E2F1 is deregulated by pRb inhibition.

3.3.5 Description of E1A truncations

E1A activities are mediated through binding to different cellular protein complexes via the E1A N-terminal domain. It is possible that E1A binding to these cellular complexes is crucial for the E2F1 induction observed with wild type E1A. That can be examined by using previously characterized functional mutants of E1A (Seger et al., 2002). These mutants retain their ability to bind to pRb but have a single mutation or lack domains within E1A that are necessary for binding to other complexes. These mutants can therefore be used to identify complexes that may be involved in E2F1 induction.

The first truncation, R2G, has the 2nd arginine mutated to glycine. This mutation abolishes the binding of E1A to the co-activator p300/CBP (Fig. 3-29). Deletion mutant Δ 2-11 lacks amino acids 2 to 11 and is also unable to bind to the p300/CBP co-activator complex. Both R2G and Δ 2-11 remain their ability to bind to the p400/TRRAP chromatin remodelling protein complex. Deletion mutant Δ 2-24 lacks amino acids 2 to 24. Deleting residues 2-24 abolishes binding to both p300/CBP and the p400/TRRAP protein complex. Deletion mutant Δ 2-36 lacks residues 2 to 36 and is also unable to bind to the p300/CBP and p400/TRRAP protein complex. Deletion mutant Δ 26-35 lacks residues 26 to 35. Δ 26-35 binds p300/CBP but has lost binding capability (or only binds weakly when overexpressed) to p400/TRRAP (Samuelson et al., 2005). Deletion mutant Δ 48-60 lacks residues 48 to 60 and has lost binding capacity to the p300/CBP complex but retains its ability to interact with the p400/TRRAP complex. The last E1A truncation, 143 has the C-terminal end removed and only consists of the first 143 amino acids. Truncation 143 has lost its binding ability to the transcriptional co-repressor CtBP but can bind both p300/CBP and p400/TRRAP complexes (Fig. 3-29).

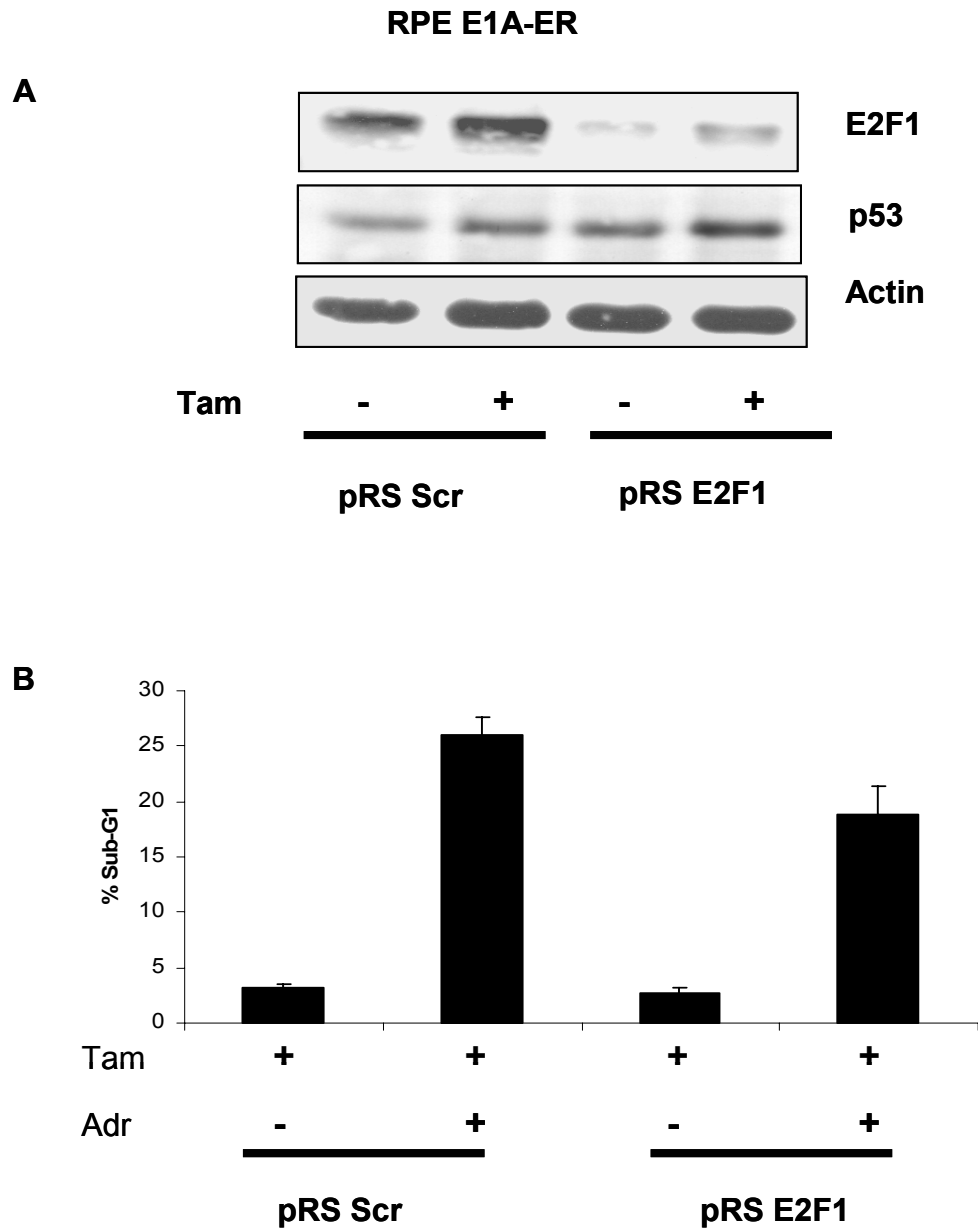
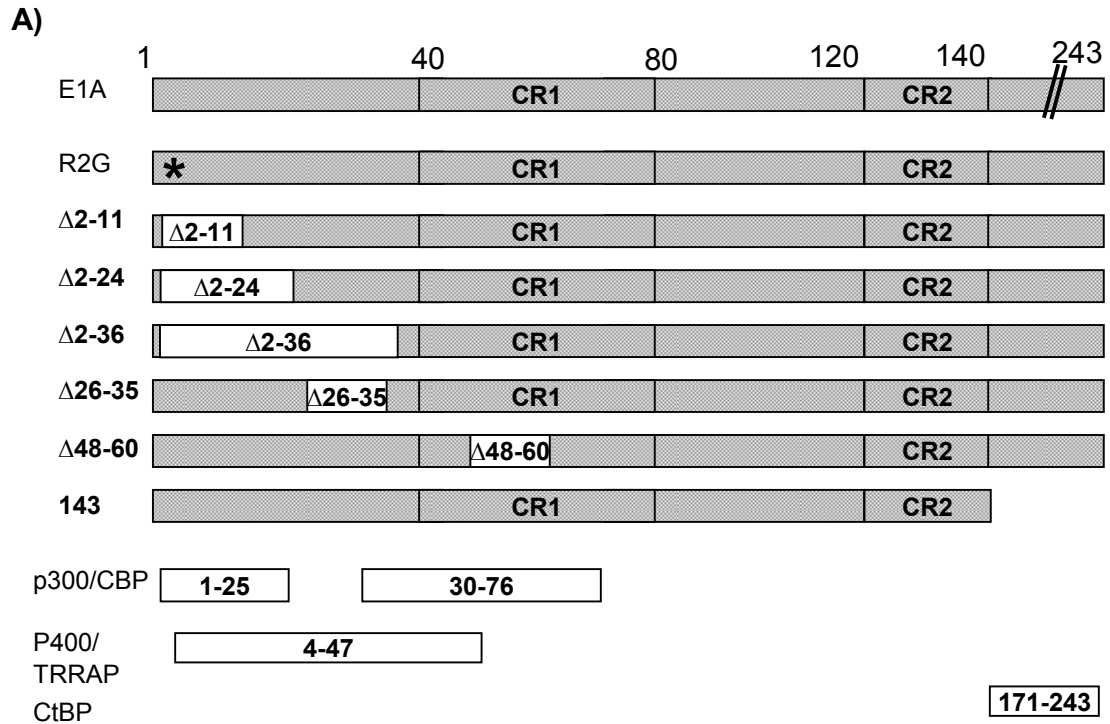


Figure 3-28 Knockdown of E2F1 reduces drug-induced apoptosis in E1A expressing

A) RPE cells expressing E1A-ER fusion protein and scrambled or E2F1 specific shE2F1 (RPE E1A-ER pRS Scr or pRS E2F1) were treated with tamoxifen (Tam) for 24 hours. The cells were then lysed in SDS lysis buffer, extracts resolved by SDS-PAGE and endogenous E2F1 and p53 levels measured by Western blotting using E2F1 (C-20) and p53 (DO1) specific antibodies. Blots were re-probed with an actin antibody to ensure equal loading. **B)** RPE E1A-ER pRS Scr and pRS E2F1 cells were treated with tamoxifen (Tam) and 0.5 µg/ml adriamycin (Adr) for 48 hours. Following treatment DNA content of cells were assessed by flow cytometry. The percentage of cells with a sub-G1 DNA content were taken as the apoptotic population.



B)

	p300/ CBP	p400/ TRRAP	CtBP	
R2G	No	Yes	Yes	E1A binding
Δ2-11	No	Yes	Yes	
Δ2-24	No	No	Yes	
Δ2-36	No	No	Yes	
Δ26-35	Yes	No	Yes	
Δ48-60	No	Yes	Yes	
143	Yes	Yes	No	

Figure 3-29 Description of E1A truncations

A) Previously described functional mutants of E1A lack domains within E1A that are necessary for binding to known protein complexes. R2G has the 2nd arginine mutated to glycine. 143 only consists of the first 143 amino acids. Other truncations lack residues indicated in white boxes. **B)** All truncations except 143 bind to the transcription co-repressor CtBP. R2G, Δ2-11 and Δ48-60 have all lost their ability to bind to the co-activator p300/CBP but remain their ability to bind to the p400/TRRAP chromatin remodelling protein complex. Deletion mutants Δ2-24 and Δ2-36 are unable to bind to both p300/CBP and the p400/TRRAP protein complex. Δ26-35 binds p300/CBP but has lost binding capability.

3.3.6 E1A binding to the p400/TRRAP is essential for E1A-induced E2F1 accumulation

All the E1A truncations were infected into RPE cells and their expression confirmed by Western blotting using an E1A specific antibody (Fig. 3-30). All truncations showed similar expression level compared to wild type E1A with the exception of truncation 143. The detection by Western blotting of truncation 143 was much lower than the other truncations, however whether this was due to truncated protein having lower affinity to the antibody has not been determined. Blots were re-probed with an actin antibody to ensure equal loading.

In order to investigate the effect of the expression of these truncations on E2F1 protein levels the blot was re-probed with E2F1 specific antibody (Fig. 3-30). Robust induction of E2F1 protein level was seen with R2G and $\Delta 2-11$ indicating that E1A binding to the p300/CBP complex was dispensable for this affect. The induction seen following expression of mutant $\Delta 48-60$ also indicated that E1A binding to the p300/CBP complex is not necessary to induce E2F1 levels. E2F1 induction was seen in cells expressing truncation 143 indicating that the C-terminal end of E1A and therefore E1A binding to the CtBP co-repressor was also dispensable for this affect.

Interestingly, truncations $\Delta 2-24$, $\Delta 2-36$ and $\Delta 26-35$ all failed to induce E2F1 levels. All of these truncations are deficient in binding to the p400/TRRAP protein complex (Samuelson et al., 2005). This therefore indicates that E1A binding to the p400/TRRAP protein complex is potentially important for E1A to induce E2F1 accumulation.

