A thesis entitled

Regulation of E2F activity by $p14^{ARF}$

Presented by

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This thesis is dedicated to my Gran, Jessie.

Abstract

The $p14^{ARF}$ product of the *ink4a/arf* locus is induced by a variety of oncogenic signals. $p14^{ARF}$ can facilitate growth arrest through the p53 pathway by hindering the down-regulation of p53 activity through its interaction with MDM2, which interferes with formation of the complex between p53 and MDM2. Here I have explored the possibility that $p14^{ARF}$ may be integrated with growth regulatory pathways other than p53, and report that $p14^{ARF}$ can modulate the activity of the cell cycle regulating E2F transcription factor.

$p14^{ARF}$ regulates E2F-1 activity in both SAOS2 cells and *p53*$^{-/-}$*/mdm2*$^{-/-}$ MEFs, excluding the possibility that the effects of $p14^{ARF}$ on E2F are influenced by MDM2. $p14^{ARF}$ down regulates E2F-dependent transcription, S-phase entry, apoptosis and colony formation. The mechanism responsible for this activity may be through regulation of E2F stability at the post-translational level. $p14^{ARF}$ forms a physical complex with E2F both *in vitro* and in cells.

$p14^{ARF}$ binds to E2F through distinct, binding domains, one of which resides in the N-terminal region and is capable of down-regulating E2F activity. These results highlight the potential interplay and cross talk between $p14^{ARF}$ and E2F-1, and establish $p14^{ARF}$ as an antagonist of cell growth that acts by targeting two of the key pathways involved in controlling proliferation, namely E2F and p53.

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Abbreviations.

A	Adenine
AAV	Adeno-associated virus
Abl	Abelson virus
ARF	Alternative reading frame
AP	Alkaline phosphatase
APC	Anaphase promoting complex
Apaf-1	Apototic protease activating factor 1
ATM	Ataxia-telengiectasia gene product.
ATR	Ataxia-telengiectasia related gene product
ATP	Adenosine tri-phosphate
ATCC	American Tissue and Cell Collection
BAX	Bcl-2 associated protein X
Bcl–2	B–cell lymphoma-2
β-gal	β-galactosidase
Bid	BH-3 Interacting Domain Death agonist
BRCA-1	Breast cancer gene 1
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
CBP	CREB-binding protein
Cdc2	Cell division cycle 2
cdk	Cyclin dependent kinase
cdi	Cyclin dependent kinase inhibitor
C/EBP	CCAAT-box/enhancer-binding protein

CERC	Cyclin E repessor complex
CH	Cysteine/histidine-rich domain
CMV	Cytomegalovirus
CpG	Cytosine-phosphate-Guandine
CRE	cAMP-response element
CREB	CRE-binding protein
CREM	Cyclin E response module
DAPI	4,6-diamidino-2-phenylindole
DBD	DNA binding domain
DHFR	Dihydrofolate reductase
DMEM	Dulbecco's modificatied Eagle's medium
DMP1	Cyclin D interacting myb-like protein 1
DNA	Deoxyribonucleic acid
DNA pol α	DNA polymerase α
dNTP	Deoxy-nucleoside triphosphate
DPE	Down-stream promoter element
DRTF	DNA transcription factor
DP	DRTF interacting protein
DTT	Dithiothreitol
E	Exon
E	Embryonic dayX
E6	Early protein 6
E7	Early protein 7
E1A	Adenovirus early protein 1A
E2F	E2 factor

EDTA	Ethylene diamine tetra-acetic acid
FADD	Fas associated death domain
FasL	Fas ligand
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
G	Guanine
G_1	Gap phase 1
G_0	Quiescence
GR	Glucocorticoid receptor
GST	Glutathione-S-transferase
GTF	General transcription factor
GTP	Guandine tri-phosphate
HA	Hemagglutinin protein (derived from influenza virus)
HAT	Histone acetylation
HBS	HEPES-buffered saline
HBV	Hepatitis B virus
HDAC	Histone deacetylase
His	Histidine
HPV	Human papilloma virus
IB	Immunoblotting
IP	Immunoprecipitation
IPTG	Isopropyl-ß-D-thiogalactopyranoside
IVT	*In vitro* translated
JMY	Junction-mediating and regulatory protein
JNK	c-Jun NH_2-terminal kinase

LB	Liquid broth
Luc	Luciferase
LXCXE	Leucine-X-Cysteine-X-Glutamic acid
M	Mitosis
MDM2	Murine double minute 2
MDMX	Mouse double minute X
MEF's	Mouse embryonic fibroblasts.
mRNA	messenger ribonucleic acid
MyoD	Myogenic HLH transcription factor
NAP	Nucleotide assembly protein
NES	Nuclear export signal
NFκB	Nuclear factor of immunoglobulin k locus in B cells
NLS	Nuclear localisation signal
NPDC	Neural
NP-40	Nonidet P-40
NuLS/NrLS	Nucleolar localisation signal
P	Proline
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
P/CAF	p300/CBP-associated factor
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PIC	Pre-initiation complex
PMSF	Phenylmethysulfonyl fluoride
Pol	Polymerase

pRb	Retinoblastoma gene product
P/S	Penicillin/Streptomycin
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
S	Serine
SCF	SKIP1/CUL1/F-Box protein complex
SDS	Sodium dodecyl sulphate
SnRNP's	Small nuclear ribonucleoproteins
S/T-P	Serine/Threonine-proline
SV40	Simian virus 40
T	Thymine
TAD	transactivation domain
TAF	TBP-associated factor
TBP	TATA binding protein
TCR	T cell receptor
TF	Transcription factors
TKO	Triple knock out
TGF-β	Transforming growth factor β
TNF	Tumour necrosis factors
TNFR	Tumour necrosis factor receptor.
TRNA	transfer RNA
TRAIL	TNF related apoptosis inducing ligand
TRITC	Tetramethylrhodamine isothiocyanate
Tris	Tris(hydroxymethyl)methylamine
TSA	Trichostatin A

TUNEL	TdT-mediated dUTP nick end labelling
Tween 20	Polyoxyethylene sorbitan monolaurate
UV	Ultraviolet light
v-Abl	Abelson murine leukaemia viral oncogene homologue 1
wt	Wild-type
w/v	Weight per volume
X	Any amino acid
(+/+)	Wild-type
(+/-)	Heterozygous mutant
(-/-)	Homozygous mutant

Declaration.

The work presented within this thesis was performed entirely by myself and in no way forms part of any other thesis. All work supported by others is acknowledged. The work was performed while I was a graduate student at the Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Science, University of Glasgow, UK. I was under the supervision of Professor Nicholas B. La Thangue.

Publications

During the course of this work, the following papers were presented for publication:

de la Luna, S, Allen, K. E, **Mason, S.L**, and La Thangue, N.B.1999. Integration of a growth-suppressing BTB/POZ domain protein with the DP component of the E2F transcription factor. *EMBO J.* **18**:212-228.

Mason, S, L., Loughran, Ö and La Thangue, N.B. 2001. $p14^{ARF}$ regulates E2F activity. Submitted for publication.

Chapter 1. Introduction

1.1.1 The Cell Cycle

Mammalian cells have developed the ability to divide and grow by a process termed the cell cycle. The cell cycle is divided into four distinct phases; G1, where growth and acquisition of proteins required for S phase occurs, S phase, where the cell replicates its DNA, G2, a second gap phase and M, mitosis, the process of cell division. A large number of cellular proteins are important for maintaining the integrity of the cell cycle, and cell cycle "check points" are employed where these proteins determine progression into the next stage. The crucial 'restriction point' in the cell cycle is at the end of G1 phase, following the first growth stage. At this point the cell has to make a commitment to proliferation, differentiation or apoptosis.

Regulatory circuits governing the cell cycle are complex and highly interlinked. Many of the proteins implicated in this process are DNA binding transcription factors and their associated proteins. Tumour cells arise when the lack of, or overexpression of, specific proteins upsets the intrinsic workings of the cell cycle and cells begin to proliferate uncontrollably. It is therefore highly important that we understand the workings of the cellular pathways which regulate cell cycle control, in order to develop new and effective cancer therapies.

1.1.2 The E2F Pathway.

The E2F pathway is frequently deregulated in tumour cells and so is an important target for anti-cancer therapies. Deregulated E2F activity leads to uncontrolled cellular proliferation and drugs which target components of the E2F pathway could be important anti-cancer agents. Although E2F itself is rarely mutated, its upstream regulators are frequently mutated or deregulated in human cancers and disruption of this pathway is thought to be a common event leading to oncogenesis (Dyson, 1998).

1.1.3 The E2F Families.

The E2F family is a heterodimeric group of transcription factors where each heterodimer consists of one E2F and one DP partner. To date, six E2Fs (termed 1-6) and three DPs (termed 1-3) have been identified (Helin *et al.,* 1992; Girling *et al* 1993; Ivey-Hoyle *et al.,* 1993; Lees *et al.*, 1993; Beijersbergen *et al.,* 1994; Girling *et al.,* 1994; Hijmans *et al.,* 1995; Ormondroyd *et al.,* 1995; Rogers *et al.,* 1996; Cartwright *et al.,* 1998). All family members share a central conserved DNA binding region and a separate heterodimerisation domain (Figure 1.1). The E2F and DP heterodimerisation domain contains a region of similarity, which has been identified as likely to form an amphipathic α-helix (La Thangue, 1994).

E2F family members have also been identified in a number of other organisms, including *Drosophila melanogaster*, which has homologues of the E2F, DP, and pRb proteins. The *Drosophila* genome encodes two E2F genes, *de2f1* and *de2f2* (Ohtani *et al.*, 1994; Frolov *et al.*, 2001), a DP gene dDP (Royzman *et al.*, 1997) and a pRb homologue RBF (Du *et al.*, 1999). dE2F and dDP are involved in proliferation and S-

phase entry (Brook *et al.*, 1996; Du *et al.*, 1996; Duronio *et al*, 1998) and provide an interesting model system in which to study the cellular roles of the E2F transcription factor.

Recent studies have identified homologues of E2F and DP in *Caenorhabditis elegans* (Ceol *et al.*, 2001, Page *et al.*, 2001). *C. elegans efl-1* is most similar to mammalian E2F-5 and *dpl-1* is most similar to DP-1. EFL-1 and DPL-1 interact with each other and with the *C. elegans* pRb homologue, LIN-35, and may act together to mediate transcriptional repression. As yet no specific role has been defined for the *C.elegans Elf-1* and *Dpl-1* in transcription or cell cycle control, however animals lacking these genes have protein asymmetries and vulval abnormalities (Ceol *et al.*, 2001; Page *et al.*, 2001), indicating the importance of these proteins in developmental regulation.

Xenopus laevis also has E2F homologues and X1 DP-1 and X1 DP2 have been cloned and shown to be expressed early in development (Girling *et al.*, 1994). This conservation of the E2F proteins across species indicates an important role for these proteins, and model systems such as *Drosophila melanogaster* and *Caenorhabditis elegans*, allow us to study these proteins in detail.

During the course of this thesis E2F-X or DP-X refers to an individual family member, while the term E2F refers to the activity of an E2F heterodimer.

1.1.4 The E2F family members

All of the E2Fs, with the exception of E2F-6, have a C-terminal *trans*activation domain, and embedded within this, a region which allows pocket proteins to bind and repress transcription (La Thangue, 1994) (Figure 1.1). E2F-6 lacks a *trans*activation domain and so is thought to act solely as a repressor of transcription (Morkel *et al.*, 1997; Cartwright *et al.*, 1998). In fact, recent data show that E2F-6 interacts with components of the mammalian polycomb transcriptional repressor complex, suggesting that a physiological role of E2F-6 may stem from its ability to recruit this repressor complex (Trimarchi *et al.*, 2001).

E2F1-3 contain an N-terminal region of approximately 100 amino acid residues, which encompasses a cyclin A-cdk2 binding domain and also a nuclear localisation signal. The presence of this N-terminal NLS places E2Fs 1,2 and 3 in a separate sub-family from E2F-4 and E2F-5. Interestingly, through the use of a second downstream promoter, the *e2f-3* gene has been shown to encode a second transcript. The two *e2f-3* gene products are referred to as E2F-3α and E2F-3β; the first is structurally more similar to E2F-1, and the second lacks the N-terminal cyclin binding domain more similarly to the second E2F sub-family (Adams *et al.*, 2000).