3.3.7 E1A binding to the p400/TRRAP complex is essential for increased drug sensitivity

Next, it was assessed what impact expressing the E1A truncations had on drug-induced apoptosis. Cells expressing the E1A mutants were treated with adriamycin for 48 hours and apoptosis measured by flow cytometry.

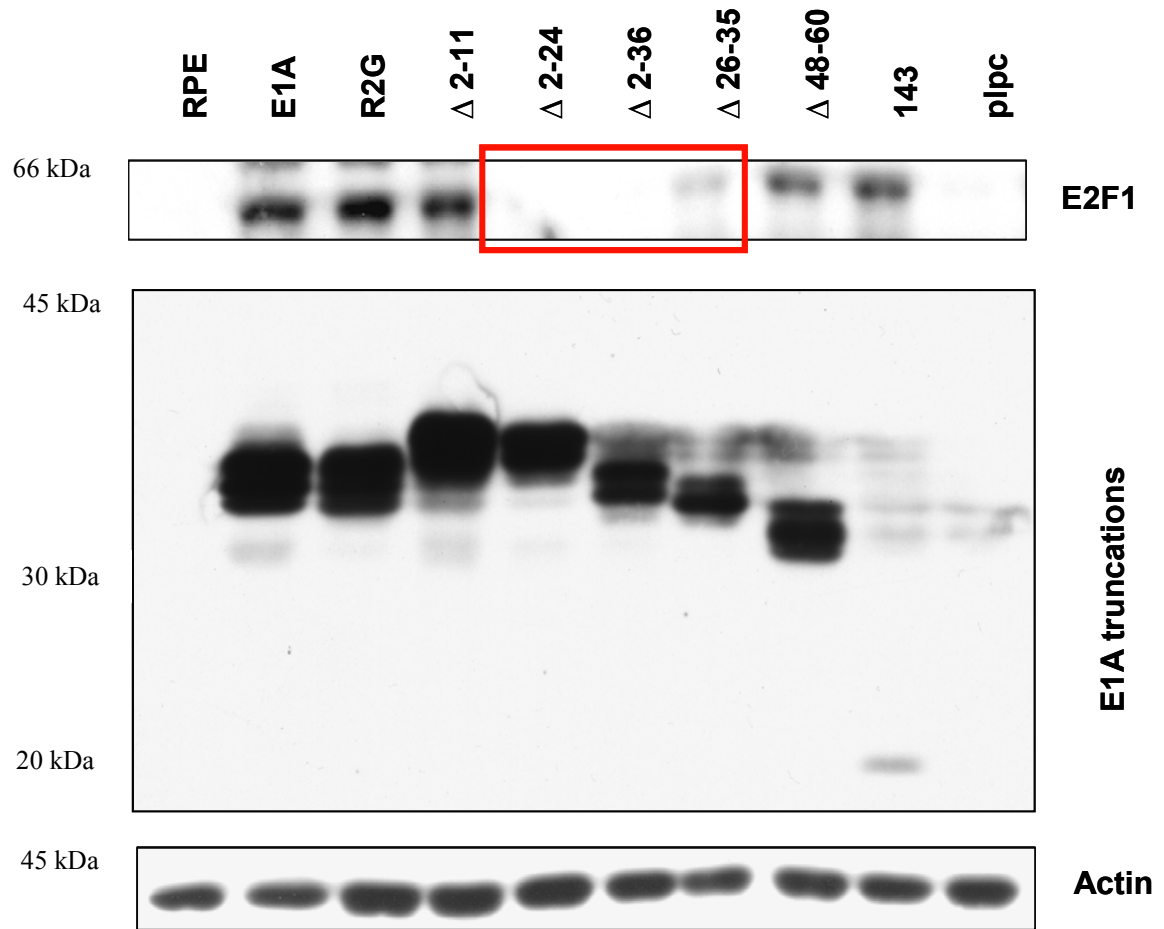


Figure 3-30 E1A truncations deficient in binding p400/TRRAP fail to induce E2F1 levels

RPE cells expressing wild type E1A, E1A truncations or empty vector (pLPC) were harvested in SDS buffer and protein levels estimated by Western blot analysis. Extracts were probed with anti-E1A (M58 +M73) and anti-E2F1 (C-20) antibodies to determine E1A and E2F1 expression. Blots were re-probed with an actin antibody to ensure equal loading. The red square indicates E1A truncations which fail to induce E2F1 levels.

This revealed that R2G, $\Delta 2-11$ and $\Delta 48-60$ mutants, which all are unable to bind to the p300/CBP complex, are as sensitive to drug induced apoptosis as wild type E1A (Fig. 3-31). This indicates that E1A interacting with the p300/CBP complex is largely dispensable for these effects. Truncation 143 which lacks the C-terminal end of E1A is also capable of increasing chemosensitivity, even though it showed much lower expression levels by Western blotting. Truncation 143 is unable to bind to the CtBP co-repressor, indicating that CtBP interaction is dispensable for E1A promoting drug induced apoptosis.

Conversely, truncations $\Delta 2-24$, $\Delta 2-36$ and $\Delta 26-35$ which are all deficient in binding to the p400/TRRAP complex, were all more resistant to adriamycin. This suggests that E1A binding to the p400/TRRAP protein complex is important in mediating increased drug sensitivity.

The mutants that failed to increase drug sensitivity are the same as failed to induce E2F1 levels following their expression. This indicates that E1A's capability in promoting drug sensitivity is not only through E2F1 deregulation by pRb inhibition, but also by increasing E2F1 levels by binding to the p400/TRRAP protein complex. However, the E1A mutants used in this study are perhaps not refined enough to exclude other E1A targets as potential reason for the effect observed. Therefore, a more extensive analysis is necessary to confirm the involvement in p400/TRRAP in E2F1 induction and increased drug sensitivity.

3.3.8 p400 knockdown induces E2F1 levels

E1A has been shown to bind and inhibit pRb but whether E1A interaction activates or inhibits the p400/TRRAP complex is not clear. The possibility of E1A inhibiting the p400/TRRAP protein complex to induce E2F1 levels was assessed.

In order to examine the effect of p400 inhibition on E2F1 levels three siRNAs designed to target p400 were used. RPE cells expressing E1A truncations $\Delta 2-36$ and $\Delta 26-35$ were transfected with siRNA oligos targeting p400 or non-targeting siRNA as control. Transfection of all three p400 specific oligos resulted in over 50 % reduction of p400 mRNA levels measured by real time qPCR (Fig. 3-32a). The effect of p400 knockdown on E2F1 proteins were next measured by Western blotting. p400 knockdown resulted in E2F1 induction with all three p400 oligos

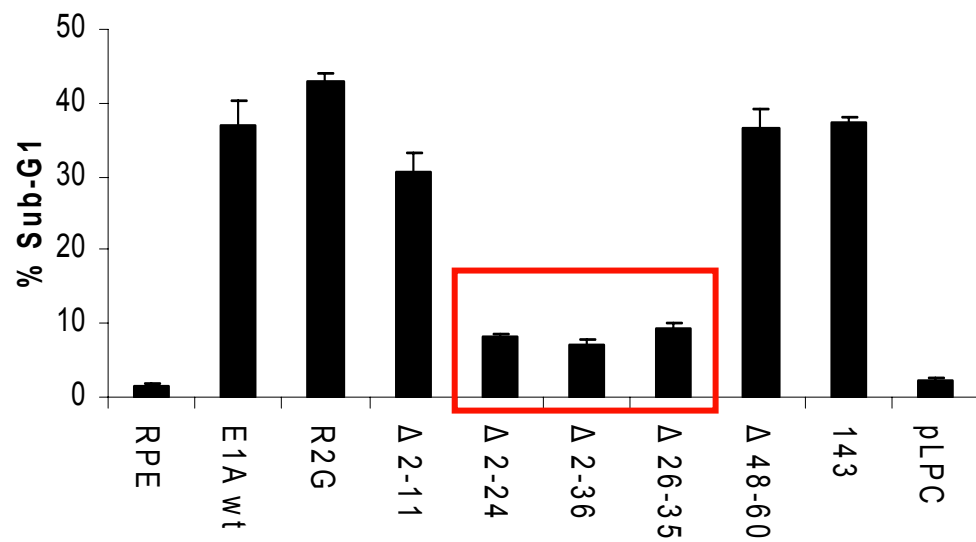


Figure 3-31 E1A mutants which fail to bind p400/TRRAP do not increase drug sensitivity

DNA content of RPE cells expressing wt E1A, E1A truncations and empty vector (pLPC) were assessed 48 hours following treatment with 0.5 μ g/ml adriamycin. The percentage of cells with a sub-G1 DNA was taken as a measure of apoptotic rate. The red square indicates E1A truncations which fail to induce apoptosis.

when compared with scrambled control (Fig. 3-32b). The effect was observed both in cell expressing $\Delta 2$ -36 and $\Delta 26$ -35 E1A truncatons.

Consistent with previous studies, knockdown of p400 had the opposite effect on p53 protein levels (Samuelson et al., 2005). Detectable reduction in p53 levels were seen following siRNA transfection with all three oligos and in both cell lines. Actin was used to ensure equal loading.

3.3.9 p400 knockdown sensitizes cells to drug-induced apoptosis

In order to investigate the effect of p400 inhibition on drug sensitivity, RPE cells expressing E1A truncations $\Delta 2$ -36 and $\Delta 26$ -35 were treated with adriamycin following p400 knockdown. Apoptosis was measured as before using flow cytometry with sub-G1 content as indicator of apoptotic cells. In line with figure 3-32 where E2F1 levels were increased following p400 knockdown, an induction of apoptosis was observed following treatment with adriamycin (Fig. 3-33).

Slight variation in apoptosis was seen between individual p400 oligos with oligo 2 showing the strongest induction of death in both cell lines. However, given that p400 knockdown decreased p53 levels, the effect of increase in E2F1 levels could have greater significance than this assay indicated. Therefore, these data strongly signify that E2F1 induction by p400 knockdown is important in sensitizing cells to drug-induced apoptosis. They also further support the hypothesis that E1A inhibits the p400/TRRAP protein complex and that inhibition is responsible for E2F1 induction and increased drug sensitivity.

3.3.10 E2F1 overexpression sensitizes $\Delta 2$ -36 expressing cells to drug-induced apoptosis

Overexpression of E2F1 allowed us to analyse the effect of E2F1 induction on chemosensitivity without decreasing p53 levels. Therefore the importance of E2F1 protein levels on chemosensitivity in cells with already deregulated E2F1 pathway was examined using adenoviral E2F1. RPE cells expressing E1A $\Delta 2$ -36 or vector as a control, were infected with adenoviral E2F1 24 hours prior to

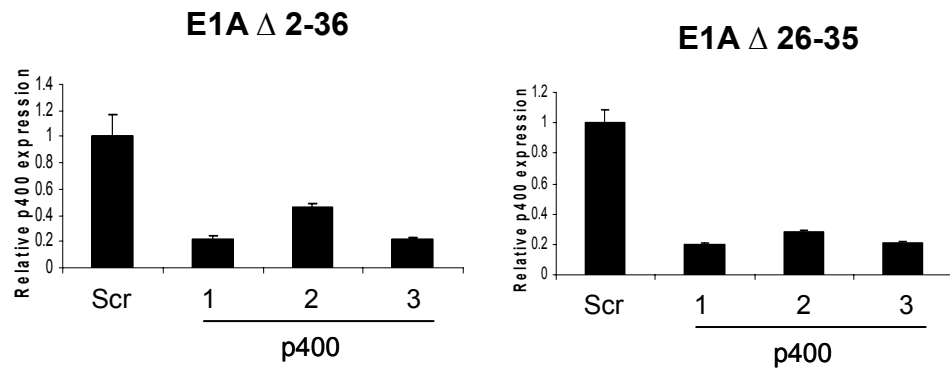
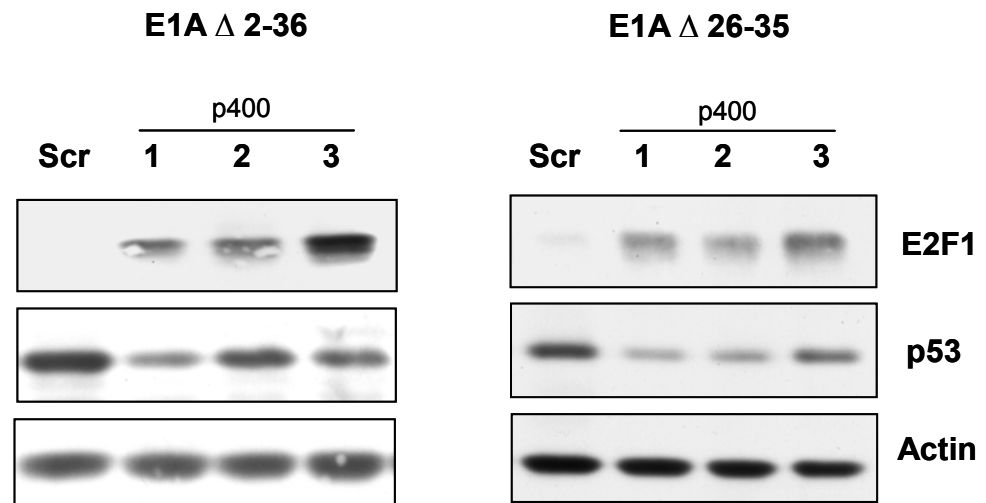
A**B**

Figure 3-32 E2F1 is induced following p400 knockdown

A) RPE cells expressing E1A Δ 2-36 or Δ 26-35 truncations were transfected with three siRNA oligos designed to target p400. Scrambled siRNA was used as control. 24 hours following transfection, RNA was extracted from samples and cDNA prepared and used for real-time qPCR analysis. p400 knockdown was measured by using p400 specific primers. 18S rRNA primers were used to normalise for total RNA control. **B)** 48 hours following siRNA transfections cells were lysed in SDS lyses buffer. Extracts were resolved by SDS-PAGE and endogenous E2F1 and p53 levels measured by Western blotting using E2F1 (C-20) and p53 (DO1) specific antibodies. Blots were re-probed with an actin antibody to ensure equal loading.

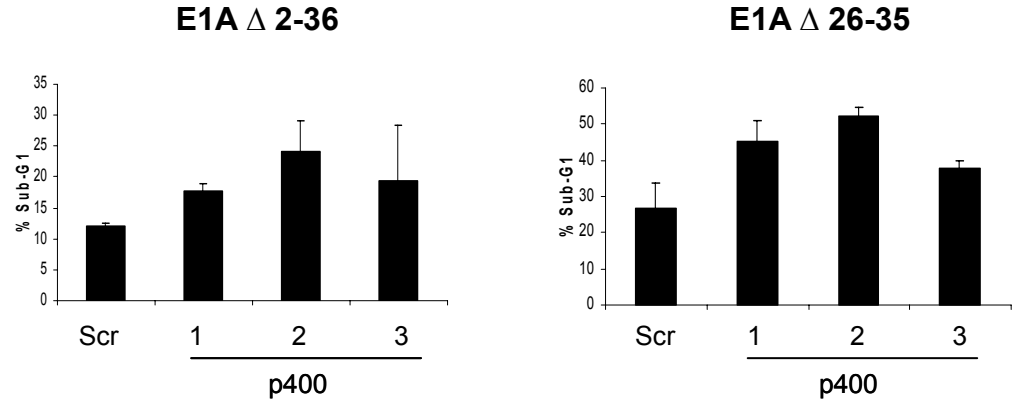


Figure 3-33 p400 silencing enhances drug-induced apoptosis

RPE cells expressing E1A Δ2-36 or E1A Δ26-35 were transfected with three siRNA oligos targeting p400 (1-3). A scrambled siRNA oligo was used as a control (Scr). 24 hours following transfection, cells were treated with 0.5 μg/ml adriamycin. 48 hours following treatment DNA content were assessed by flow cytometry. The percentage of cells with a sub-G1 DNA was taken as a measure of apoptotic rate.

adriamycin treatment. Adenovirus expressing empty vector were used as an expression control.

Following adenoviral and adriamycin treatment, apoptosis was measured using flow cytometry. Overexpression of E2F1, without adriamycin treatment, had no effect on apoptosis in $\Delta 2$ -36 expressing cells or control cell line (Fig. 3-34a). Consistent with previous data, adriamycin treatment had little effect on apoptosis in RPE control cells and showed slight increase in apoptosis in the $\Delta 2$ -36 expressing cell line. However, a robust increase in apoptosis was observed when E2F1 was overexpressed prior to adriamycin treatment in the $\Delta 2$ -36 expressing cells, whereas E2F1 overexpression had little effect on chemosensitivity on control cell line.

Equal expression of adenoviral E2F1 was verified by Western blotting using E2F1 specific antibody (Fig. 3-34b). p53 levels were also analysed by Western blotting and revealed robust induction in response to adriamycin treatment but no changes were observed between the different cell lines. E1A $\Delta 2$ -36 expression was confirmed using E1A specific antibody and actin was used to ensure equal loading of proteins.

These results demonstrate that high levels of E2F1 sensitize cells to drug-induced apoptosis in cells where pocket proteins are inhibited by the E1A oncoprotein. These effects are independent of p53 status since similar accumulation of p53 was observed in both cell lines following DNA damage.

3.3.11 E1A is dependent on binding to pRb to induce E2F1 levels

E1A, in addition to many viral oncoproteins such as the HPV protein E7, binds to the pRb pocket domain via a Leu-x-Cys-x-Glu (LxCxE) binding motif (Singh et al., 2005). E1A contains two conserved regions, CR1 and CR2 and E1A binds to hypophosphorylated form of pRb, primarily through the motif that is located in CR2. The interaction of E1A with pRb deregulates E2F1 and results in a stimulation of E2F1-dependant transcription (Frisch and Mymryk, 2002).

In order to investigate if E2F1 induction by wild type E1A was dependent on E1A binding to pRb, a pRb binding deficient mutant form of E1A, which lacks the CR2 domain (E1A Δ CR2) was used. Following infection of virus expressing E1A Δ CR2

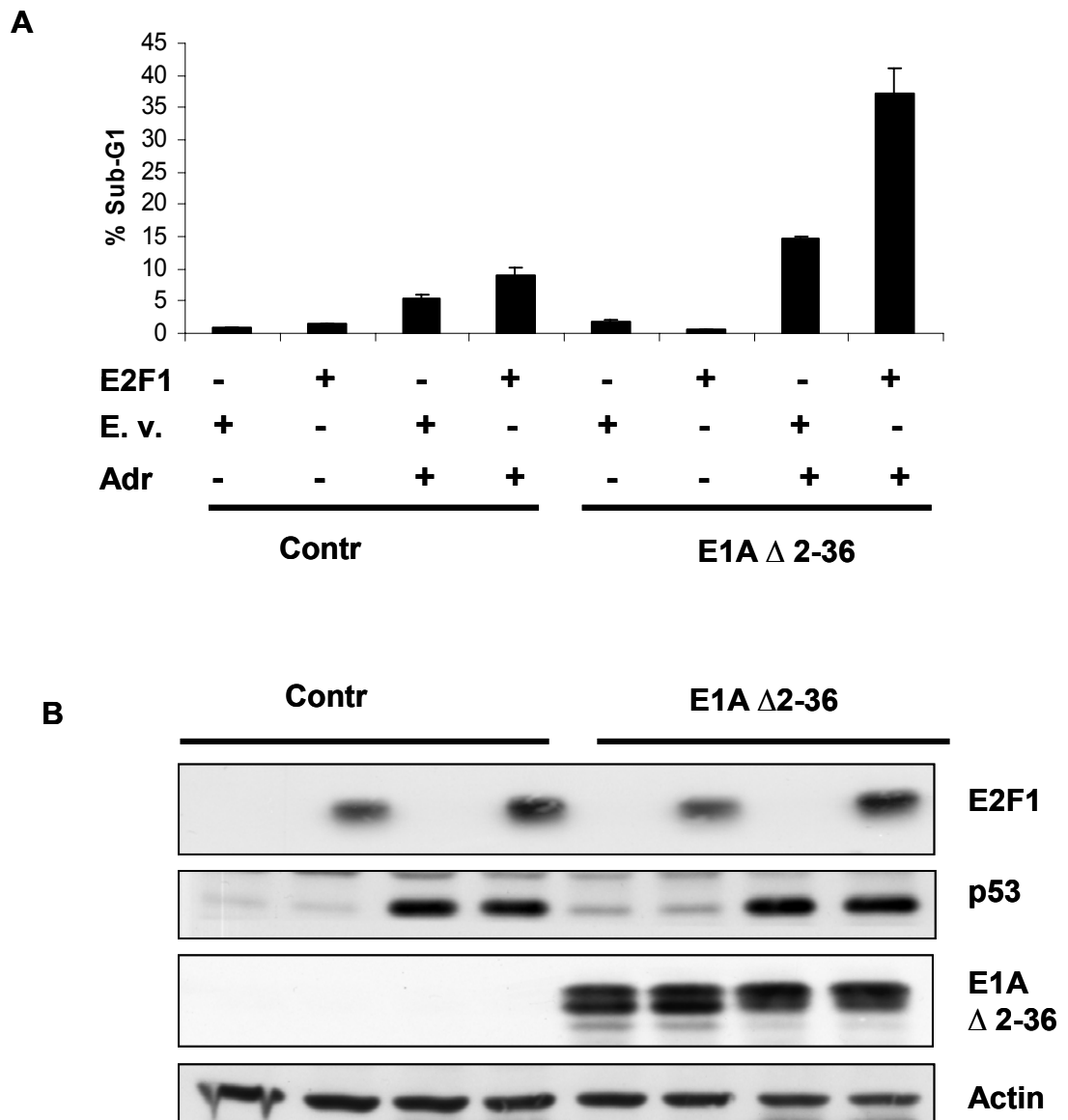


Figure 3-34 E2F1 overexpression sensitizes E1A Δ 2-36 expressing cells to drug-induced apoptosis

A-B) RPE cells expressing E1A Δ 2-36 truncation or empty vector (contr) were infected with adenoviral E2F1 or empty virus (E.v.) (MOI 3.8 IFU/cell). **A)** 24 hours following infection cells were treated with 0.5 μ g/ml adriamycin Adr). 48 hours following adriamycin treatment, the DNA content of cells were assessed. The percentage of cells with a sub-G1 DNA content was taken as a measure of apoptotic rate. **B)** 24 hours following adenoviral infection cells were treated with 0.5 μ g/ml adriamycin. 24 hours following adriamycin treatment cells were lysed in SDS lyses buffer. Extracts were resolved by SDS PAGE and endogenous E2F1, E1A and p53 levels measured by Western blotting using E2F1 (C-20), E1A and p53 (DO1) specific antibodies. Blots were re-probed with an actin antibody to ensure equal loading.

into RPE cells, E2F1 levels were measured by Western blotting. This revealed that E1A Δ CR2 failed to induce E2F1 levels to the same degree as wild type E1A despite being expressed at similar levels. This indicates that E1A is dependent on binding to pRb to induce E2F1 levels (Fig. 3-35a).