E2F-4 and E2F-5 both lack the N-terminal region, and so are distinct from the other family members in that they are localised to the cytoplasm and are only able to enter the nucleus when bound to a DP partner or pocket protein; DP3 α and δ proteins have a central, bi-partite nuclear localisation signal which mediates this nuclear

localisation (de la Luna *et al.,* 1996; Allen *et al.,* 1997). Recently E2F-7 has been cloned, and resembles E2F-6 in that it lacks a *trans*activation domain (La Thangue. Personal Comm.).

1.1.5 The DP family members

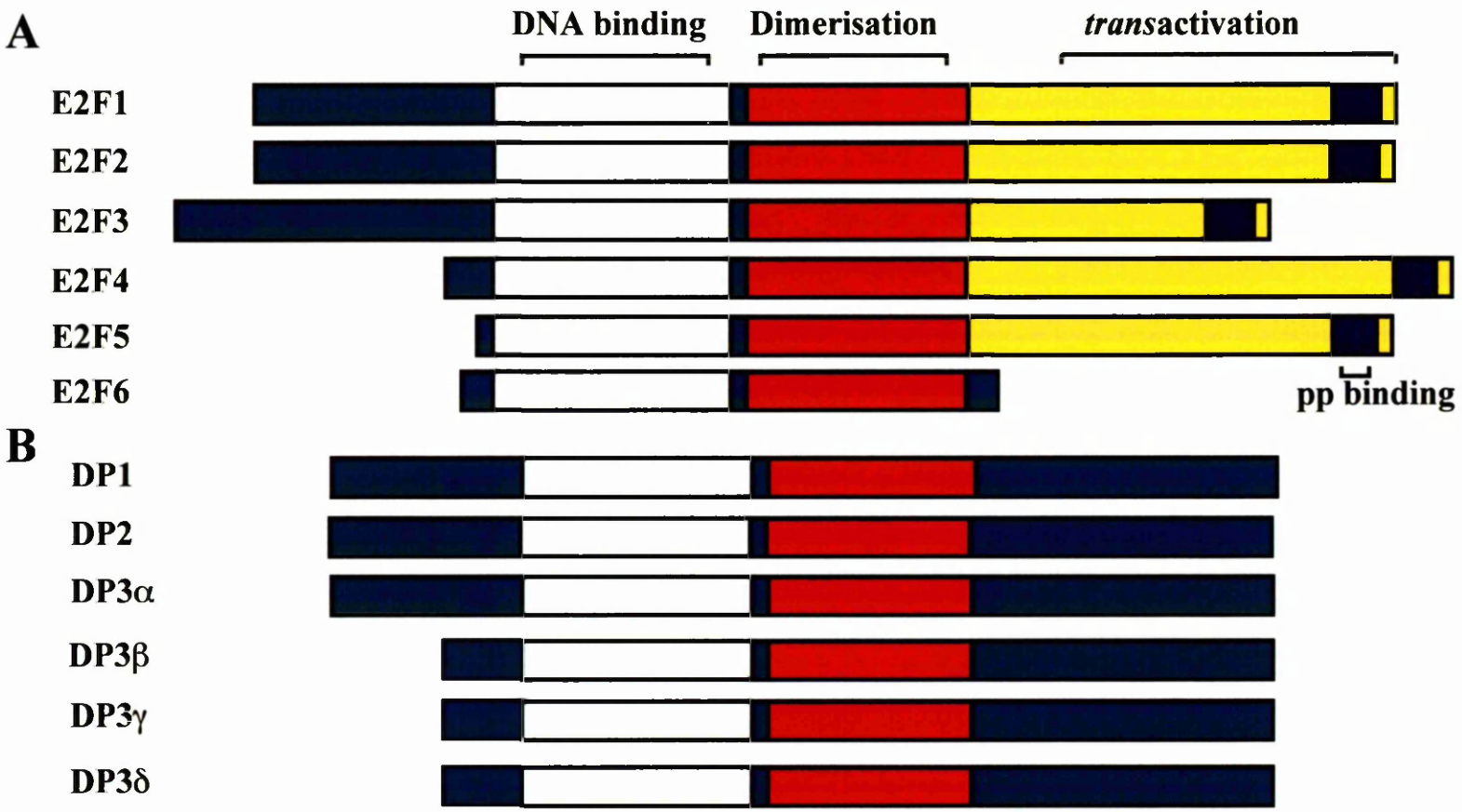
Originally, the E2F-DP heterodimeric transcription factor was referred to as DRTF1/E2F, where E2F was an activity induced in HeLa cells after adenovirus infection and DRTF was defined as a transcription factor down-regulated during the differentiation of F9 cells (Partridge and La Thangue, 1991; Girling *et al.*, 1993).

DP-1 was the first member of the DP family to be identified and was isolated from cellular DRTF complexes (Girling *et al.,* 1993). The most characterised function of the DP protein family is their ability to co-operate with E2F in DNA binding and transcriptional activation, little else is known about their function. However, a DP-1 polypeptide has been shown to be phosphorylated in a cell cycle dependent fashion and to undergo a phosphorylation-dependent mobility shift during the cell cycle, indicating that its level of phosphorylation is probably regulated during cell cycle progression (Bandara *et al.,* 1993; Bandara *et al.,* 1994). DP-1 and DP-2 are integrated with another growth regulating pathway, which involves signal transduction emanating from activated Ras protein (Jooss *et al.*, 1995). Both DP-1 and DP-2 are able to replace myc and co-operate with Ha-*ras* in a transformation assay (Jooss *et al.*, 1995). These data suggest an E2F independent effector function for the DP family members (Jooss *et al.*, 1995).

Figure 1.1 The E2F and DP family members.

(A) Summary of the structures of the E2F family members, showing the location of the conserved DNA binding (white), dimerisation (red), pocket protein binding (blue) and transactivation (yellow) domains.

(B) Summary of the structures of the DP family members.


A
DNA binding
Dimerisation
transactivation
E2F1
E2F2
E2F3
E2F4
E2F5
E2F6
pp binding
B
DP1
DP2
DP3α
DP3β
DP3γ
DP3δ

DP-2 was cloned in *Xenopus*, as a DP-1 homologue, and shown to have greater than 70% protein similarity to DP-1 (Girling *et al.*,1994). Generally, in the literature DP-2 refers to the human form and DP-3 to the mouse form of what is thought to be the same protein. Human DP-2 was identified as an E2F binding protein in a yeast two-hybrid screen of a HeLa cell library and exists as at least three DP-2 related proteins that are regulated by alternative splicing of the five prime region (Rogers *et al.*, 1996). DP-3 was cloned from a murine library and is present in four different splice forms termed alpha (α), beta (β), delta (δ) and gamma (γ), determined by their different N terminal organisation (Ormondroyd *et al.*, 1995).

The four DP-3 isoforms vary in their cellular localisation (de la Luna *et al.*, 1996). An N terminal basic region, that encompasses a bi-partite NLS controls whether the protein is nuclear or cytoplasmic (de la Luna *et al.*, 1996). DP-3α and DP-3δ contain the NLS, while the family members DP-3β and γ are normally found in the cytoplasm, unless bound to a pocket protein which can take them into the nucleus (de la Luna *et al.*, 1996). DP-4, a new member of the family has recently been identified which shows high homology to DP-1 (La Thangue. Personal Comm.).

1.1.6 The Pocket Proteins

E2F transcriptional activity is tightly regulated during the cell cycle through binding to members of the pocket protein family; pRb, p107 and p130 (Dyson, 1998). pRb and its related family members, p107 and p130 contain a C terminal "pocket" region, which binds to members of the E2F family (La Thangue 1994; Dyson 1998).

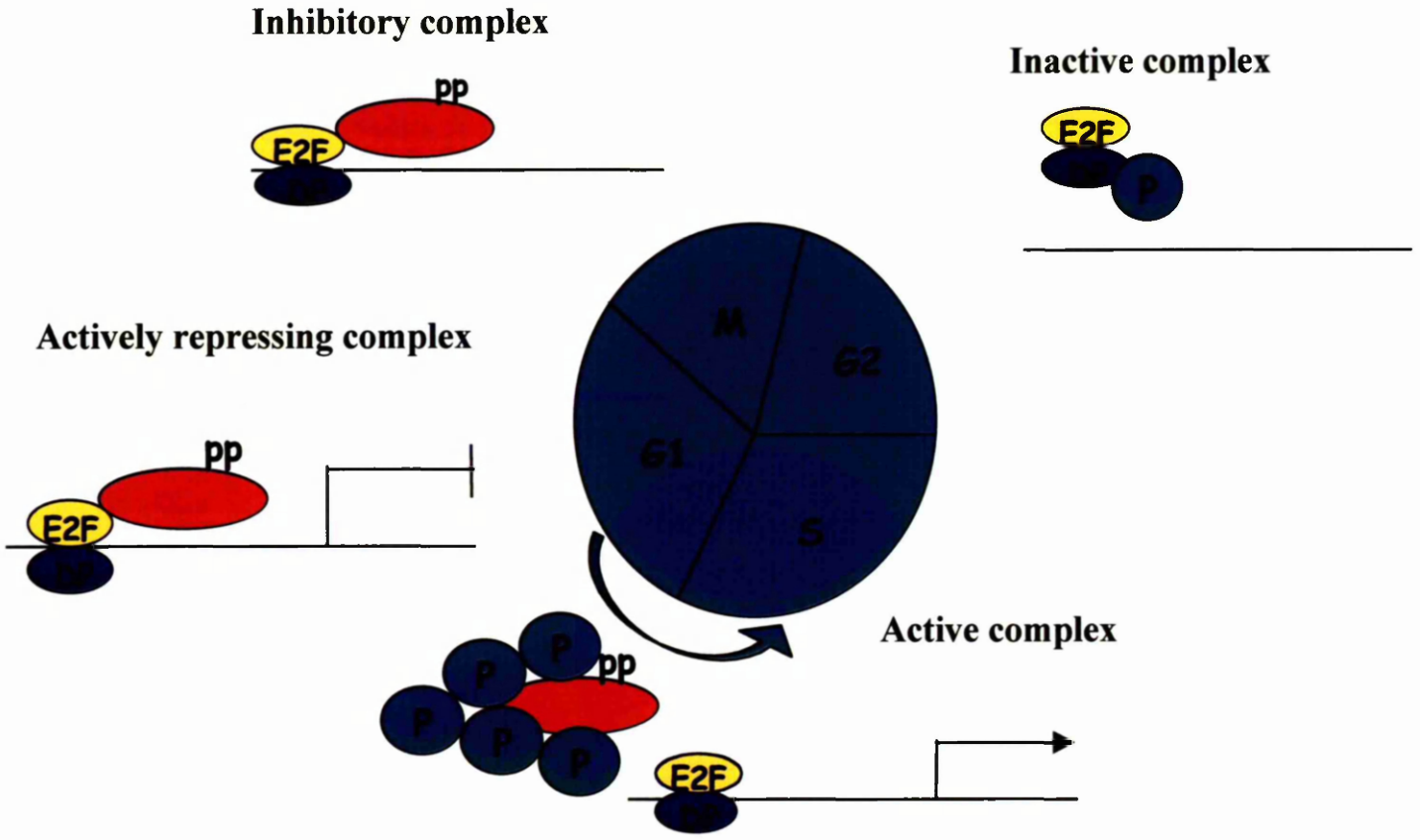
The individual pocket protein family members seem to bind preferentially to different E2F family members. E2F-1, E2F-2 and E2F-3 bind almost exclusively to pRb, while E2F-4 binds to all three pocket proteins and E2F-5 interacts preferentially with p130. E2F-pocket protein complexes are found at different stages of the cell cycle. pRb-E2F complexes can be found in quiescent or differentiated cells but are most evident at the G1-S phase boundary. p130-E2F complexes are found in quiescent or differentiated cells while p107-E2F complexes are found mainly in S-phase (Dyson, 1998; Takahashi *et al.*, 2000.).

There are thought to be three possible ways by which this pRb/E2F mediated repression can occur. Firstly that pRb directly blocks the E2F transcriptional activation domain through binding, or secondly that pRb indirectly prevents E2F from contacting the basal transcription machinery (Dyson, 1998). Thirdly, pocket proteins are able to repress transcription through the binding and recruitment of histone deacetylases, which deacetylate histones, thereby promoting chromatin condensation that in turn prevents transcription factors from contacting DNA and therefore, transcription from occurring (Ferreira *et al.,* 1998, Luo *et al.,* 1998).

Different types of E2F complexes are thought to exist during the cell cycle (Figure 1.2). Repressive complexes, where a pocket protein is bound to a heterodimer in its hypophosphorylated form, thus actively preventing transcription. Inhibitory complexes, in which a pocket protein is bound to a heterodimer, but is preventing, as opposed to actively repressing transcription. Active complexes, where the pocket protein is hyperphosphorylated and the heterodimer is free to activate transcription

Figure 1.2 E2F complexes during the cell cycle

Inhibitory complexes occur when pocket proteins physically block the E2F *trans*activation domain and repressive complexes result when the pocket protein is recruited to the DNA. Activating complexes occur when the pocket protein becomes phosphorylated by CDKS and loses affinity for the heterodimer. Inactive complexes exist when the DP partner becomes phosphorylated and the heterodimer loses affinity for the DNA.


Inhibitory complex
pp
E2F
Inactive complex
E2F
P
Actively repressing complex
pp
E2F
M
G2
G1
S
Active complex
P
P
P
P
P
pp
E2F

(Dyson 1998), and inactive complexes where the DP partner becomes phosphorylated by cellular kinases, and prevents the heterodimer from binding DNA (Figure 1.2) (Bandara *et al.,* 1994).

Regulation of E2F transcription is controlled primarily through phosphorylation of the pocket protein member, and the importance of this regulation is highlighted by the fact that single point mutations in E2F-1 which abolish pRb binding lead to enhanced S phase entry, and development of tumours (Shan *et al.,* 1996; Conner *et al.,* 2000). pRb phosphorylation during the cell cycle is initiated by cyclin D/cdk4 and maintained by cyclin E/cdk2 (Muller and Helin, 2000). Only hypophosphorylated pRb is able to bind to E2F, and in this situation the E2F-pocket protein complex is repressive and no transcription is able to occur. However, when pRb becomes phosphorylated by cellular cyclin-cdk complexes at the G1 stage of the cell cycle, the E2F heterodimer is released and is able to activate transcription.

The phosphorylation of pRb is a highly orchestrated process and corresponds with an increase in the expression of cyclins and an up-regulation of cyclin dependent kinase activity (Hatakeyama and Weinberg, 1994; Lundberg and Weinberg, 1998). pRb is phosphorylated on a number of potential S/T-P motifs throughout its sequence, and the *in vivo* phosphorylation of pRb by individual cyclin/cdk complexes is important for the cellular function of pRb. Cell cycle progression and loss of pRb repressive function involves the sequential activation of cyclin D/cdk4, cyclinE/cdk2 and cyclinA/cdk2 complexes during late G1 and S phases (Hatakeyama and Weinberg, 1994; Lundberg and Weinberg, 1998; Sherr and Roberts, 1999). Attempts have been

made to elucidate the mechanism of pRb phosphorylation and studies on the cumulative effect of phosphorylation on pRb have shown that disruption of transcriptional repression results from an accumulation of phosphate groups on the S/T residues in the pRb molecule and that no one particular site is responsible (Brown *et al.,* 1999).

Recently, the mechanism by which Rb acts to repress transcription has been analysed in an *in vitro* transcription assay, using order of assembly experiments (Ross *et al.*, 2001). Data presented suggested that active pRb dependent repression (independent of E2F binding) was observed on a chromatin template but not on a naked templete, and that this transcription occured independently of histone deacetylases. These experiments also showed that pRb mediated repression was able to occur after assembly of the transcriptional pre-initiation complex, in contrast with E2F inhibition by pRb which is thought to occur before assembly of the pre-initiation complex (PIC) (Ross *et al.,* 2001). These data suggest discrete mechanisms by which pRb is able to directly inhibit an activator such as E2F or actively repress other transcription factors which are bound to the DNA. pRb also represses transcription by recruiting histone methyltransferase enzymes to DNA (Ferreira *et al.*, 2001), however the mechanism by which these act has not yet been fully elucidated.

c-mos is a protein kinase which is able to transform cells and disrupt E2F-p130 complexes (Afshari *et al.*, 1997). Loss of these E2F-p130 complexes may be an important step in allowing alteration of cell cycle control and conferring a growth advantage to these cells (Afshari *et al.*, 1997).

The importance of the pocket protein-E2F regulation is highlighted by the fact that viral oncoproteins such as adenovirus E1A, SV40 large T and papilloma virus E7 protein are able to block the Rb/E2F interaction, through binding to the pocket region of pRb, therefore allowing un-regulated proliferation to occur (Bandara *et al.*, 1991; Zamanian and La Thangue, 1992 ; Dyson, 1998).

1.1.7. Upstream Regulators.

Progression through the cell cycle is dependent on phosphorylation of key regulatory proteins by cyclin-dependent kinases (CDKs), which are themselves regulated by subunits known as cyclins. Whilst the levels of cyclin proteins oscillate throughout the cell cycle, regulated through the actions of cell cycle regulated transcription and proteolysis, the levels of CDKs are relatively stable (Lees, 1995). Cyclin/cdk complexes are regulated by the cyclin dependent kinase inhibitors (CDIs) that sequester CDKs to halt cell cycle progression in response to a variety of signals. Signalling pathways activate the p53 transcription factor, in response to stress and up-regulate its downstream CDI targets such as $p21^{CIP1/WAF1}$. CKIs include two distinct families, the INK4 (INhibitors of CDK4) family, that specifically inhibit certain cdk/cyclin species and consists of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$, and the CIP/KIP family whose three members $p21^{CIP1/WAF1}$, $p27^{KIP1}$ and $p57^{KIP2}$ inhibit the activity of all the CDKs via binding to the cyclin fold (Roussel,1999).

The G1 regulator pRb, is regulated by a number of upstream cyclins/CDK complexes and CDIs, and cell cycle progression normally occurs when pRb is inactivated by

phosphorylation, catalyzed by a CDK in complex with their cyclin partner. Most of the studies of pRb phosphorylation have relied on overexpresion of cyclins and/or CDKS, so it is still unclear which cyclin-cdks normally phosphorylate pRb *in vivo*. However, it is thought that cyclin D/cdk4/6 phosphorylates pRb early in G1, cyclin E/cdk2 phosphorylates the protein near the end of G1, and cyclin A/cdk2 may maintain phosphorylation of pRb during S-phase (Harbour and Dean, 2000). *In vitro* studies have shown that E2F recruits the pre-initiation complex factor, TFIID to gene promoters, and that pRb blocks this E2F mediated recruitment of the pre-initiation complex (Ross *et al.*, 1999).

1.1.8. Importance of E2F

The importance of the E2F pathway is highlighted by the fact that it is so frequently de-regulated in transformed cell lines and human cancers. Although E2F itself is not frequently mutated, mutations in pRb, cyclin D, CDK4 and $p16^{INK4a}$ are frequent in a range of human tumours (Sherr, 1996). The pRb gene *rb1* is mutated in approximately 30% of human cancers, and the p16 (INK4a) gene is methylated, deleted or mutated in a majority of gliomas, leukaemias and melanomas (Kamb *et al.*, 1994). The *cyclin D1* locus is amplified in a wide range of tumours, including lymphomas, squamous cell tumours and breast carcinomas (Pines, 1995).

The E2F family members themselves are a multi-functional group of proteins, which are involved in gene activation, repression, cell cycle proliferation or cell death and are able to inhibit tumorigenesis. E2F also has important roles in cells, which are not

actively proliferating, indicating a wider role than just regulator of the restriction point (Dyson, 1998). The dual function of E2F as both a tumour suppressor and oncogene is particularly interesting, and may be tissue specific, or dependent on as yet undefined extrinsic factors (McLeod,1999). Depending on the tissue studied, loss of E2F-1 has opposite consequences in terms of regulation of proliferation and apoptosis, and in the same organ, loss and gain of E2F function can have similar consequences (Muller and Helin, 2000).

1.1.9 Transcriptional regulation

Transcription is the process by which DNA is transcribed into RNA by one of three cellular RNA polymerases. RNA polymerase I synthesises large ribosomal RNA (Paule and White, 2000), RNA polymerase II transcribes messenger RNA (Hirose and Manley, 2000) and RNA polymerase III transcribes tRNA and some ribosomal RNAs (Paule and White, 2000). As RNA polymerase II mediates the transcription of mRNA, which is then translated into proteins, it is important to understand Pol II mediated transcription in order for us to know how the transcription of cellular genes is regulated.

The promoters of protein encoding genes consist of an initiator element, where transcriptional initiation occurs and frequently a down-stream TATA box (so called for the TATA nucleotide sequence) (Figure 1.3). Additionally promoters may contain other DNA elements, referred to as enhancer elements, which mediate binding of DNA binding transcription factors, or sites for DNA site-specific transcription factors

to bind to. Promoter DNA can be packaged into inactive chromatin and in this situation transcription can be prevented through DNA binding sites being inaccessible to transcription factors.

The basal, or general transcription factors (GTFs) are typically defined as the minimal proteins necessary to reconstitute accurate transcription from a minimal promoter. Basal transcription is mediated by GTFs, which associate with RNA polymerase on the DNA to form the PIC, and activate basal transcription (Roeder, 1996) (Figure 1.3). In the case of Pol II transcription, these transcription factors are referred to as TFIIA-TFIIH and have a number of properties ranging from stabilisation of the TBP-DNA complex to phosphatase and helicase activities (Liu, *et al.*, 1999; Vandel and Kouzarides., 1999).