3.3.12 E2F1 deregulation by pRb inhibition is essential in increasing drug sensitivity

In order to examine the importance of pRb inhibition and E2F1 induction by E1A on drug sensitivity, RPE cells expressing E1A Δ CR2 were treated with adriamycin. 48 hours following treatment, apoptosis was measured and compared with cells expressing empty vector or wild type E1A. Cells expressing E1A Δ CR2 were more resistant to adriamycin induced apoptosis (12% sub-G1) than cells expressing wild type E1A (46% sub-G1) (Fig. 3-35b). This data confirms the importance of E2F1 deregulation by pRb inhibition in drug sensitivity and also advocates the importance of E2F1 induction in the response. However, E1A Δ CR2 expressing cells showed a minor increase in apoptosis compared to empty vector control. This suggest that either E1A Δ CR2 can weakly bind to pRb via CR1 and deregulate some E2F1 proteins, or E1A binding to other proteins can alter cells drug sensitivity.

3.3.13 E1A is dependent on pRb binding to complex with E2F1

As discussed previously, E1A binds and inhibits pRb function, which results in activation of E2F1. The results presented in this chapter suggest that the ability of E1A to induce E2F1 levels depends on E1A binding to the p400/TRRAP protein complex and to pRb. It was therefore hypothesized that pRb acts as a scaffold between E1A and E2F1, and allows E1A to complex with E2F1 which leads to the E2F1 accumulation. To test this hypothesis, it was investigated if E2F1 can be found in a complex with E1A and pRb. In addition it was tested if E1A binding to pRb is essential for E2F1 to complex with E1A. In order to do so, E1A and E1A Δ CR2 were immunoprecipitated with anti-E1A antibody from RPE cells expressing these proteins. An anti-HA antibody was used as an immunoprecipitation control. Immunoprecipitated proteins were then probed with anti-pRb and E2F1 antibodies proteins to assess if they co-immunoprecipitate with E1A. As expected, pRb co-immunoprecipitated with wild type E1A but not with E1A Δ CR2

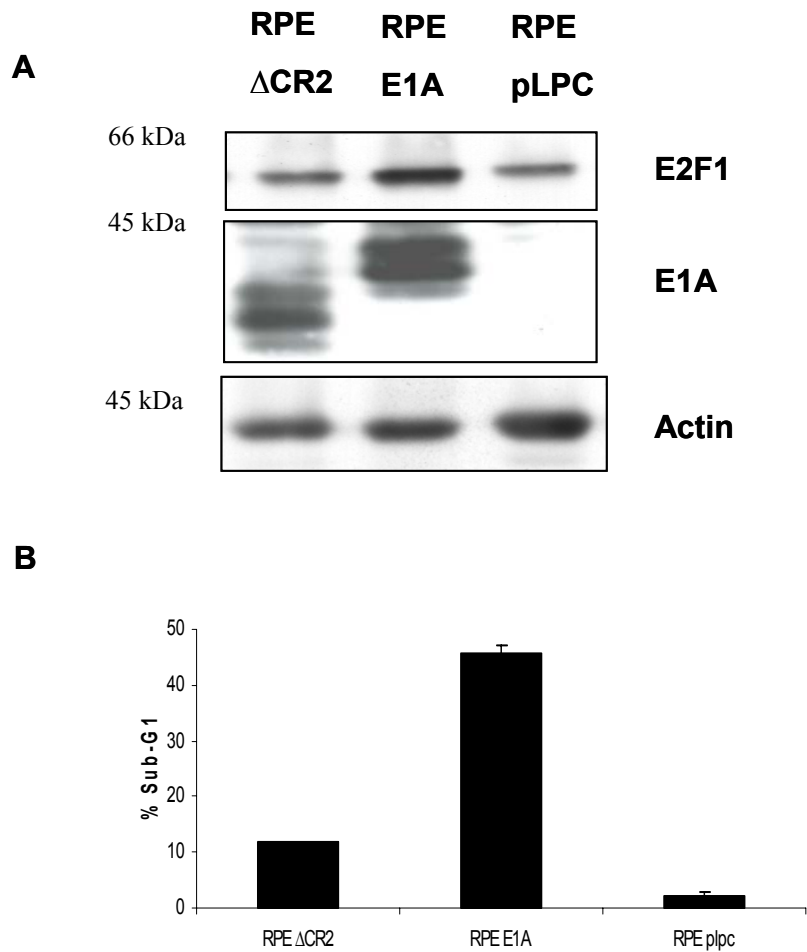


Figure 3-35 Deregulation of E2F1 by pRB inhibition is necessary for increased drug sensitivity

A) RPE cells expressing wild type E1A, pRB binding deficient mutant of E1A (Δ CR2) and empty vector (pLPC) were harvested in SDS lysis buffer and protein levels estimated by Western blot analysis. Extracts were probed with an anti-E2F1 antibody (C-20) to determine E2F1 expression and anti-E1A antibody (M58 + M73) to determine E1A expression. Blots were re-probed with an actin antibody to ensure equal loading.

B) DNA content of RPE cells expressing wt E1A, Δ CR2 or empty vector were assessed by flow cytometry 48 hours following treatment with 0.5 μ g/ml adriamycin. The percentage of cells with a sub-G1 DNA was taken as a measure of apoptotic rate.

truncation (Fig. 13-36). Re-probing blots with E2F1 antibody revealed that E2F1 also co-immunoprecipitated with wild type E1A, but not E1A Δ CR2, suggesting that E1A is dependent on binding to pRb to complex with E2F1.

However, it can not be excluded that E1A can directly bind E2F1 via the CR2 domain in a pRb independent manner. Therefore, the Δ CR2 mutant would fail to co-immunoprecipitate E2F1, but independently of pRb binding. In addition, it is a possibility that increased E2F1 expression in wild type E1A expressing cells results in increase in co-immunoprecipitated E2F1.

3.3.14 E2F1 is induced following E1A activation in pRb^{-/-} cells

In order to assess if E1A is dependent on pRb binding to induce E2F1 levels, E1A-ER fusion protein was virally infected into wild type and pRb knockout (pRb^{-/-}) MEFs (te Riele et al., 1992). Cells were treated with tamoxifen and lysed 24 hours following treatment. Endogenous E2F1 protein levels were measured by Western blotting which revealed noticeable induction in treated cells of both cell lines (Fig. 3-37). No induction was seen in actin levels which were used to ensure equal loading of proteins.

These data indicate that E1A is able to induce E2F1 accumulation without binding to E2F1 via pRb. However, it is possible that the other pRB family proteins, p107 or p130, substitute for pRb and bind to E2F1 in pRb^{-/-} cells. Therefore p107 or p130 could provide the platform for E1A-E2F1 interaction, resulting in E2F1 accumulation following E1A expression. In support of this possibility results from Gao et al have shown that in Saos2 cells, which are pRb^{-/-}, p130 formed complexes with E2F1 (Gao et al., 2002).

3.3.15 E2F1 is not induced following E1A expression in pRB family knockout cells

In order to assess if E1A is dependent on binding to any of the members of the pRB family to induce E2F1 levels, E1A-ER fusion protein was virally infected into triple knockout (pRb^{-/-}, p107^{-/-} and p130^{-/-}) MEFs (TKO) (Dannenberg et al., 2000). Cells were treated with tamoxifen and lysed 24 hours following treatment. Endogenous E2F1 protein levels were measured by Western blotting

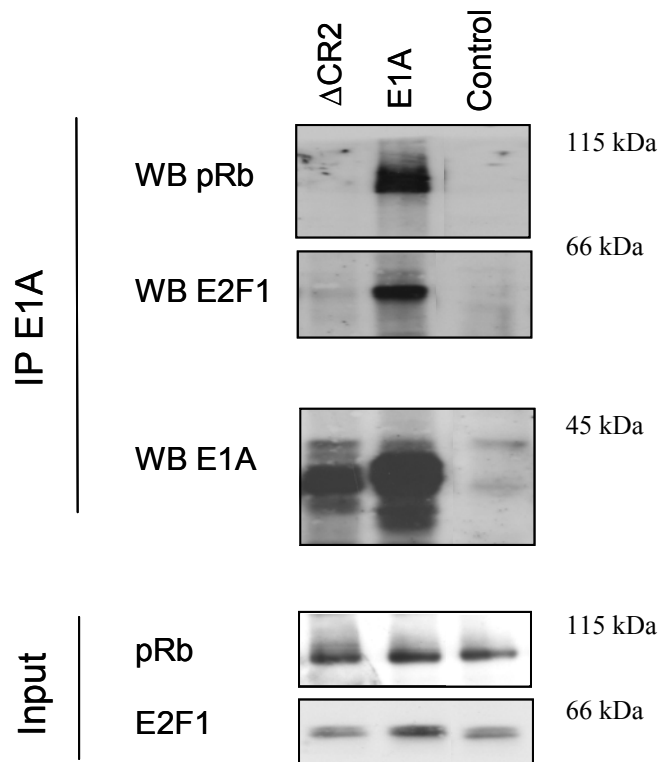


Figure 3-36 E1A is dependent on pRb binding in order to form a complex with E2F1

RPE cells expressing wild type E1A or the E1A Δ CR2 truncation were immunoprecipitated with an E1A specific antibody or anti-HA antibody as negative control. Extracts were then resolved by SDS PAGE as detailed in the materials and methods. Co-immunoprecipitated E2F1 and pRb protein levels were measured by Western blotting using E2F1 (C-20) and pRb (C-15) specific antibodies. Blots were re-probed with E1A antibody to measure precipitated protein levels.

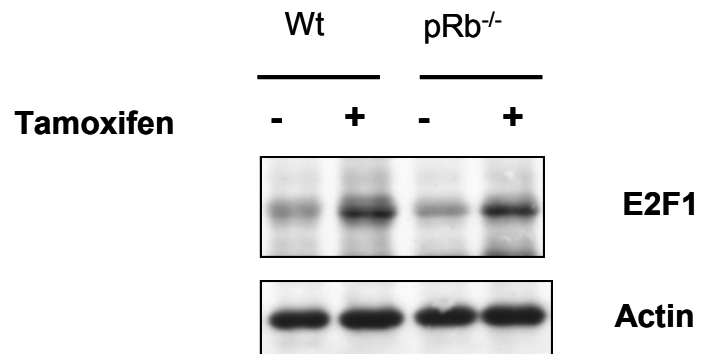


Figure 3-37 Endogenous E2F1 protein levels are induced following E1A activation in an E1A-ER system following treatment with tamoxifen in wild type and pRb^{-/-} MEFs

Wild type and pRb-null MEFs expressing E1A-ER were treated with tamoxifen. 24 hours following treatment, cells were harvested in SDS buffer and protein levels estimated by Western blot analysis. Extracts were probed with anti E2F1 antibody (C-20) to determine E2F1 protein levels. Blots were re-probed with an actin antibody to ensure equal loading.

which revealed no induction in treated TKO cells whereas pRb^{-/-} cells showed noticeable E2F1 induction (Fig. 3-38). No induction was seen in actin levels which were used to ensure equal loading of proteins.

This data indicate that E1A is dependent on binding to any of the pRB family members to induce E2F1 levels.