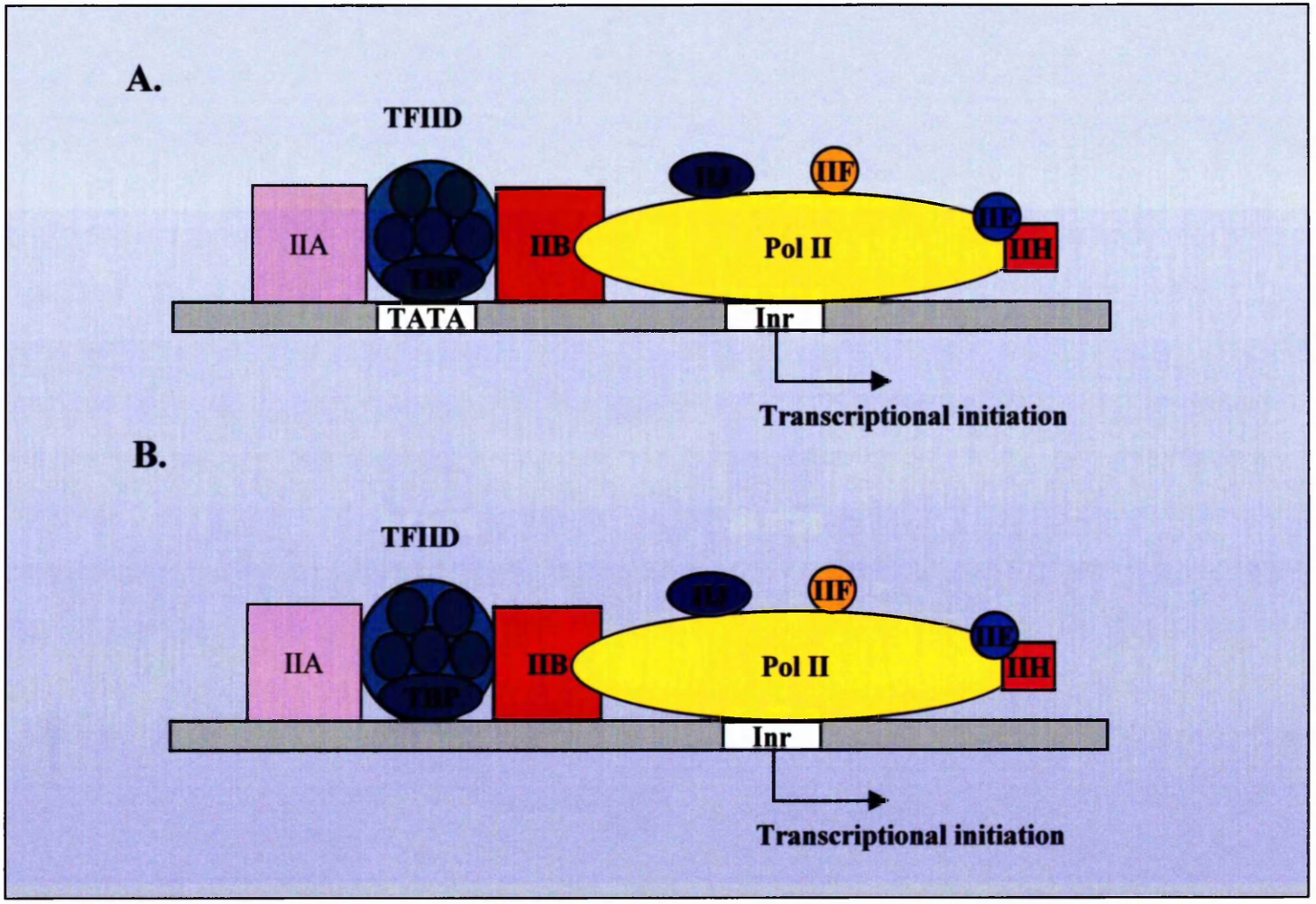
TATA Binding Protein (TBP) is the universal transcription factor which is involved in transcription of RNA pol I, II and III genes (Comai, *et al.*, 1994). TBP binds to the TATA box in TATA driven promoters (Figure 1.3A). The transcription factors SL1, TFIID and TFIIID are composed of TBP and a number of TBP associated factors (TAFS), and different promoters have different requirements for transcription by TBP or the TBP containing complexes (Comai *et al.*, 1994). Recently it has been shown that acetylation of core histones can facilitate the binding of TBP to a nucleosomal TATA sequence (Sewalk *et al.*, 2001). Positioning of a nucleosome over the TATA region appears to be a common mechanism for repressing basal transcription, and these data show that one consequence of induced histone

Figure 1.3. The Pre-initiation Complex

Assembly of the pre-initiation complex on TATA and TATA less promoters.

(A) The pre-initiation complex forms after recruitment of TBP to the TATA box. The general transcription factors assemble on the promoter of RNA pol II responsive genes. The general transcription factors assemble step-wise on the promoter.

(B) The pre-initaiation complex also forms on TATA less genes although in this situation Pol II is thought to bind prior to the general transcription factors


A.
TFIID
IIA
TBP
IIB
IIF
IIE
Pol II
IIH
TATA
Inr
Transcriptional initiation
B.
TFIID
IIA
TBP
IIB
IIF
IIE
Pol II
IIH
Inr
Transcriptional initiation

acetylation at a native promoter is the alleviation of nucleosome mediated repression of the binding of TBP (Sewal *et al.*, 2001).

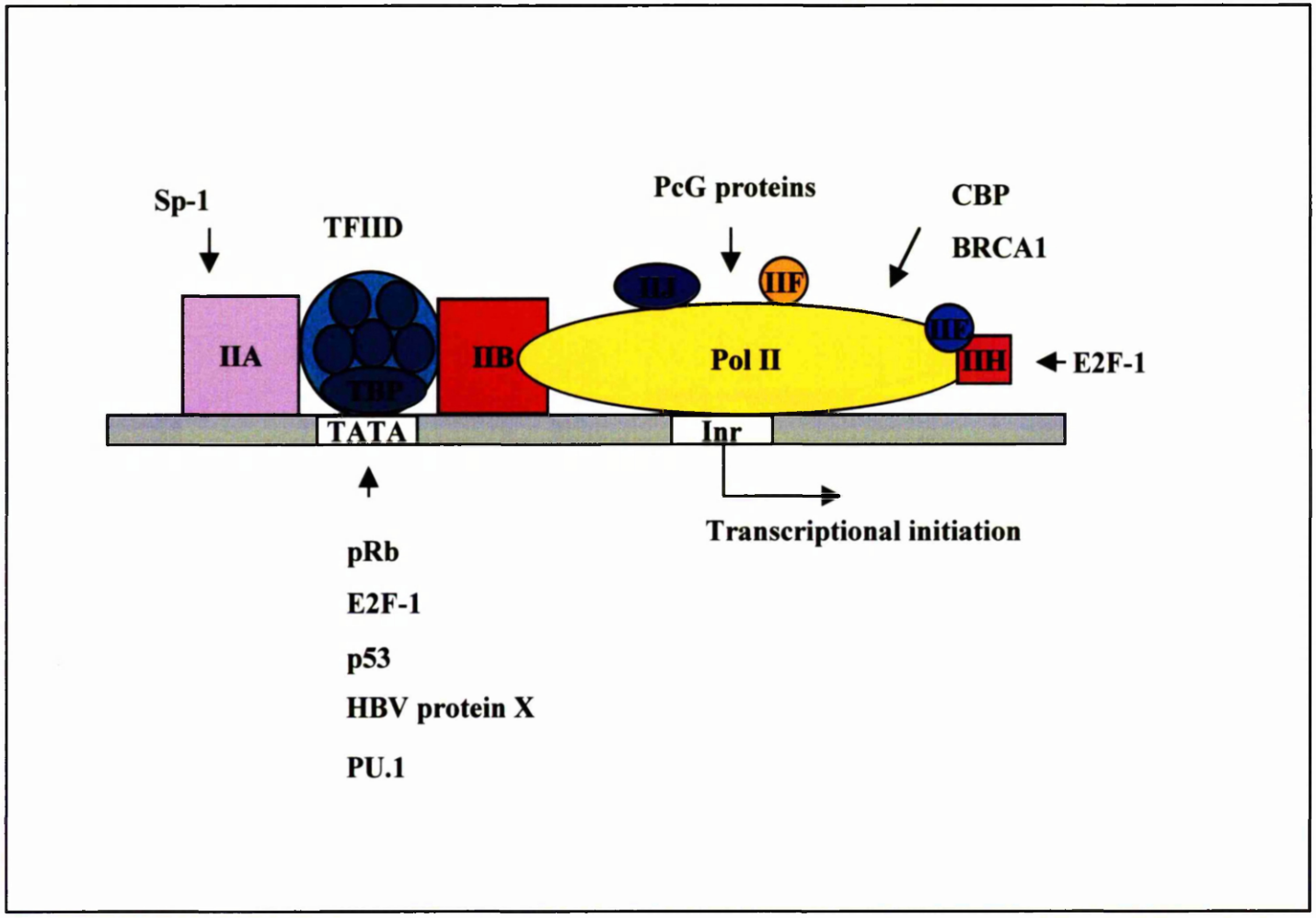
Initiation of mRNA synthesis by RNA pol II depends on the controlled and co-ordinated activities of a large number of proteins (Figure 1.4). RNA polymerase II and the general transcription factors assemble into a pre-initiation complex at the core promoter elements of pol II regulated genes (Roeder, 1996) (Figure 1.3). TFIID consists of the TATA binding protein TBP, and a number of other TBP associated factors, and TFIID plays a central role in transcriptional initiation as it binds specifically to the TATA box and nucleates PIC assembly.

The multisub-unit transcription factor TFIID is an essential component of the RNA polymerase II machinery that works in concert with TFIIA and TFIIB to assemble initiation complexes at eukaryotic promoters (Andel *et al.*, 1999). As the pre-initiation complex forms, TBP binds to the TATA box in TATA containing promoters, and then sequentially the GTFs and RNA polymerase bind to the DNA, and the downstream initiator element (Figure 1.3). Non TATA containing promoters also exist and in these cases the PIC is thought to assemble from RNA Pol II binding at the initiator element (Fig 1.3B).

The crystal structure of the IID-IIA-IIB complex shows that TFIID is a trilobed, horseshoe structure with TFIIA and TFIIB bound on opposite lobes and flanking a central cavity. TBP is also shown to be in between TFIIA and TFIIB in this crystal structure (Andel *et al.*, 1999). Studies in yeast have shown that activator dependent

Figure 1.4 Pre-initiation Complex Associated Factors

Many proteins bind and regulate components of the pre-initiation complex. The assembled PIC is represented and transcription factors which bind to the PIC are indicated by arrows pointing to the appropriate binding partners (Chen *et al.*, 1993; Hagemeier *et al.*, 1993; Quadri *et al.*, 1995; Scully *et al.*, 1997; De Luca *et al.*, 1998; Vandel and Kouzarides, 1999).


Sp-1
TFIID
PcG proteins
CBP
BRCA1
IIA
TBP
IIB
IIJ
IIF
Pol II
IIE
IIH
E2F-1
TATA
Inr
Transcriptional initiation
pRb
E2F-1
p53
HBV protein X
PU.1

alterations of chromatin structure occur and that the association of TBP with promoters is stimulated by other, transcriptional activators (Kuras and Struhl., 1999). The general transcription factors play varying roles in transcriptional initiation. TFIIA binds to TBP and is thought to be involved in stabilisation of the TBP-TATA complex (Lui *et al.*, 1999). TFIIH has been shown to have both phosphatase and helicase activity (Vandel and Kouzarides., 1999)

A large number of transcription factors have been identified. Many are DNA binding, site specific transcription factors (Roeder, 1996). The diversity of these transcriptional proteins is important for the regulation of gene expression. As well as these DNA binding factors, other transcriptionally associated proteins exist which bind to transcription factors and cause either repression or activation of target genes. These proteins are termed co-activator or co-repressor proteins.

Many transcription factors bind directly to TBP, including cellular and viral *trans*activators and repressors (Figure 1.4). One such viral protein is the hepatitis B virus transactivator protein X, which activates a number of cellular genes (Qadri *et al.*, 1995). Protein X binds specifically to TBP *in vitro*, and this interaction requires ATP (Qadri *et al.*, 1995). PU.1, a sequence specific transcription factor with an Ets-like DNA-binding domain can bind to both pRb and TFIID (Hagemeier *et al.*, 1993a). These data then showed that sequence similarities exist between the pRb pocket and the general transcription factors TFIID and TFIIB, and the authors discuss the possibility that pRb may carry out functions similar to TFIID and TFIIB (Hagemeier *et al.*, 1993a). The site-specific DNA binding transcription factors E2F-1

(Hagemeier *et al.*, 1993b) and p53 (Chen *et al.*, 1993) also bind directly to TBP, and interestingly E2F-1 has also been shown to interact with another component of the pre-initiation complex TFIIH (Vandel and Kouzarides, 1999). pRb can down-regulate transcription mediated by all three RNA polymerases and can also bind to TBP (De Luca *et al.*, 1998).

Transcription factors can activate and repress transcription or can be bi-functional, for example NC2. NC2 is the *Drosophila* homologue of the transcriptional repressor Dr-1 and can activate DPE-driven (down-stream promoter element) promoters and repress TATA driven promoters (Willy *et al.*, 2000). DNC2 acts at the time of PIC assembly, suggesting that dNC2 may either be a component of the PIC or, may be required for PIC assembly (Willy *et al.*, 2000). These data indicate that dNC2 could be an important target of regulatory factors (for example, sequence-specific transcriptional factors and co-activator proteins) for recruitment to core-promoters. Even-skipped is another *Drosophila* protein which has been shown to repress transcription through binding to TBP and blocking the TFIID-TATA box interaction (Li and Manley, 1998).

Cell-specific and spatially localised patterns of gene expression depend on combinations of sequence-specific activators and repressors that bind to extensive *cis*-regulatory elements. Co-regulatory proteins are thought to be recruited to the DNA template by sequence-specific transcription factors (Mannervik, *et al.*, 1999). Co-activator proteins and complexes can link upstream activators to the core RNA polymerase II complex (Mannervik *et al.*, 1999). CBP is a component of large

protein complexes containing additional histone acetyltransferases, including p/CAF (p300/CBP associated factor) and steroid receptor co-activators. CBP itself may have dual roles in chromatin decondensation and Pol II recruitment and has been shown to interact with a sub-unit of RNA pol II (Mannervik *et al.*, 1999) (Figure 1.4).

Polycomb group proteins (PcG) act as repressive multiprotein complexes that may render target genes inaccessible to the transcriptional machinery, inhibit chromatin remodelling, influence chromosome domain topology and recruit histone deacetylases (Breiling *et al.*, 2001). PcG proteins have been found to bind to core promoter regions and have been shown to bind to general transcription factors (GTFs) *in vitro*. Chromatin immunoprecipitation experiments showed that binding of PcG proteins to core promoters included GTFs, and that depletion of PcG proteins led to de-repression of developmentally regulated genes (Breiling *et al.*, 2001). These data suggest that PcG proteins may maintain gene silencing by inhibiting GTF-mediated activation of transcription.

1.1.10 The role of E2F as a transcription factor.

All of the E2F and DP family members have DNA binding activity, and have been shown to bind to promoters which contain the consensus E2F site TTTc/gGCGCg/c (Lees *et al.*, 1993, Buck *et al.*, 1995). Recently, the crystal structure of an E2F-4-DP-2-DNA complex has been resolved (Zheng *et al.*, 1999). Analysis of this complex suggests that protein heterodimerisation is required before DNA binding and hence this paper increases our insight into the mechanism of DNA recognition by E2F.

Although E2F and DP are able to form homodimers that bind weakly to DNA, the heterodimer binds DNA much more efficiently (Krek *et al.*, 1993; Bandara *et al.*, 1994). *In vitro* studies have shown that different heterodimer complexes preferentially bind specific E2F sequences within the promoters of E2F regulated genes, implying that the sequence within a promoter may specify its role in transcriptional regulation (Tao *et al.*, 1997). These authors showed that E2F, DP and pRb proteins are all capable of influencing the choice of promoter sites, an observation which suggests that architectural attributes of the heterodimer/pocket protein complexes play a role in the regulation of E2F dependent promoters. Although these studies give us an interesting insight into potential mechanisms underlying the diverse effects of different E2Fs, further work is required to determine the relationship to promoter activation by individual E2Fs.

Much of the data on E2F transcription compiled to date relies on *in vitro* experiments, which may not exactly mimic the situation *in vivo*. Presently however, our ability to look at physiological promoter situations is restricted by the techniques available. One technique, which does allow us to look at these physiological situations, is described by Takahashi *et al* (2000). In this study, the authors used a cross-linking approach in synchronised living cells in order to examine the E2F and pocket protein presence on promoters (Takahashi *et al.*, 2000).

It was found that promoter repression in quiescent cells is associated with p130-E2F-4 recruitment and low histone acetylation, while in late G1 E2F-1 and E2F-3

replaced E2F-4 and histones became acetylated (Takahashi *et al.*, 2000). The authors propose a model in which E2F-4 may function on certain promoters as a dedicated repressor in G0 and G1, then in late G1 and S-phase E2F-4 is replaced by E2Fs 1 and 3. In this situation, inhibition of one E2F may lead on to binding of another and individual E2Fs may play a role in distinguishing the timing of gene expression (Takahashi *et al.*, 2000).

E2F has been shown to be important for the regulation of a number of genes involved in cell cycle control (*cyclin A*, *e2f1*,), DNA replication (*dhfr*, *replication protein a* (Kalma *et al.*, 2001)) and apoptosis ($p14^{ARF}$ (Bates *et al.*, 1998), *apaf-1* (Moroni *et al.*, 2001)) (Table 1.1, adapted from Helin 1998). More recent technology, exploring the use of micro arrays has revealed a large number of genes to be regulated by E2Fs1-3 (Muller *et al.*, 2001). Bioinformatics has also been used as a tool to identify new E2F target genes; a computer program which identified binding sites for members of the E2F family was used to find the promoters of genes containing an E2F binding site (Kel *et al.*, 2001). The genes so far identified are implicated in a wide range of cellular processes including differentiation and development, and these studies further highlight the broadening importance of the E2F pathway in cell cycle control and determination of cell fate.

Formation of the E2F-DP heterodimer is important for transcription, as E2F-1 and DP-1 together are able to *trans*activate exogenous reporter genes much more efficiently than E2F-1 alone (Krek *et al.*, 1993; Bandara *et al.*, 1994). It is widely accepted that the E2F family members 1-5 have overlapping abilities to form

Table 1.1 Genes regulated by E2F.

Table showing some genes involved in cell cycle control and DNA synthesis which are regulated by E2F binding to sites within their promoter regions. Adapted from Helin (1998), and including data from:(Ohtani et al., 1995, Schulze et al., 1995, Irwin et al., 2000, Muller 2001)). Recent data from Moroni *et al.*,(2001) increases greatly the number and variety of genes regulated by E2F.

Gene	Role in Cell Cycle
cyclin A, *cyclin E*, *cyclin D*	Act with a cdk partner to phosphorylate other cellular proteins. eg, pRb, DP-1
e2f-1, *e2f-2*, *e2f-3*, *e2f-4*, *e2f-5*	Family of DNA binding, site specific transcription factors. Have roles in cellular proliferation
DNA polymerase α, *thymidine kinase*, *dihydrofolate reductase*	Involved in DNA replication
PCNA	Proliferating Cell Nuclear Antigen
p21$^{WAF1/CIP1}$	Cyclin dependant kinase inhibitor (CDI)
p14/p19^{ARF}	Regulators of tumour suppressor pathways
topoisomerase1	DNA supercoiling
mcm	Involved in DNA synthesis
cdc6, *cdc2*, *cdk2*, *cdc25A*, *cdc25C*	Mediate phosphorylation of cell cycle proteins in partnership with cyclins
orc-1	Involved in DNA replication
p107, *pRb*	Pocket protein family members. Regulators of E2F transcription.
c-myc, *N-myc*, *B-myb*	Involved in cell proliferation
DP-1	Heterodimeric partner of E2F.
p73	Member of the p53 family of transcription factors. Involved in apoptosis.
apaf-1	Apoptosis causing factor

activating complexes and repressive complexes, depending on cellular conditions. Further support for this comes from studies in *Drosophila melanogaster*, where identification of dE2F2 showed that dE2F1 acts as an activator while dE2F2 acts as a repressor (Frolov *et al.*, 2001). An interesting *in vitro* study however has shown that an E2F-4/DP-1 complex which is repressing one consensus promoter site in an *in vitro* CASTing assay, a repetitive immuno-precipitation and PCR procedure, is able to activate on another, different E2F site. This suggests that the DNA composition of promoter sites rather than transcription factors can determine whether activation or repression occurs (Tao *et al.*, 1997).

A novel E2F site has been identified in the cyclin E promoter, termed CREM (Cyclin E Repressor Module). It consists of a variant E2F binding site and upstream A/T rich region. This promoter region is able to delay the expression of cyclin E until G1 phase by preferentially binding a high molecular weight complex called, CERC, (Cyclin E Repressor Complex) consisting of E2F, DP, a pocket protein and an as yet unidentified number of low molecular weight components (Le Cam *et al.*, 1999). CERC2 has recently been identified as binding to this region and having a TSA-sensitive histone deacetylase activity (Polanowska *et al.*, 2001). These data indicate that the cell-cycle dependent control of the cyclin E promoter is embroiled in acetylation pathways via the CREM-like E2F element (Polanowska *et al.*, 2001).

Interestingly, it has been shown that E2F-1 levels are upregulated in response to UV and γ-irradiation, and that in these cases the E2F-1 DNA binding activity is up-regulated (Hofferer *et al.*, 1999). This indicates a role for E2F-1 in the DNA damage

pathway, particularly as it is involved in transactivating many genes which are required for DNA replication and repair, such as *dhfr, mcm* and *thymidine kinase* (Helin 1998).

Methylation is a process by which methyl groups are attached to cytosine residues in DNA. The promoter regions of many cancer-associated genes are methylated and transcriptionally silenced (Baylin and Herman, 2000). Studies on synthetic DNA segments have shown that none of the E2F family members are able to bind, or *trans*activate, the methylated *dhfr*, *e2f-1* and *cdc2* promoters, while all family members except E2F-1 are able to bind and *trans*activate the *c-myc* and *c-myb* promoters (Campanero *et al.,* 2000). These data suggest a role for methylation in the regulation of the transcription of E2F target genes by E2F-1.

1.1.11. E2F Co-activators.

Many molecules have been identified which act as co-activators, proteins which enhance the transcriptional activity of some transcription factors by bridging their interactions with other transcriptional components. E2F-1 transcription, and apoptosis can be enhanced by the co-activator protein p300 (Lee *et al.,* 1998). A p300 binding protein involved in nucleosome assembly (NAP) has also been shown to augment E2F-1 transcriptional activity, possibly through its interaction with p300 (Shikama *et al.,* 2000). These data suggest that E2F may exist in a large multi-protein complex within the cell and regulate transcription through a variety of different mechanisms.

1.1.12. Co-operation

Another DNA binding site specific transcription factor, Sp-1 has been shown to interact directly with E2F-1 and together E2F-1 and Sp-1 act synergistically in activation of *dhfr* gene transcription (Lin *et al.,* 1996). Interestingly the cdk inhibitor $p21^{CIP1/WAF1}$, a protein which blocks CDK activities upstream of pRb, to keep pRb in its hypophosphorylated form and preventing the release of transcriptionally active E2F has been shown to regulate E2F-1 in a non-pRb dependent fashion. $p21^{CIP1/WAF1}$ is able to bind directly to both E2F-1 and DP-1 and this binding abrogates the transcriptionally repressive effect of $p21^{CIP1/WAF1}$ both in a reporter assay system in cells and in an *in vitro* transcription assay (Delavaine and La Thangue, 1999).

1.1.13. Regulators of E2F activity.

Recently some cellular proteins other than members of the pocket protein family have been identified that suppress E2F-1 transcriptional activity. Pur α, a sequence specific single stranded DNA binding protein which has been implicated in both transcriptional regulation and DNA replication has been shown to bind to E2F-1 and decrease its binding to DNA and the transcriptional activation of the *dhfr* promoter (Darbinian *et al.,* 1999). NPCD-1 a neural factor involved in the control of proliferation and differentiation binds to the hetero-dimerisation domain of both E2F-1 and DP-1 and is able to also reduce DNA binding and transcriptional activation of E2F target genes, through disruption of the heterodimeric complex (Sansal *et al.,*

2000). This suggests that the role of NPCD-1 in proliferation and differentiation may be through the E2F interaction.

Another protein which has been described as being able to down-regulate E2F transcription is p202. p202 is an interferon inducible murine protein which can bind to pRb, and whose over-expression inhibits proliferation (Choubey *et al.*, 1996). p202 can inhibit E2F stimulated transcription of both exogenous reporter genes and endogenous genes, in a pRb independent manner (Choubey *et al.*, 1996).

Recently, E2F-1 activity has been shown to be inhibited by the Rep78 protein of Adeno-associated virus (AAV), a virus which inhibits the transforming potentials of DNA tumour viruses (Batchu *et al.*, 2001). Rep78 binds to E2F-1 to stabilise the pRb-E2F-1 complex, and also binds directly to the E2F-1 promoter and down-regulates adenovirus induced transcription (Batchu *et al.*, 2001). This paper outlines an important molecular mechanism for the anti-oncogenic property of AAV Rep78.

1.1.14 E2F in Proliferation

Knock-out mouse models have provided us with an insight into the importance of the individual E2F family members in proliferation. It has been shown through a variety of studies that some E2F family members are essential for development and cell cycle regulation, while others are dispensable for these but still exhibit some defects. Over-expression of E2F-1 promotes cell cycle progression and S phase entry, and quiescent cells can be driven into S phase by the introduction of E2F-1 (Johnson *et*

al., 1993). Over-expression of cyclinE-cdk2 can also induce S-phase when over-expressed in quiescent cells and together E2F-1 and cyclin E/cdk2 can collaborate to induce S-phase progression (Leone *et al.*, 1999). Interestingly, in this study, inhibition of cyclinE-cdk2 blocks S-phase progression in normal, but not in $rb^{-/-}$ cells implying an important role for cyclinE/cdk2 in the regulation of cell cycle control. In contrast to these observations, over-expression of dominant-negative DP-1, which retains E2F binding but lacks DNA binding activity, creates inactive E2F-1 complexes, which are unable to bind DNA and can cause cell cycle arrest in G1 (Wu *et al.*, 1996). Mitogen stimulated cells deprived of E2F activity can still maintain physiologically relevant levels of cyclin E protein, through ectopic expression of the transcription factor Myc (Santoni-Rugiu *et al.*, 2000). These data indicate the involvement of a parallel pathway to E2F/Rb in the G1-S-phase transition, and implies that cyclin E is particularly important for S phase progression.