3.3.16 E2F1 induction following E1A activation does not require the transactivation domain of E2F1

Recent observations have defined an alternative E2F1 binding site within pRb. Previously it has been shown that the large pocket domain (amino acids 379-928) is sufficient for stable interaction with E2F (Qin et al., 1992). The second E2F interaction site is located entirely within the C-terminal domain (amino acids 792-928) and is specific for E2F1 (Dick and Dyson, 2003). E2F1/pRb complexes formed through this site have low affinity for DNA, but the interaction is sufficient for pRb to regulate E2F1-induced apoptosis, and interestingly, E2F1 loses the ability to interact with this site following DNA damage. This indicates that interaction through this E2F1-specific site is regulated separately from other pRb-E2F interactions.

The previously reported pRb binding site in E2F1 is located in the C-terminal transactivation domain (amino acids 409-426). However, with the identification of the new E2F1 binding domain in pRb, it was shown that the C-terminus of pRb is able to interact with E2F1 truncation that lacks the transactivation domain (E2F1 1-374) (Dick and Dyson, 2003). It was further suggested that C-terminus of pRb likely requires the marked box region of E2F1 for binding and makes contact with multiple places in the N terminus as well. The low DNA binding affinity of the E2F1/pRb complexes formed through this site may explain why the interaction was overlooked in previous studies.

In order to assess if E1A expression is able to induce levels of E2F1 that lack the “classic” pRb binding domain, viruses expressing either full length E2F1 or a truncated version which lacks the transactivation domain of E2F1 (E2F1 ^{Δ374}) were infected into E2F1^{-/-} MEFs expressing E1A ER. Both cell lines showed similar basal expression levels (Fig. 3-39). Cells were then treated with

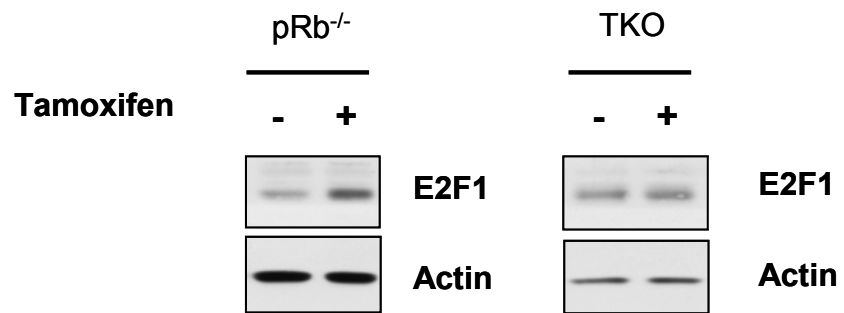


Figure 3-38 Endogenous E2F1 protein levels are not induced following E1A activation in an E1A-ER system following treatment with tamoxifen in triple knockout (pRb^{-/-}, p017^{-/-} and p130^{-/-}) MEFs

pRb-null and triple knockout (TKO) MEFs expressing E1A ER were treated with tamoxifen. 24 hours following treatment, cells were harvested in SDS buffer and protein levels estimated by Western blot analysis. Extracts were probed with anti E2F1 antibody (C-20) to determine E2F1 protein levels. Blots were re-probed with an actin antibody to ensure equal loading.

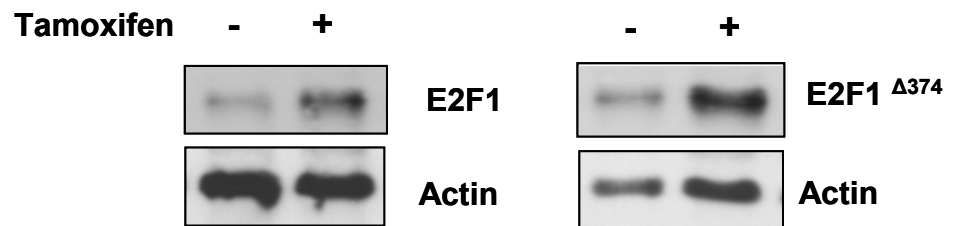


Figure 3-39 E2F1 $\Delta 374$ protein levels are induced following E1A activation in an E1A-ER system following treatment with tamoxifen

E2F1^{-/-} MEFs expressing E1A-ER fusion protein and full length or E2F1 lacking the transactivation domain (E2F1 $\Delta 374$) were treated with tamoxifen. 24 hours following treatment cell were harvested in SDS buffer and protein levels estimated by Western blot analysis. Extracts were probed with anti E2F1 antibody (C-20) to determine full length E2F1 protein levels and E2F1 antibody (KH95/20) to detect E2F1 $\Delta 374$ protein levels. Blots were re-probed with an actin antibody to ensure equal loading.

tamoxifen and lysed 24 hours following treatment. E2F1 protein levels were measured by Western blotting. That revealed noticeable induction following treatment in both cell lines. Actin levels were used to ensure equal loading of proteins.

This shows that E1A can induce E2F1 levels independent of E2F1 binding pRb via the originally identified pRb binding domain. It therefore remains a possibility that E1A binds pRb, which then uses the recently identified binding domains to interact with E2F1. That interaction could allow E1A to complex with and induce levels of E2F1.

It was next investigated if E2F1 binding to DNA was necessary for E1A-induced accumulation. Virus expressing DNA binding deficient E2F1 132E was infected into E2F1^{-/-} MEFs expressing E1A ER. Cells were then treated with tamoxifen and lysed 24 hours following treatment. E2F1 protein levels were measured by Western blotting. In line with an interaction with the new E2F1 binding domain, this revealed noticeable induction following treatment, indicating that E1A-induced accumulation of E2F1 is independent of E2F1 binding DNA (Fig. 3-40). Actin levels were used to ensure equal loading of proteins.

3.3.17 Acetylation mutant is induced following E1A activation

As previously discussed, E2F1 has also been shown to be acetylated on lysine 117, 120 and 125 leading to increased stability of the protein following DNA damage (Martinez-Balbas et al., 2000; Pediconi et al., 2003). One possibility is that acetylation on these lysines are involved in the E2F1 induction seen following E1A activation. In order to test that, the mutant with all three lysine residues substituted to arginine (K117R/K120R/K125R) was used. Virus expressing the acetylation mutant was infected into E2F1^{-/-} MEFs expressing E1A ER. Cells were then treated with tamoxifen and lysed 24 hours following treatment. E2F1 protein levels were measured by Western blotting. This revealed noticeable induction following treatment, indicating that E1A-induced accumulation of E2F1 is independent of acetylation on lysines 117, 120 and 125 (Fig. 3-41). Actin levels were used to ensure equal loading of proteins.

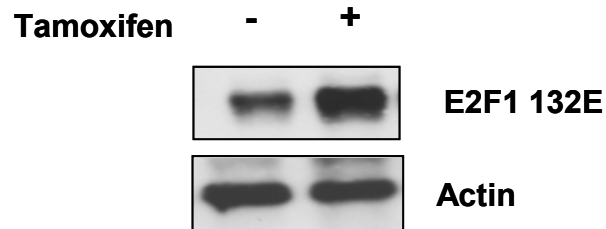


Figure 3-40 DNA binding deficient mutant of E2F1 is induced following E1A activation in an E1A-ER system following treatment with tamoxifen

E2F1^{-/-} MEFs expressing E1A-ER fusion and DNA binding deficient mutant of E2F1 (E2F1 132E) were treated with tamoxifen. 24 hours following treatment harvested in SDS buffer and protein levels estimated by Western blot analysis. Extracts were probed with anti E2F1 antibody (C-20) to determine E2F1 protein levels. Blots were re-probed with an actin antibody to ensure equal loading.

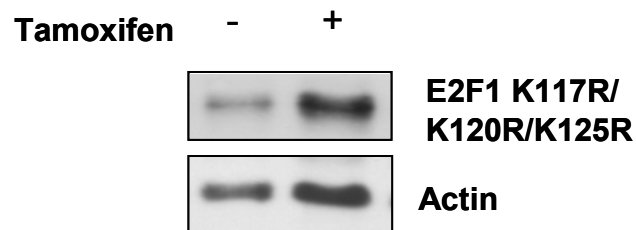


Figure 3-41 Acetylation mutant of E2F1 is induced following E1A activation in an E1A-ER system following treatment with tamoxifen.

E2F1^{-/-} MEFs expressing E1A-ER fusion protein were infected with virus expressing mutant of E2F1 with three lysine residues substituted to arginine (E2F1 K117R/K120R/K125R). Cells were treated with tamoxifen and 24 hours following treatment harvested in SDS buffer and protein levels estimated by Western blot analysis. Extracts were probed with anti E2F1 antibody (C-20) to determine E2F1 protein levels. Blots were re-probed with an actin antibody to ensure equal loading.

3.3.18 *Chapter summary*

It was demonstrated in the previous chapters that E2F1 is induced following DNA damage and is involved in drug-induced apoptosis. The results in this chapter illustrate that E2F1 is also induced following oncogene activation. E1A expression induces E2F1 levels in a post-transcriptional manner. This induction also contributes to increased chemosensitivity.

E1A is dependent on binding both to the pRB family of proteins and the p400/TRRAP chromatin remodelling complex to induce E2F1 accumulation. E1A binding to these proteins is also crucial to sensitize cells to drug-induced apoptosis. Truncated forms of E1A that are deficient in binding to either the pRB family of proteins or the p400/TRRAP complex fail to induce E2F1 levels. They are also impaired in their ability to increase drug sensitivity.

E1A induced E2F1 levels in wild type MEFs and pRb^{-/-} MEFs, indicating that E1A is not dependent of pRb binding to mediate the effect. However, E2F1 is not induced in triple knockout MEFs (pRb^{-/-}, p107^{-/-} and p130^{-/-}), indicating that E1A is dependent on expression of at least one of the pRB family members to induce E2F1 accumulation.

Using p400 specific siRNAs to silence p400 resulted in an increase in E2F1 levels. This indicates that E1A also inhibits the p400/TRRAP complex to mediated E2F1 induction. p400 knockdown also sensitized cells to drug-induced apoptosis.

Overexpression of E2F1 in cells with deregulated E2F1 sensitizes cells to drug-induced apoptosis. RPE cells expressing E1A Δ 2-36, which deregulates E2F1 through pRb binding but does not bind the p400/TRRAP complex and therefore not induce E2F1 levels, were infected with adenovirus expressing E2F1. Overexpression of E2F1 sensitized these cells to death following adriamycin treatment. The increased chemosensitivity is independent of p53 changes, since p53 accumulation is similar in both E1A Δ 2-36 expressing cells and control cells.

Chapter 4: Discussion

4 Chapter 4: Discussion

4.1 Regulation of E2F1 stability is important for DNA damage-induced apoptosis

E2F1 is tightly controlled during the normal cell cycle. The abundance of E2F1 is highest in G₁/S phase but kept relatively low during the remainder of the cell cycle. The mechanisms that control the accumulation of E2F1 activity during the cell cycle are complex involving multiple mechanisms including the ubiquitin-mediated degradation pathway. Furthermore, studies have shown that E2F1 is upregulated in response to DNA damage caused by ionising radiation or UV exposure (Blattner et al., 1999; Hofferer et al., 1999; Huang et al., 1997). Chemotherapeutic drugs that lead to DNA damage have also been shown to induce E2F1 levels in cells (Meng et al., 1999). It is unclear if the same mechanisms that regulate E2F1 stability during the cell cycle also regulate E2F1 stability following DNA damage. Given E2F1's important role in inducing apoptosis, it is important to understand the mechanism(s) responsible for E2F1 stabilization.

The present studies show that E2F1 is induced by three different chemotherapeutic drugs; adriamycin, actinomycin D and etoposide in Mouse Embryonic Fibroblasts (MEFs). These drugs all cause DNA damage by interfering with DNA replication or transcription and have been used in treatment of many tumour types. E2F1 in primary human Retinal Pigment Epithelial (RPE) cells showed similar induction in response to adriamycin. The induction is rapid and easily detectable after 6 hours following treatment in both cell lines. No changes were observed in RNA levels following adriamycin treatment in RPE cells, indicating that the induction is post-transcriptional. This result is consistent with previous studies which showed that E2F1 upregulation is due to an increase in the half life of the protein (Blattner et al., 1999; Lin et al., 2001; Stevens et al., 2003). Studies have also indicated that the accumulation of E2F1 in response to DNA damage is not a result of cell cycle arrest, since the other activating E2Fs, which also accumulate in G₁/S phase, show no induction following DNA damage (Lin et al., 2001).

The studies here show that regulation of E2F1 stability is important for apoptosis caused by DNA damage. MEFs in which E2F1 has been deleted (E2F1^{-/-}) show increased drug resistance compared to wild type MEFs. Re-introducing full length human E2F1 into E2F1^{-/-} MEFs rescued the drug sensitivity observed in wild type MEFs. Furthermore, E2F1 induction following DNA damage is also important for apoptosis induction in RPE cells, since adriamycin-induced apoptosis is inhibited by siRNA of E2F1. Previously it has been shown that, in line with the selective induction of E2F1 protein in response to DNA damage, drug-induced apoptosis is inhibited by an siRNA targeting E2F1 but not by siRNAs specially targeting E2F2 or E2F3 (Wang et al., 2004). This finding underlines the specific role of E2F1 in this response. In line with these findings, the results presented here demonstrating reduction in death when either E2F1 is deleted or silenced further support the critical role of E2F1 in DNA damage-induced apoptosis.

It is well documented that p53 is induced following DNA damage and p53 levels have been shown to rise during E2F1 mediated apoptosis, which corresponds with an increase in ARF expression (Bates et al., 1998). However, it is demonstrated here that the ability of induced E2F1 to sensitize RPE cells to drug-induced apoptosis is independent of p53. The p53 protein is induced following adriamycin treatment. This induction is however not affected by E2F1 knockdown and is therefore unlikely to be contributing to the decrease in apoptosis observed in E2F1 knockdown cells. This raises the possibility that downstream E2F1 apoptotic targets are activated following adriamycin and this activation is crucial for the response. That possibility could be tested by knocking down potential candidates with siRNA. Many genes, such as p73 or Apaf1 may mediate this response, since many of them have been shown to contribute to p53-independent E2F1-induced apoptosis (Furukawa et al., 2002; Stiewe and Putzer, 2000). In addition, in order to search for the E2F1 target genes that mediate the response following adriamycin treatment a microarray study could be carried out using the two different cell lines. That should give indications which genes are affected by the E2F1 knockdown and respond differently to the DNA damage, leading to the decrease in apoptosis observed.

4.2 E2F1's response beyond known mechanisms

Recent studies have shown that E2F1 accumulation following DNA damage is mediated by the stress-responsive kinases ATM/ATR and Chk2. ATM phosphorylates E2F1 at serine 31, while Chk2 is responsible for serine 364 phosphorylation (Lin et al., 2001; Stevens et al., 2003). Both these phosphorylation events have been shown to lead to E2F1 stabilization. Using E2F^{-/-} MEFs the contribution of these events to E2F1 stabilization were tested. These mechanisms were analysed in MEFs because, perhaps in contrast with many tumour cell lines, they might have all possible mechanisms leading to E2F1 stabilization intact. The results revealed that neither of these phosphorylation events are required for E2F1 induction since an E2F1 double mutant harbouring the mutations S31A/S364 was shown to accumulate following adriamycin treatment.

The results show human E2F1 to be induced following DNA damage in wild type MEFs. However, it is not clear if human E2F1 can be phosphorylated at serine 364 in MEFs. While serine 31 is conserved between human and mouse E2F1, serine 364 is not present in mouse E2F1. If mouse Chk2 has the ability to recognise Chk2 consensus phosphorylation site in the human protein is not known (Fig. 4-1a).

The three lysine residues that have previously been shown to be acetylated in human cells (K117, K120 and K125) are conserved in mice (Fig 4-1b). Nonetheless, a mutant carrying arginine substitution for these lysine residues did not completely inhibit E2F1 induction following treatment with adriamycin. This indicates that acetylation at these sites is not required for E2F1 induction following DNA damage. However, other acetylation events can not be excluded to be involved. Both wild type and the triple acetylation mutant of E2F1 are induced following HDAC inhibition with TSA indicating that other acetylation events might play a role. Due to the low affinity of the anti-acetylated lysine antibody for acetylated E2F1 (data not shown) it has not been possible to verify if the acetylation mutant is acetylated following DNA damage.

The C-terminal activation domain of E2F1 contains two separate domains that are necessary for pRb and Mdm2 binding. Both of these proteins bind E2F1

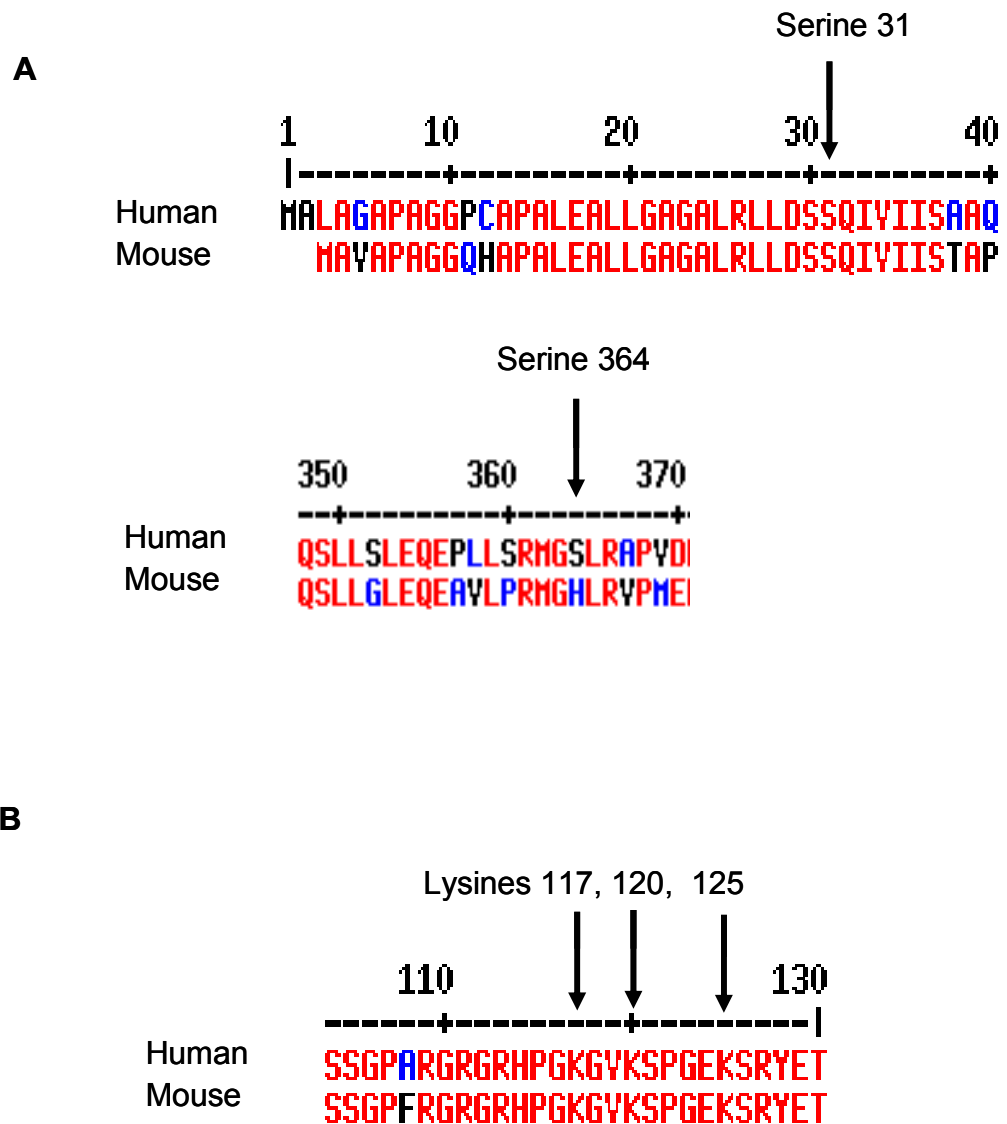


Figure 4-1 Comparison of human and mouse E2F1 sequences

A) Comparison of human and mouse E2F1 sequences indicate that serine 31 is conserved between human and mouse E2F1. On the other hand, serine 364 is not present in mouse E2F1. **B)** Comparison of human and mouse E2F1 sequences indicate that the three lysine residues that have previously shown to be acetylated in human cells (K117, K120 and K125) are conserved in mouse E2F1.

directly and have been shown to block ubiquitination and stabilise E2F1 by protecting it from degradation (Campanero and Flemington, 1997; Hateboer et al., 1996b; Hofmann et al., 1996; Zhang et al., 2005). However MDM2's effect on E2F1 stability may be context specific. In addition to mediating E2F1's stabilization, Mdm2 has also been shown to promote E2F1 degradation (Blattner et al., 1999; Loughran et al., 2000). The results presented here indicate that DNA damage-induced accumulation of E2F1 occurs also via inhibition of proteasomal degradation. However, both pRb and Mdm2 can be excluded from playing an essential role in the DNA damage response. A MDM2 binding deficient mutant of E2F1 is induced to comparable levels as wild type following DNA damage. This is also further supported by the response of truncation 1 (tr. 1 Δ^{374}), which lacks the entire transactivation domain and the regions that bind pRb and MDM2, but is stabilized following DNA damage. In addition, E2F1 has been shown to be induced following DNA damage in pRb^{-/-} MEFs (O'Connor and Lu, 2000).

What is the mechanism leading to E2F1 induction following DNA damage, if it does not involve the known phosphorylation sites, the known acetylation sites or interaction with proteins within the transactivation domain? One possibility is modification changes that inhibit Skp2 mediated E2F1 degradation. Skp2 binds to the N-terminal end of E2F1 and promotes its ubiquitination (Marti et al., 1999). Serine 31 is positioned close to the Skp2 binding site and has been suggested to alter E2F1 degradation by inhibiting Skp2 binding (Mundle and Saberwal, 2003). Direct Mdm2-E2F1 binding has also been suggested to displace Skp2 from E2F1 and inhibit its ubiquitination (Zhang et al., 2005). The responses of the S31A mutant and tr. 1 Δ^{374} rule these possibilities out. However, it is still possible that other modifications regulated Skp2 binding to E2F1 and effect E2F1 stability following DNA damage. The contribution of Skp2 to E2F1 accumulation following DNA damage could be tested in cells where Skp2 is knocked down by siRNA or in Skp2^{-/-} MEFs.

ARF has also been shown to interact with E2F1 and target it for protein degradation (Martelli et al., 2001). It appears that multiple, independent domains within E2F1 serve as targets for ARF-mediated degradation. That makes the identification of the mechanism hard to define. It is also not clear if the interaction of ARF and E2F1 is direct or not. ARF-mediated E2F1 degradation is

inhibited by the proteasome inhibitor LLnL and may therefore be connected with ubiquitination of E2F1. However, to date, no known ubiquitin sites within E2F1 have been defined. Further analysis is therefore needed to define the biochemical mechanism by which ARF targets E2F1 for degradation. Also, whether ARF plays an essential role in E2F1 induction following DNA damage is not known but could be tested in cells with siRNA mediated ARF knockdown or in ARF^{-/-} MEFs.