Studies on *Drosophila* have further highlighted the importance of E2F, DP and Cyclin E in proliferation. dDP and dE2F are necessary for viability and mutations cause lethality at late larval/pupal stage (Royzman *et al.*, 1997). Mutations in dDP and dE2F distinguish G1-S progression from an associated transcriptional program and show that dcyclin E is essential for S phase late in *Drosophila* development (Royzman *et al.*, 1997). Mutations in dE2F can compromise DNA replication by eliminating transcriptional activation of several essential replication factors at G1-S phase (Duronio *et al.*, 1998). Mutations in dDP1 however, cause a block of cyclin E transcription indicating that, while lack of certain replication factors as a result of

mutations in either dE2F or dDP can cause graded effects on replication, cyclin E is required for an "all or nothing" S phase transition (Duronio *et al.*, 1998).

Studies on transgenic mice that over-express E2F-1 have shown that E2F-1 over-expression leads to a lack of hair growth, probably due to hyper-proliferation in the epidermis (Peirce *et al.*, 1998b). Over-expression of E2F-1 has also been implicated in liver dysplasia (Conner *et al.*, 2000).

Surprisingly, the E2F-1 knockout mouse is viable and fertile, although these mice do develop a broad and unusual range of tumours, and experience testicular atrophy and endocrine gland dysplasia (Yamasaki *et al.*, 1996). These observations highlight the role of E2F-1 as a tumour suppressor and also suggest a role for E2F-1 in the development of some tissue lineages. The fact that E2F-1 mice are viable suggests that some of its roles can be compensated for by other family members. Indeed, in *e2f-1*$^{-/-}$ mouse embryonic fibroblasts a delayed S-phase is observed, suggesting an important, but not unique role for E2F-1 in progression through the restriction point. (Wang *et al.*, 1998). E2F-1 has also been shown to have oncogenic properties (Johnson *et al.*, 1994), and together with DP-1 is able to co-operate with activated Ras to form transformed foci and tumours in nude mice.

Embryos mutant for both E2F-1 and pRb demonstrate a significant suppression of apoptosis and impaired S phase entry (Tsai *et al.*, 1998). *rb*$^{-/-}$ embryos die at E14.5 and mutation of *e2f-1* in these animals resulted in an extended survival (Tsai *et al.*, 1998). Loss of E2F-1 in *rb*$^{+/-}$ mice leads to a reduction in tumours and increased life-

span as a result of E2F-1 interference with the apoptosis observed in the *rb*$^{-/+}$ animals (Yamasaki *et al.*, 1998). These data indicate that E2F-1 regulation is not the sole function of pRb in development, and that mutation of *e2f-1* is not sufficient to rescue the lethality caused by the *rb*$^{-/-}$ mutation (Tsai *et al.*, 1998; Yamasaki *et al.*, 1998).

Interestingly, E2F-3 has been shown to be critical for full cell viability. *e2f-3*$^{-/-}$ MEFs demonstrate a proliferation defect, and a down-regulation of the expression of E2F responsive genes. However, the loss of E2F-3 does not affect the regulation of other family members and re-introduction of exogenous E2F-3 or over-expression of E2F-1 is able to compensate for this loss (Humbert *et al.*, 2000a). Both E2F-1 and E2F-3 activity is cell cycle regulated, mostly disappearing by G2. E2F-3 activity has been shown to be required for S-phase entry, as well as E2F-1 and immuno-depletion experiments have shown that lack of E2F-3 inhibits S-phase entry in proliferating cells. More recent studies demonstrated that E2F-3 is able to partially alleviate some of the defects in *rb*$^{-/-}$ embryos, including inappropriate proliferation in the developing lens and CNS. It has been shown that *rb*$^{-/-}$;*e2f3*$^{-/-}$ mutant mice are more viable than *rb*$^{-/-}$, although noticeably they are smaller and embryonic lethal, probably due to cardiac failure (Zeibold *et al.*, 2001). Interestingly, these embryos showed the presence of the developmental defects which had previously been observed in viable late stage *rb*$^{-/-}$;*e2f1*$^{-/-}$ embryos (Tsai *et al.*, 1998).

More recent studies have centred on the roles of E2F-4 and E2F-5 in proliferation. E2F-4 is the most abundant member of the family found in cultured cells and E2F-4 knock out mice die within the first few weeks of life. The offspring from these

animals suffer from a number of developmental defects including; haematopoietic lineage development and defects in gut epithelium development (Rempel *et al.*, 2000). Also, a defect in erythrocyte maturation, craniofacial defects and an increased susceptibility to bacterial infections, perhaps as a direct result of these developmental defects, is observed (Humbert *et al.*, 2000b). The E2F-5 knock-out mouse embryos develop normally but new-born mice develop nonobstructive hydrocephalus (Lindeman *et al.*, 1998). These mice excrete excessive cerebrospinal fluid and cell cycle kinetics were not found to be disturbed in MEFs from these animals. This indicates a role for E2F-5 in the secretory behaviour of a differentiated neural tissue (Lindeman *et al.*, 1998).

The double knock out *e2f-4*$^{-/-}$;*e2f-5*$^{-/-}$ mouse is embryonic lethal, suggesting an overlapping essential role for the two proteins in development. MEFs from these mice are able to proliferate normally, but are unable to undergo cell cycle arrest in response to $p16^{INK4a}$. Introduction of exogenous E2F-4 or E2F-5 into these cells is able to reintroduce the p16 response, however E2F-1 cannot compensate for loss of E2F4 and 5, indicating that the two sub-families have different functions (Gaubatz *et al.*, 2000). These data suggest that E2F-4 and 5 make an essential contribution to pocket protein dependant G1 arrest, and together with the E2F-1 and E2F-3 results further support the model of different roles for the E2F family members in development. The DP-1 knockout mouse has also been shown to be embryonic lethal, possibly due to a lack of proliferation in developing cells (Yamasaki., 2001).

Although the main role for E2F is thought to be in regulation of the G1-S phase transition, E2F has also been identified as being involved in the co-ordination and timing of S-phase entry. E2F activity during S phase was shown to be required for the accumulation of the mitotic regulatory cyclin, cyclinB, and also to keep the anaphase promoting complex (APC) molecule cdh1 phosphorylated in order to prevent degradation of important mitotic regulatory factors (Lukas., *et al.* 1999). The APC is a multi-protein complex, which has ubiquitin ligase activity and is essential for mitotic cyclin proteolysis (Lukas *et al.*, 1999).

1.1.15 The Role of E2F in Apoptosis.

Apoptosis is the process of programmed cell death. Cells are able to self-destruct in response to specific signals from the cellular proteins. In response to specific, intracellular signals, nuclear condensation occurs, followed by fragmentation and destruction of nuclear DNA, loss of cell volume and membrane integrity (Bates and Vousden 1999). Two main apoptotic pathways have been identified, and a variety of diverse extra and intra-cellular signals have been shown to be able to regulate cell death (Figure 1.5).

The first pathway is mediated by death receptors and occurs as a response to ligand binding to receptors, such as members of the TNFR family. TNF receptors bind ligands such as TNFα, and activation of these receptors leads to a number of responses including apoptosis and induction of anti-apoptotic signals such as NF-κB (Phillips *et al.*, 1999). Ligand binding stimulates recruitment of a group of proteases

called caspases, which act as signal transducers and death effectors (Guo and Hay, 1999). Pro-caspases cleave down-stream caspases, and caspases are thought to have a major role in cleaving cellular substrates that lead to cell death (Guo and Hay, 1999).

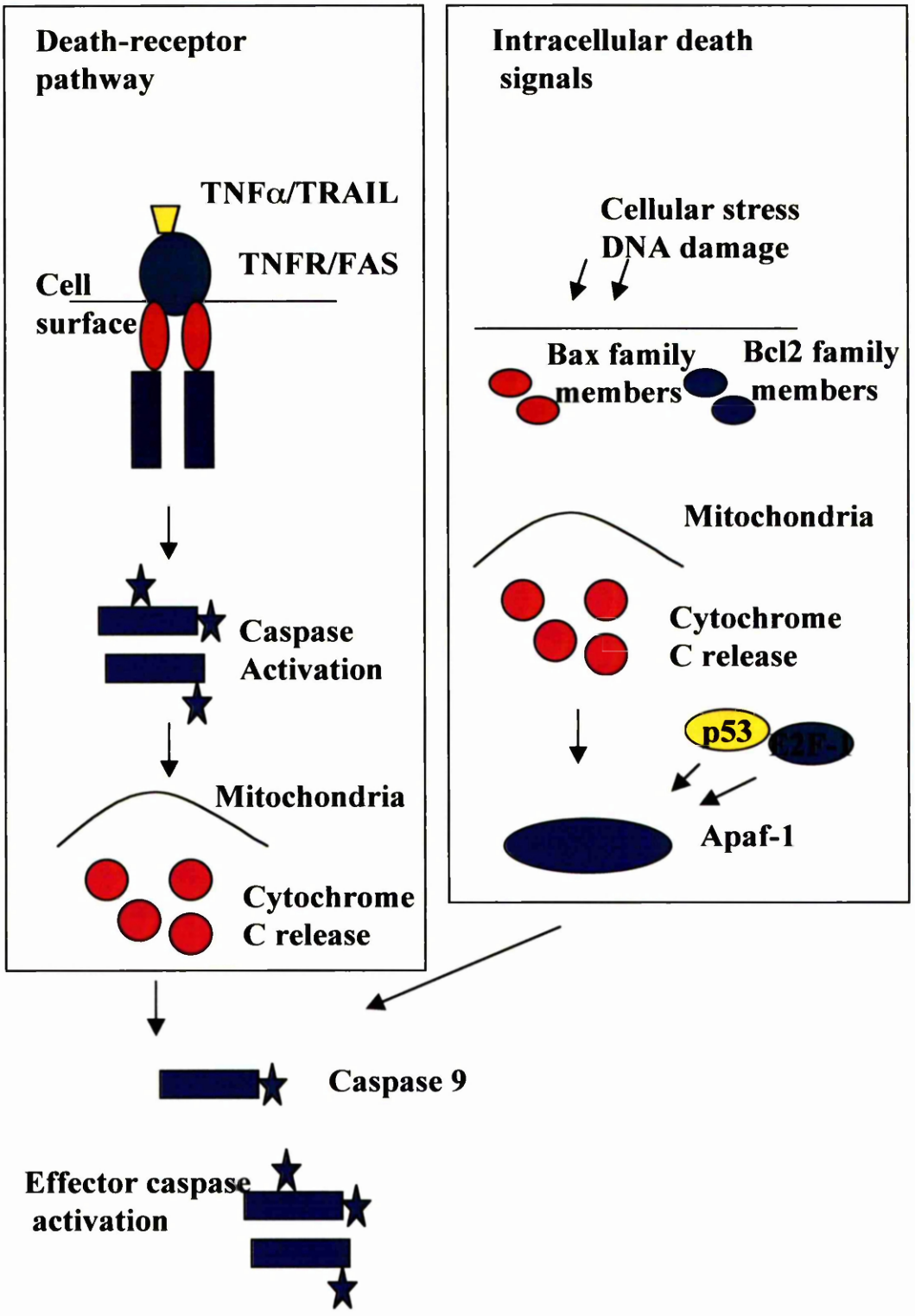
The second apoptotic pathway works through different caspase molecules, and a protein termed Apaf-1, which can bind and activate caspase-9 in a cytochrome C dependant manner. Cytochrome C is released from the mitochondria in response to intracellular signals and can be regulated by the Bax/Bcl2 family of apoptotic factors that includes Bax, Bcl2 and Bid (Bates and Vousden, 1999; Ming and Hay, 1999) (Figure 1.5).

Cellular growth and development involves competing pathways of proliferation and apoptosis. The key to effective tumour therapies may well be found through understanding the link, and switch between apoptosis and cellular proliferation. Transcription factors such as p53 and c-myc are inducers of apoptosis (Ming and Hay, 1999), particularly p53 which has been shown to activate the downstream apoptosis promoting factor Bax. Interestingly the p53 apoptotic effect can be overcome by over-expression of its negative regulator, MDM2 (Chen *et al.,* 1996).

The E2F family of transcription factors are well characterised regulators of cellular proliferation and have also been found to have important roles in apoptosis. Induction of E2F-1 in quiescent fibroblasts leads to S-phase entry followed by apoptosis (Qin *et al.,* 1994). The importance of E2F-1 in apoptosis is highlighted by the observation that *e2f-1*$^{-/-}$ mice exhibit a defect in T lymphocyte development due to

Figure 1.5. Apoptosis pathways.

Two main apoptotic pathways have been identified, and a variety of diverse extra and intra-cellular signals have been shown to be able to regulate cell death. In the death receptor pathway cell surface receptors are activated by ligand binding and caspases become activated. This leads to cytochrome C release and the subsequent activation of effector caspases which instigate apoptosis. Intracellular death signals also lead to release of cytochrome C through activation of the Bax and Bcl2 family members and the apoptotic factor Apaf-1.


Death-receptor pathway
TNFα/TRAIL
TNFR/FAS
Cell surface
Caspase Activation
Mitochondria
Cytochrome C release
Intracellular death signals
Cellular stress DNA damage
Bax family members
Bcl2 family members
Mitochondria
Cytochrome C release
p53
Apaf-1
Caspase 9
Effector caspase activation

a maturation stage specific defect in thymocyte apoptosis (Field *et al.,* 1996). Tumour cell proliferation has also been shown to be impaired in the absence of E2F-1, suggesting that E2F-1 is required for tumour development (Pan *et al.*, 1998).

E2F-1 promotes apoptosis in both p53 dependent and independent manners (Wu and Levine, 1994; Lee, 1998). Initially, various studies concluded that E2F-1 is the only family member with this apoptotic inducing activity (DeGregori *et al.,* 1997, Wang *et al.,* 2000), however it has been shown that E2F-4 is also able to induce apoptosis through a caspase dependant mechanism, independently of E2F-1 (Chang *et al.,* 2000). Recently, E2F-2 and E2F-3 have also been described as having some apoptotic properties (Moroni *et al.,* 2001; Zeibold *et al.,* 2001).

The ability of E2F-1 to induce apoptosis has been shown to be separable from it's ability to promote DNA synthesis. In a study which shows that the DNA binding domain, but not the *trans*activation domain of E2F-1 is required for apoptosis (Hsieh *et al.,* 1997; Phillips *et al.*, 1997). Analysis of E2F mutants indicates that although DNA-binding is required, transcriptional activation is not necessary for the induction of apoptosis by E2F-1, suggesting that it may be mediated through alleviation of E2F-dependent transcriptional repression (Phillips *et al.*, 1997).

This E2F-1 induced apoptosis can be overcome by expression of Bcl2, an anti-apoptotic factor, suggesting that in part, the caspase dependent pathway may be involved. Interestingly, it has been shown that over-expression of pRb is also able to overcome E2F-1 mediated apoptosis. However this is probably not due to direct

suppression of the E2F *trans*activation domain, due to the TAD not being required for the apoptotic phenotype (Hsieh *et al.,* 1997, Phillips *et al.,* 1997). MDM2, the oncogene that regulates p53 activity, also binds to E2F-1 (Martin *et al* .,1995) and inhibits the apoptosis induced by E2F-1 (Loughran and La Thangue, 2000), indicating a role for MDM2 as a regulator of the E2F pathway.

An increase in the levels of p53 protein has been observed in response to apoptotic induction by E2F-1 (Kowalik *et al.,* 1998). This information, together with the observation that a deficiency of E2F-1 leads to a decrease in the amount of p53 dependent apoptosis, indicates that E2F-1 may act upstream of p53 in the apoptotic response (Pan *et al.,* 1998). It has also been suggested that the induction of the $p14/19^{ARF}$ tumour suppressor is important for stabilisation of p53 and it's ability to induce apoptosis (Irwin *et al.,* 2000). E2F-1 has been shown to induce apoptosis through a death receptor mechanism, through the inhibition of anti-apoptotic pathways, partially due to the down-regulation of TRAF2 (Philips *et al.,* 1999). This down-regulation of TRAF2 is due to increased protein degradation in response to E2F activity, and this TRAF2 down-regulation inhibits normal induction of anti-apoptotic signals such as NF-κB (Phillips *et al.*, 1999).

More recent studies have highlighted a role for p73, the p53 homologue, in E2F-1 mediated apoptosis (Irwin *et al.,* 2000, Lissy *et al.,* 2000). E2F-1 is able to *trans*activate p73, and induce apoptosis in the absence of p53. TCR-activation-induced cell death, an apoptotic response in peripheral T cells is blocked by expression of a dominant negative E2F-1, or p73 protein and from this the authors

conclude that TCR-activation-induced cell death is dependent on both E2F-1 and p73 activities (Lissy *et al.*, 2001).

Interestingly, recent studies have identified a role for E2F-3 in apoptosis. $rb^{-/-};e2f3^{-/-}$ embryos, show a rescue of the apoptosis found in $rb^{-/-}$ embryos, suggesting that endogenous E2F-3 makes a major contribution to the apoptosis resulting from the loss of the pRb tumour suppressor (Zeibold *et al.*, 2001). It may be possible that E2F-3 acts as an upstream regulator of E2F-1, contributing indirectly to the induction of apoptosis *in vivo*. However, the effect of E2F-1 is much less than that of E2F-3. It may be more likely that both E2F-1 and E2F-3 contribute to the activation of apoptosis, or that the two family members have overlapping functions.

Recently, a model has been proposed by Moroni *et al.*, (2001) for E2F mediated p53 independent apoptosis. In this study the authors show that the Apaf-1 gene is a direct transcriptional target of E2F (Moroni *et al.*, 2001). E2F-1 is able to preferentially activate Apaf-1 over E2F-2 or E2F-3 and this provides a direct link between deregulation of the Rb pathway and apoptosis (Moroni *et al.*, 2001). This model proposes a mechanism by which E2F-1 directly up-regulates $p14^{ARF}$, p73 and Apaf-1, leading to transcriptional activation of Bax via p53 and p73 and at the same time, accumulation of Apaf-1 and subsequent release of cytochrome C (Moroni *et al.*, 2001).

1.1.16 Post-translational modifications.

Most cellular proteins are regulated by a combination of a number of post-translational mechanisms including, phosphorylation, acetylation, methylation and ubiquitination. It is of particular importance that cell cycle protein levels are tightly regulated to allow for specific cellular transitions and to establish a base for the next round of the cell cycle. Many of these cellular mechanisms are important for regulation of the E2Fs, particularly E2F-1, which has been shown to undergo a number of post-translational modifications.

1.1.17 Cell Cycle Regulation.

E2F activity is regulated by cell-cycle dependent changes in sub-cellular localisation of the E2F and DP sub-units (Verona *et al.,* 1997). In cycling cells the majority of E2F-p107, E2F-p130 and free E2F complexes are found in the cytoplasm, while E2F-pRb complexes are found mainly in the nucleus. These nuclear E2F-pRb complexes are found to be high in G1 and then decrease as the cell cycle progresses. The induction of S-phase entry by E2F family members is dependent on their nuclear localisation (Muller *et al.,* 1997). Using chimeric proteins it was shown that the N-terminus of E2F-1 was sufficient to induce nuclear accumulation and S-phase entry. Under these conditions chimeric E2F-4 was able to behave as E2F-1 (Muller *et al.*, 1997). The NLS in E2Fs 1,2 and 3 is important for their nuclear accumulation, however E2F-4 and E2F-5 depend on the DP or pocket proteins to bind in the cytoplasm and take them into the nucleus (de la Luna *et al.,* 1996). E2F-1 binds

MDM2 and localises to the nucleus. However MDM2 targeted degradation of the E2F and DP sub-units probably occurs in the cytoplasm (Loughran and La Thangue, 2000).

1.1.18 Phosphorylation.

The Rb protein and E2F are both regulated by various signalling cascades, including the cdk, stress induced kinases JNK-1 and p38 kinase (Wang *et al.,* 1999). JNK-1 inhibits E2F-1 activity by phosphorylation and reducing its DNA binding affinity while divergently, p38 reverses pRb repression of E2F through pRb phosphorylation (Wang *et al.*, 1999). E2F-1 activity is regulated by the direct binding of cyclinA/cdk2 to the N terminus of E2F-1 (Krek *et al.*, 1994; Xu *et al.,* 1994). This cyclinA/cdk2 phosphorylation occurs *in vitro* and *in vivo* and inhibits the DNA binding activity of the E2F-1/DP-1 heterodimer. Interestingly phosphorylation of DP-1 by cyclin A bound to E2F-1 also results in the elimination of E2F DNA binding. The importance of this phosphorylation by cyclin A is highlighted by the fact that disruption of cyclin A-E2F binding resulted in an S-phase delay followed by either regrowth or apoptosis (Krek *et al*, 1995), indicating an important role for E2F-1 phosphorylation in S phase entry. Recent studies have shown that E2F-1 is selectively induced in response to DNA damage and this induction is mediated by ATM-dependent phosphorylation (Lin *et al.*, 2001). The amino terminus of E2F-1 contains a site for ATM/ATR phosphorylation and mutational studies showed that this site is required for the observed DNA-damaged induced apoptosis in mouse thymocytes (Lin *et al.*, 2001).

Studies in yeast showed that phosphorylation of E2F-1 is important for it's interactions with other proteins, including pRb (Fagan *et al.,* 1994). Phosphopeptide mapping experiments show that E2F-1 is phosphorylated by a number of as yet unidentified kinases, and it is likely that E2F is a focal point for multiple signalling pathways that affect growth and cell cycle regulated transcription (McLeod, 1999).

E2F-5 has also been shown to be phosphorylated, by the cyclinE/cdk2 complex (Morris *et al* ., 2000). This phosphorylation was also shown to stimulate its binding to the co-activator protein p300. As E2F-5 is known to *trans*activate the cyclin E gene, this indicates an auto-regulatory mechanism by which E2F transcription switches on cyclin E expression and in turn this phosphorylates E2F-5 to augment transcriptional activation.

1.1.19 Acetylation.

E2F-1 has been shown to be acetylated both *in vitro* and *in vivo*, by the acetyltransferase p300, and much more efficiently by the p300/CBP associated enzyme P/CAF (Martinez-Balbas *et al.,* 2000). The acetylation of lysine residues within E2F-1 was shown to increase E2F-1 DNA binding, transcriptional activation and protein stability, although the observed increase in *trans*activation could be a result of either increased DNA binding or protein stability (Martinez-Balbas *et al.,* 2000). The acetylated residues have been mapped mainly to the DNA binding domain and although are not directly involved in binding may allow a conformational change in the E2F/DNA complex, which would allow increased

*trans*activation. Interestingly, the acetylated residues are only conserved in the 'activating' E2F family members suggesting that this novel regulatory mechanism may have physiological implications for activation of E2F target genes (Martinez-Balbas *et al.,* 2000).

Acetylation has recently been identified as a new type of modification and level of control in pRb function (Chan *et al.*, 2001). Adenovirus E1A, which binds p300/CBP through an amino-terminal transformation-sensitive domain, stimulates the acetylation of pRb by recruiting p300 and pRb into a multi-protein complex (Chan *et al.*, 2001). Furthermore, the acetylation of pRb is cell cycle regulated and hinders the phosphorylation of pRb by cyclin dependent kinases. These data define a new level of cell cycle control mediated by HAT activity and identifies a new cellular target for acetylation (Chan *et al.*, 2001).