The DNA damage-induced accumulation of truncation 3 (tr. 3^{Δ284}), which lacks both the transactivation domain and the Chk2 phosphorylation site at S364, indicates that the last 53 N-terminal residues are not required for the DNA damage response. This also excludes the requirement of the known cell cycle dependent phosphorylation events on E2F1. E2F1 is phosphorylated at serines 332 and 337 residues by Cdk4-cyclin D which prevents its interaction with pRb, and at serine 375 by cdk2-cyclin A, which inhibits the DNA-binding activity of E2F1 (Dynlacht et al., 1994; Fagan et al., 1994; Krek et al., 1995; Xu et al., 1994). Therefore, it is unlikely that cell cycle dependent phosphorylation events, which can affect E2F1 stability, are involved in DNA damage-induced E2F1 stabilization.

The recently identified E2F1 binding domain in pRb requires the marked box of E2F1 as well as making contact with multiple places in the N terminal region of E2F1 (Dick and Dyson, 2003). The two distinct E2F binding sites offer pRb the ability to distinguish between E2F proteins and provide it with the opportunity to differentially regulate E2F functions. In addition, DNA damage causes a change that prevents E2F1 from binding to this unique interaction site on pRb. Therefore, it can be suggested that pathways leading to DNA damage-induced E2F1 modification could control the interaction of E2F1 and pRb, which could affect E2F1 stability. However, as discussed previously, E2F1 is induced in pRb^{-/-} MEFs, which indicates that E2F1 induction following DNA damage does not require pRb (O'Connor and Lu, 2000).

Most previous studies, which have identified mechanism leading to E2F1 stabilization following DNA damage, have been carried out in cancer cells. It is probable that cancer cells have already have lost some of the mechanisms leading to E2F1 accumulation DNA damage, and therefore make it easier to

inhibit the induction of the remaining mechanism. This cell specificity can explain why E2F1 still responds to DNA damage even if it carries mutations in the known DNA damage responsive phosphorylation and acetylation sites.

4.3 Two short E2F1 truncation respond to DNA damage

The present studies have led to the identification of two E2F1 truncations that respond to DNA damage in a manner not involving previously described mechanisms. One truncation only contains the first 120 amino acids of E2F1 (tr. 6^{Δ120}). Given that tr. 6^{Δ120} with serine 31 and lysines 117 and 120 mutated (tr. 6^{Δ120} S31A/K117A/K120A) is still induced following DNA damage, indicates that this region of E2F1 contains residues that are important for the DNA damage response. Whether the response involves protein-protein interaction, phosphorylation, acetylation or other forms of post-transcriptional modifications is unknown. It is possible that Skp2 binding to the N-terminus also regulates the degradation of this truncation. It is also possible that this region of E2F1 binds to the previously identified C-terminal region in pRb and that interaction is abolished following DNA damage, which could lead to increased stability. The potential interaction between tr. 6^{Δ120} and these proteins could be tested by co-immunoprecipitation experiments, using specific antibodies to these proteins.

It is also possible that ARF mediated ubiquitination is inhibited in tr. 6^{Δ120} following DNA damage and that inhibition is responsible for the accumulation. The regions within E2F1 that are able to interact with ARF are not known, and if tr. 6^{Δ120} is able to interact with ARF was not tested.

The second minimal truncation identified tr. 11¹²⁰⁻¹⁹¹, consists only of the DNA binding domain, and does not include the known ATM and Chk2 phosphorylation sites. This domain comprises two of the known DNA damage responsive acetylation sites (K120 and K125) that could be responsible for the induction. However, that was not considered to be a likely explanation since substitution of all three lysines on full length E2F1 did not alter the induction and neither did substitution of two of them on tr. 6^{Δ120}. The data therefore indicate that the DNA binding domain of E2F1 can respond to DNA damage by a mechanism not previously described. This domain is unable to bind DNA but retains the ability to induce apoptosis when overexpressed (Bell et al., 2006). If this domain can

interact with the C-terminus of pRb was not established. It is also not known if tr.11¹²⁰⁻¹⁹¹ is able to bind ARF, which as previously discussed could be involved in the DNA damage response.

The possibility that DNA damage leads to changes in ubiquitination on 6^{Δ120} or tr.11¹²⁰⁻¹⁹¹ could be tested in ubiquitination assays. The truncations could be co-expressed in cells with ubiquitin and then immunoprecipitated following DNA damage and ubiquitination measured by Western blotting. That could reveal if any changes occur with regard to ubiquitination of the truncation following DNA damage.

DNA damage responses often involve phosphorylation events. An example is the many DNA damage-induced phosphorylation sites which have been identified in p53, and contribute to its stability (Lavin and Kozlov, 2007). It was therefore decided to search for potential phosphorylation sites in tr. 6^{Δ120} and if tr.11¹²⁰⁻¹⁹¹. Using NetPhos 2.0 (<http://www.cbs.dtu.dk/services/NetPhos/>), a network-based method for predicting potential phosphorylation sites at serine, threonine or tyrosine residues in protein sequences, several potential phosphorylation sites were identified in tr. 6^{Δ120} and tr.11¹²⁰⁻¹⁹¹. However, when these potential phosphorylation sites were mutated to residues that can not be phosphorylated, both truncations showed induction following DNA damage. That does not rule out the possibility that a combination of mutations would be required to inhibit the induction. Further studies are therefore required to fully understand the mechanism involved in the induction of tr. 6^{Δ120} and tr.11¹²⁰⁻¹⁹¹. To investigate if DNA damage leads to changes in phosphorylation of the truncations mass-spectrometry could be used. Phospho-peptide mapping is a technique that allows identification of phosphorylated residues on immunoprecipitated proteins. Therefore, changes in phosphorylation on the truncation following DNA damage could be revealed using phospho-peptide mapping on full length E2F1 protein or on the truncations.

In summary, the data presented here indicate that there are many pathways leading to E2F1 stability following DNA damage, some of which remain to be discovered. E2F1's response to DNA damage is complex and may be overlapping and involve many different modifications. It will be interesting to observe if any of the mechanisms are lost during tumour development, which could prevent

E2F1-induced apoptosis in tumours where mutations and DNA damage are continuously occurring.

4.4 E1A deregulates and induces E2F1 levels

E1A, the oncogene from type 5 adenovirus, is known to deregulate E2F by targeting the pRB family of pocket proteins (Chellappan et al., 1992; Whyte et al., 1988). Present results show that E1A also induced E2F1 levels in a post-transcriptional manner. This is perhaps unexpected because E1A is known to disrupt E2F-pocket protein complexes, and should therefore generate free, but unstable E2F1, since pRb has been shown to prevent E2F1 degradation (Campanero and Flemington, 1997; Hateboer et al., 1996b; Hofmann et al., 1996). However, it is possible that this stabilization by E1A serves to activate the transcriptional machinery and enhance S-phase entry of adenovirus-infected cells.

4.4.1 E1A induces E2F1 level in a pRB dependent way

In 1996 Hateboer et al. showed that adenovirus early region 1 (E1) proteins cause stabilization of E2F1 and DP (Hateboer et al., 1996b). These effects were dependent on simultaneous expression of both E1A and E1B and required the transactivation domain of E2F1. Present data indicate that the E1A-induced E2F1 accumulation is mediated by a different mechanism. The induction does not require the transactivation domain of E2F1 since E2F1 Δ^{374} is induced following E1A activation and E1B expression is dispensable.

However, E1A is dependent on binding to any one of the pRB family proteins to cause E2F1 induction. A pRB binding deletion mutant of E1A (E1A Δ CR2) fails to bind to pRB family proteins and fails to induce E2F1 levels. The results in pRb^{-/-} MEFs, where E2F1 is induced following E1A expression, indicate that the other pRB family members can substitute for pRb when pRb does not exist. In pRb^{-/-} MEFs it is possible that either p107 or p130 provide a scaffold for E1A forming a ternary complex with E2F1. That could be tested by immunoprecipitation experiment in the wild type and pRb^{-/-} MEFs. However, the results from the TKO MEFs support the dependence of the pRB family proteins for the effect by E1A (figure 4-2). In order to test if one of the pRB family members is sufficient for

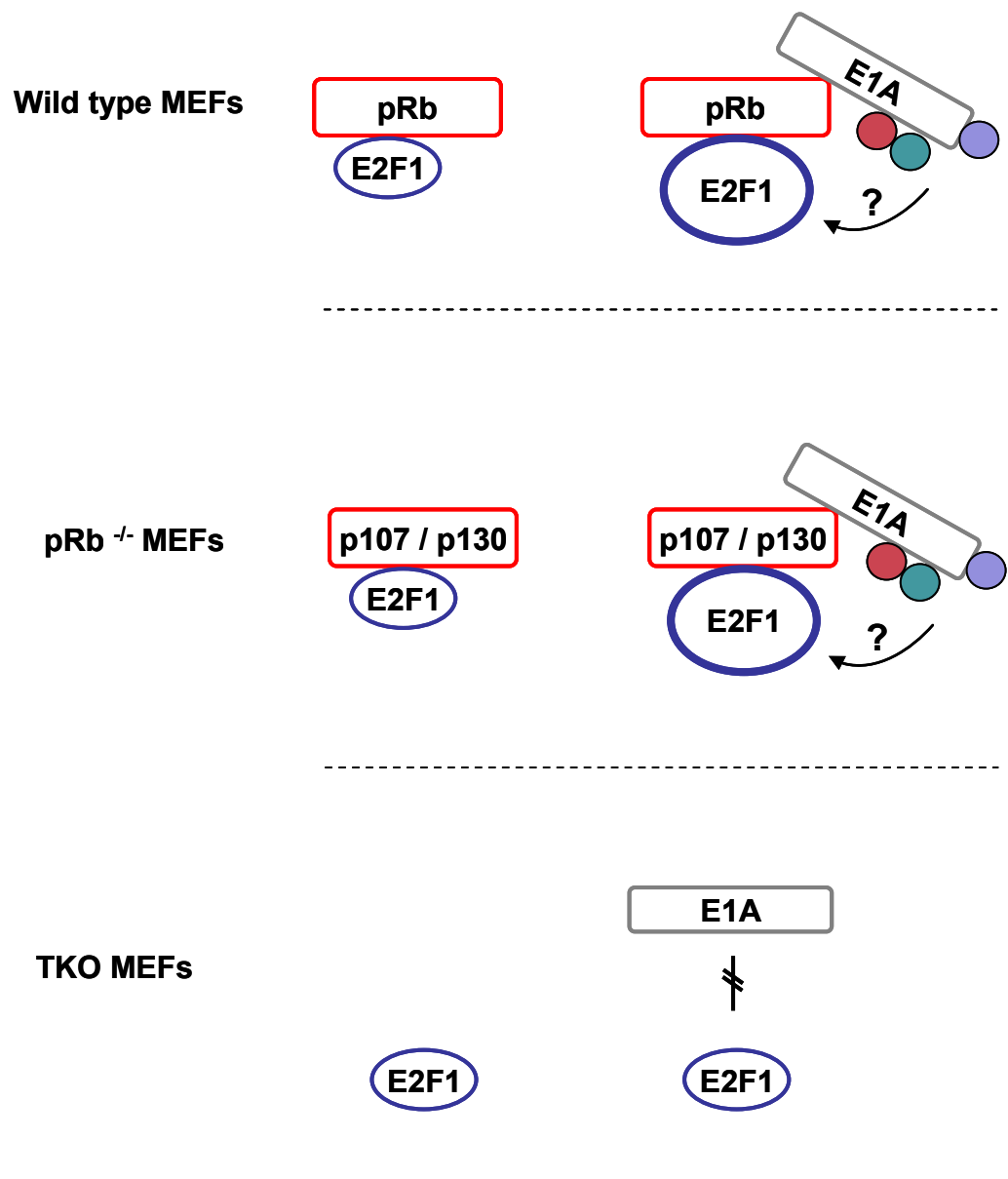


Figure 4-2 E1A is dependent on presence of the pRB family proteins to induce E2F1 accumulation.

E1A deregulates E2F activity by binding to the pRB family of pocket proteins. pRb binds preferably to E2F1 and could therefore bring together E1A and E2F1 in wild type cells. However, the other family members can compensate for pRb in pRb-null cells and allow for a ternary complex with E1A and E2F1 in pRb-null cells. In TKO cells (null for all pRB family proteins) E1A fails to induce E2F1 levels, possibly by failing to interact with E2F1 as a result of lack of all the pRB proteins.

E1A to interact with E2F1, pRb, p107 and p130 could be transfected separately into the TKO MEFs and investigated which of them could rescue the E2F1 accumulation effect by E1A. In addition, immunoprecipitation experiments could reveal if all members could compensate for pRb knockout and interact with E2F1 to provide the platform for E1A interaction.

But how then can E1A induce E2F1 Δ^{374} levels? This can be explained by the recently identified pRb binding domain within E2F1. pRb binding to E2F1 via this domain is not dependent on the transactivation domain, but requires the marked box region of E2F1 for binding and makes contact with multiple regions within the N terminus as well. Therefore, E1A could interact with pRb which then binds E2F1 via the alternative interaction. This could be tested by immunoprecipitation experiments in the MEFs expressing wild type and tr.1 Δ^{374} , where the interaction between tr. 1 Δ^{374} , E1A and pRb could be investigated. In addition, a mutant form of pRb which only binds E2F1 via the alternative binding site could be tested for its ability to rescue the E2F1 accumulation following E1A activation in the TKO MEFs.

The recently identified E2F1-pRb complex was shown to have low affinity for DNA binding (Dick and Dyson, 2003). Present data show that E2F1 induction following E1A activation does not require E2F1 to bind DNA. E2F1 132E, a DNA binding deficient mutant is induced to similar levels as wild type E2F1 following E1A expression.

4.4.2 E1A is dependent on binding to the p400/TRRAP protein complex to induce E2F1 levels

A sequence within the E1A N-terminus (amino acids 4-47) interacts with a proteins of 400 kDa, which has previously been identified as p400 and TRRAP (transactivation/transformation-domain-associated protein) (Barbeau et al., 1994; Howe and Bayley, 1992; McMahon et al., 1998). This p400 binding region has been shown to be vital for E1A-mediated transformation in mouse cells (Fuchs et al., 2001). p400 is related to the yeast SW12/SNF2 chromatin remodelling complex and modulates both transcriptional activation and repression. ATP-dependent nucleosome remodelling complexes control the access of transcription factors to promoter region by modifying chromatin

structures and nucleosome positioning (Sudarsanam and Winston, 2000). p400 has been found in several large multisubunit complexes, including P/CAF complex and Tip60 histone acetyltransferase (HAT) complex which is involved in DNA repair and apoptosis (Ikura et al., 2000). Consistent with the finding that Tip60 contains p400, immunoprecipitation (IP) experiments have showed that p400 associates with a number of proteins also present in the Tip60 complex, including the DNA helicase TAP54 (McMahon et al., 1998).

TRRAP is a component of various large HAT complexes including Tip60 and complexes containing GCN5 or P/CAF acetyltransferases. Thus, since many complexes contain TRRAP it appears that only a fraction of endogenous TRRAP is bound to p400. By contrast, analysis of IPs generated with p400 specific antibodies suggest that the majority of endogenous p400 molecules exists in complex with TRRAP (McMahon et al., 1998). Studies have shown that E1A binds a TRRAP complex that contains GCN5 acetyltransferase during normal adenoviral infection which may facilitate infection by deregulating cellular transcriptional programs (Lang and Hearing, 2003). Studies with Myc indicate that TRRAP acts as a bridge to recruit HAT activity catalyzed by the GCN5 protein and stimulate transcription (McMahon et al., 2000).

In contrast to repression, the mechanisms of transactivation by E2F have not been established in molecular details. It has been suggested that E2F proteins must reverse the pRb-imposed chromatin structure to stimulate transcription. In favour of direct role of E2Fs in histone acetylation, they interact with HATs, including P/CAF and GCN5, which enhances E2F-dependent transcription, possibly through acetylation of E2F itself (Lang et al., 2001; Martinez-Balbas et al., 2000; Marzio et al., 2000; Pediconi et al., 2003) (Fig 4-3). E2F1 has also been shown to bind TRRAP through the E2F1 transactivation domain (Lang et al., 2001; McMahon et al., 1998).

In spite of these observations, there is no direct proof of how E1A binding to p400/TRRAP effects or mediates E2F1 dependent transcription. In addition, it is not clear what HAT if any, plays a part in the E1A interacting p400/TRRAP complex and whether the p400/TRRAP complex can directly affect E2F1 stability.

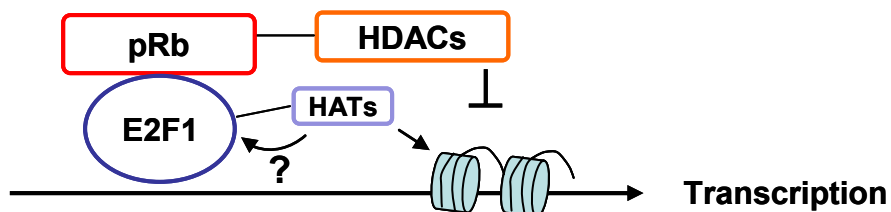


Figure 4-3 pRb inhibits E2F1 mediated transcription by recruiting histone deacetylases (HDACs)

It has been suggested that E2F1 reverses the pRb inhibition by interacting with histone acetyltransferases (HATs), such as P/CAF and GCN5, which enhances transcription, possibly through acetylation of E2F1 itself.

The data presented here indicate that E1A binding to p400/TRRAP is crucial in increasing E2F1 levels (Fig 4-4). Moreover, the p400 knockdown experiments suggest that E1A mediates this effect by inhibiting the complex, since p400 knockdown also leads to increased E2F1 levels. However, if these effects are dependent on HAT activity was not tested. Due to the low affinity of the anti-acetylated lysine antibody for acetylated E2F1 (data not shown) it has not been possible to verify if E2F1 is acetylated or de-acetylated following E1A expression.

It remains a possibility that p400 plays a vital role in a complex that promotes E2F1 down-regulation and E1A inhibits this leading to increased E2F1 stability. It is also possible that p400 knockdown promotes E2F1 stability by increasing the access of other HATs that acetylate E2F1 following p400 removal.

These questions could be answered in *in vitro* acetylation assays where acetylated E2F1 could be more easily detected. Knockdown experiments could also be carried out in order to assess if E2F1 induction is dependent on the HATs known to mediate HAT activity in p400 complexes. That could reveal if the accumulation is dependent on p/CAF, Tip60, GCN5 or other known p400 interacting HATs.

4.4.3 E2F1 overexpression sensitizes cells with de-regulated pRb pathway to drug-induced apoptosis

The data presented, using the E1A mutant $\Delta 2-36$ indicate that adenoviral mediated induction of E2F1 increases chemosensitivity in cells where the E2F1 pathway is already deregulated (Fig. 3-34). The increased chemosensitivity is independent of p53 changes, since p53 accumulation is similar in both E1A $\Delta 2-36$ expressing cells and control cells.

That indicates that activation of the E2F1 pathway in tumours could be a way to increase chemotherapy-induced cell death either in the presence or absence of p53. Defects in the pRb pathway may be universal in human cancers and include deletion or mutation of the pRb gene itself (Harbour and Dean, 2000). In addition, viral oncoproteins such as the E7 protein from high-risk papilloma virus

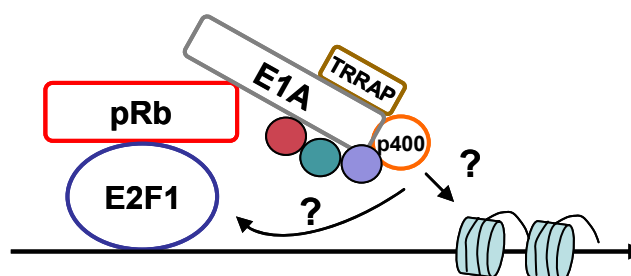


Figure 4-4 E1A binding to the p400/TRRAP chromatin remodelling complex is essential to induce E2F1

p400 knockdown also induces the levels of E2F1 indicating that E1A mediates the effect by inhibiting p400 activity. Whereas p400 promotes acetylation on chromatin when bound to E1A is not clear and if p400 affect acetylation directly on E2F1 is not known.

(HVP) also target the pRb protein leading to de-regulated E2F1 activity (Chellappan et al., 1992)

Therefore these tumours should have high E2F1 activity. However, it is not known if other viral oncoproteins known to target the pRB family proteins also bind the p400/TRRAP complex. Furthermore it is not clear if E2F1 levels are high specifically in tumours expressing viral oncogenes. Could p400 inhibition lead to increased E2F1 levels and increased chemosensitivity in these tumours? Thorough investigation is needed to define p400/TRRAP expression and activity in tumour and how it correlates with E2F1 protein levels and activity.

4.4.4 Conclusion

The results presented here demonstrate that DNA damaging agents that are regularly used to treat cancer induce E2F1 levels by a post-transcriptional mechanism. This induction is important for drug-induced apoptosis and is in line with the notion that E2F1 plays a role in drug induced apoptosis in a p53-independent manner. This connection between DNA damage and E2F1-dependent apoptosis following drug treatment is of particular significance.

Using adriamycin as a DNA damaging agent, studies revealed that the previously identified mechanisms leading to E2F1 induction could not singularly or in combination be fully responsible for the E2F1 induction following DNA damage. Therefore, other mechanism(s) must play a role in E2F1's response to DNA damage under these conditions.

The results describe two minimal domains of E2F1 that respond to adriamycin treatment which could not be explained by previously identified mechanisms. That indicates that the E2F1's response to DNA damage is complex and perhaps overlapping and may involve numerous pathways and different modifications. Whether these mechanisms are inhibited in tumour development will need thorough investigation.

In addition, E2F1 protein levels are induced following oncogene activation. E1A expression induces E2F1 levels in a post-transcriptional manner which contributes to increased chemosensitivity. E1A is not only dependent on deregulating E2F1 by binding the pRB family of proteins to induce E2F1 levels

and increase chemosensitivity. E1A is also dependent on binding to the p400/TRRAP chromatin remodelling complex to induce E2F1 accumulation and sensitize cells to drug-induced apoptosis.

Overexpression of E2F1 in cells with deregulated E2F1 sensitizes cells to drug-induced apoptosis in a way that is independent of p53 activity. That provides the potential that activation of the E2F1 pathway in tumors with wild type or mutated p53 can be considered as a therapeutic option.

These results contribute to the understanding of how activation of the E2F1 pathway can be targeted therapeutically leading to enhanced chemotherapy-induced tumour cell death.

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