1.1.20 Degradation

E2F-1 and E2F-4 have been observed to be unstable proteins and studies have shown that they are degraded by the ubiquitin-proteasome pathway (Hateboer *et al.,* 1996). E2F stability is mediated by a C terminal region close to the pocket protein binding domain, and E2F bound pocket protein complexes are found to be stable. Interestingly, the E1A virus transforming protein E6 also increases stability of the E2F, a result which would not have been expected as these proteins disrupt the E2F-pocket protein interaction (Hateboer *et al.,* 1996). Viral proteins may have evolved

this mechanism to ensure S-phase entry and replication of the virus infected cell, while simultaneously preventing apoptosis.

Further studies have identified the SCF/SKP2 complex as being specifically involved in the cell-cycle dependent destruction of E2F-1. E2F-1 is rapidly degraded in late S/G2 phase, around the same time as the SCF component $p45^{SKP2}$ accumulates (Marti *et al.*,1999). Binding of $p45^{SKP2}$ to the N terminal of E2F-1, in a region distinct from cyclin A binding was required for ubiquitination and targetting for degradation and implies a model in which the SCF complex regulates the destruction of E2F-1 towards the end of S-phase while phosphorylation of E2F-1 by cyclin A is still required to regulate the duration of S-phase (Marti *et al.*, 1999).

Both the E2F-1 and DP-1 sub units have also been shown to be down-regulated by the MDM2 protein (Loughran and La Thangue 2000). MDM2 has been described as having E3-ligase activity, suggesting that multiple E3-ligases may be involved in degradation of the E2F components (Honda *et al.*, 1997). Interestingly recent work has shown E2F-1, 2 and 3, levels are also down-regulated by over-expression of the $p19^{ARF}$ tumour suppressor protein (Martelli *et al.*, 2001), indicating a broader range of regulation involving regulatory feedback loops and cross-talk between pathways. DP-1 sub-units which are unable to bind E2F end up poly-ubiquitinated in the cytoplasm, and degradation of these peptides is required for cell cycle progression, indicating that DP-1 is probably also targeted for degradation *in vivo* (Magae *et al.*, 1999).

1.2 The p53 Pathway

1.2.1 Introduction

A key regulator of cellular growth is the tumour suppressor p53. The gross over-expression of mutated forms of p53 witnessed in a variety of tumours regardless of the transforming agent and cell type underlies the vital importance of p53 in genome stability (Rotter *et al.*, 1981; Rotter *et al.*, 1983). The p53 pathway is frequently targeted for de-regulation in human cancer and p53 itself is the most commonly mutated gene in tumour cells (Friend, 1994). The high frequency of p53 mutations observed in human tumours clearly helps define p53 as the "guardian of the genome" (Friend, 1994)

1.2.2 p53

p53 is a sequence specific transcription factor that forms tetramers and binds to DNA (Fields and Jang 1990). p53 has the ability to both activate and repress the target promoters of a large number of cellular genes (Budde and Grummt, 1998; Murphy *et al.*, 1999; Yu *et al.*, 1999; Woods and Vousden, 2001). Many of the genes targeted by p53 are cell cycle arrest, DNA repair or apoptotic associated genes (Momand *et al.*, 2000 ; Woods and Vousden, 2001). Normally, p53 levels in the cell are low, however in response to most stress situations such as DNA damage, or heat shock, p53 levels in the cell increase (Momand *et al.*, 2000). This increase in p53 levels is due to a combination of an increase in p53 translation and its extended half-life (Momand *et al.*, 2000).

p53 is a negative regulator of cellular proliferation and transformation. p53 is a phosphorylated protein and can be targeted by many cellular kinases, however phosphorylation of p53 is not essential for DNA-damage induced stabilisation of p53 (Ashcroft *et al.*, 1999). DNA damage leads to the stabilisation of p53, increased transcriptional activity, followed by cell cycle arrest through induction of the CDKIs such as $p21^{WAF1/CIP1}$ and DNA repair (Prives 1998). When DNA damage is beyond repair, p53 initiates apoptosis through activation of apoptotic inducing genes, including *apaf-1* (Moroni *et al.*, 2001) and *Bax* (Miyashita *et al.*, 1994).

p53 is a nuclear protein and contains three nuclear localisation signals in the C-terminal region. Mutation of these signals leads to p53 accumulation in the cytoplasm and loss of p53 growth-inhibitory activities (Woods and Vousden, 2001). p53 also contains a nuclear export signal within its tetramerization domain (Stommel *et al.*, 1999). Mutation of residues in the NES prevents p53 export and hampered tetramer formation (Stommel *et al.*, 1999).

1.2.3 MDM2

MDM2 was first identified as an amplified gene in a transformed mouse 3T3 cell line (Cahilly-Snyder *et al.,* 1987). Since then it has been shown that the human form of MDM2 is overexpressed in a number of human tumours, particularly sarcomas, leukaemias, breast carcinomas and malignant gliomas (Ladanyi, *et al.,* 1993; Leach *et al.,* 1993; Sheikh *et al.,* 1993; Cordon-Cardo *et al.,* 1994). MDM2 has been shown

to exist as alternatively spliced transcripts in tumour cells (Sigalis *et al.*, 1996). These alternatively spliced *mdm2* proteins show loss of p53 binding, consistent with partial deletion of sequences encoding the p53 binding domain (Sigalas *et al.*, 1996).

A high proportion of transgenic mice overexpressing MDM2 develop breast tumours implying that deregulation of MDM2 is important for oncogenesis (Lundgren *et al.*, 1997). MDM2 binds to and regulates a number of cell cycle regulatory proteins. Of particular interest, the transcriptional co-activator p300 (Grossman *et al.*, 1998), pRb (Xiao *et al.*, 1995) and $p14^{ARF}$ (Quelle *et al.*, 1995).

MDMX is a homologue of MDM2, and is structurally almost identical (Jackson and Berberich, 2000). However, MDMX cannot substitute for MDM2 in early embryonic development (Shvarts *et al.*, 1996). MDMX can form stable heterodimers with MDM2 through its ring finger domain and is also able to associate with p53 (Tanimura *et al.*, 1999). Although MDMX is able to associate with p53, it is unable to facilitate nuclear export or induce p53 degradation (Jackson and Berberich, 2000). However, expression of MDMX can reverse MDM2 mediated degradation of p53 whilst maintaining suppression of p53 *trans*activation (Jackson and Berberich, 2000).

1.2.4 The MDM2-p53 Feedback loop

MDM2 is involved in a negative feedback loop with p53 (Wu *et al.*, 1993). p53 upregulates MDM2 expression and in turn, MDM2 can export p53 from the nucleus, and target it for ubiquitin mediated degradation (Haupt, *et al.*, 1997; Honda *et al.*

1997). MDM2 binds directly to the p53 *trans*activation domain and downregulates p53 dependent transcription and apoptosis (Haines *et al.*, 1994). The functional relationship between MDM2 and p53 is dramatically illustrated by the finding that p53-null mice can completely rescue the early embryonic-lethal phenotype of MDM2 deficiency (Jones *et al.*, 1995), demonstrating that the p53-MDM2 pathway is essential in development.

1.2.5 p300-MDM2

Control of p53 turnover is critical to p53 function. Adenovirus E1A binding to p300/CBP translates into enhanced p53 stability, implying that these co-activator proteins normally operate in p53 turnover control (Grossman *et al.*, 1998). Most endogenous MDM2 is bound to p300 in the cell, suggesting that at least one MDM2 function operates on, or with p300 and specific interactions of both p53 and MDM2 with the CH-1 region of p300 are important steps in p53 turnover (Grossman *et al.*, 1998). Elements of the p300 N terminal region, including the CH-1 region are homologous to HPV E6 protein, suggesting a role for p300-MDM2 complexes in p53 degradation. Furthermore, these data taken together highlight a role for specific p300/CBP, CH-1, p53 and MDM2 interactions in the MDM2 mediated control of p53 abundance (Grossman *et al.*, 1998). p300 also acts in an MDM2 negative feedback loop to regulate p53, and is required for *mdm2* induction by p53 and the subsequent inhibition of p53 stabilisation (Thomas and White, 1998). This p300 dependent stabilisation of p53 also results in apoptosis (Thomas and White, 1998), hence, p300 regulation of *mdm2* expression controls the apoptotic activity of p53.

1.2.6 The pRb and p53 relationship

There are numerous points of interaction between p53 and pRb mediated tumour suppression, some of which are mediated by E2F. Co-operation between the p53 and pRb pathways has been amply demonstrated. Classic examples involve the oncoproteins encoded by the DNA tumour viruses, which cancel pRb function to drive cells into S-phase and neutralise p53 to prevent host cell suicide. pRb loss induces E2F and p53 dependent apoptosis (Sherr 1998). pRb can regulate the stability and the apoptotic function of p53 via MDM2, and a pRb-MDM2-p53 complex can exist in cells (Hsieh *et al.*, 1999). pRb specifically rescues the p53 apoptotic function but not the transcriptional activity of p53, so transactivation by p53 is not required for the apoptotic function of p53 (Hsieh *et al.*, 1999).The recent observation that both E2F and p53 can independently activate apaf-1, shows a further degree of cross talk between the two pathways (Moroni *et al.*, 2001)

1.3 The INK4a/ARF Tumour Suppressor.

1.3.1 Introduction

The INK4a/ARF locus is one of the most frequently mutated genes in human cancer, irrespective of the tumour type (Sharpless and DePhino, 1998). This unique genetic locus encodes two major tumour suppressor proteins, $p16^{INK4a}$, which is an inhibitor of cyclin D-dependent kinases and so feeds into the pRb/E2F pathway, and $p14/p19^{ARF}$, which regulates the p53 pathway through inhibition or regulation of MDM2 (Chin *et al.*, 1998). As loss of cell cycle control through inactivation of the pRb and p53 pathways appears to be a vital step in the development of cancer cells, this places the INK4a/ARF locus and its two gene products at the nexus of these two growth controlling pathways.

1.3.2 The INK4a/ARF locus.

The INK4a/ARF locus is located at position p21 on the short arm of chromosome 9 in humans (9p21), and the cognate loci on chromosome 4 in mouse and 5 in rat (Sharpless and DePhino, 1998; Stott *et al.*, 1998). The locus extends over approximately 20Kb and the promoters for the two gene products are widely spaced (Robertson and Jones, 1998). The 9p21 position is a chromosomal hot spot, frequently subject to deletions and rearrangements in a wide range of human cancers, including T cell acute lymphoblastic leukaemia, mesothelioma, bladder and nasopharyngeal carcinomas (Gardie *et al.*, 1998; Yang *et al.*, 2000).

The *ink4a*$^{-/-}$/*arf*$^{-/-}$ knock out mice exhibit a cancer prone phenotype, developing spontaneous tumours and have high sensitivity to carcinogenic treatments. Fibroblasts from these mice proliferate rapidly, have high colony forming efficiency and can be transformed by introduction of activated Ha-Ras (Serrano *et al.,* 1996). Together, these results establish the INK4a/ARF locus as a *bona fide* tumour suppressor (Chin *et al.,* 1998). Recent papers detailing the phenotype of the p16^{INK4a} knock-out mouse show that these p16$^{INK4a-/-}$ mice are more susceptible to tumours than wild-type littermates (Krimpenfort *et al.*, 2001; Sharpless *et al.*, 2001). However, the observed phenotype is mild compared to that of the *ink4a*$^{-/-}$/*arf*$^{-/-}$ locus knock out mice, indicating that although p16^{INK4a} is a tumour suppressor gene, the strong phenotypes observed in the double knock-out mice was probably due to loss of *arf* (Krimpenfort *et al.*, 2001).

1.3.3 Genomic Organisation of the INK4A/ARF Locus

The INK4a/ARF locus has an interesting genomic organisation, in that two proteins are encoded in distinct reading frames within a common coding sequence. This is a common feature in bacteria and viruses but exceedingly rare in eukaryotes. Two transcripts, which are driven by distinct promoters that encode two functionally separate tumour suppressor proteins, are expressed from the INK4a/ARF locus, p16^{INK4a} and p14/19^{ARF} (Quelle *et al.,* 1995). Within the INK4a/ARF locus are four exons, E1α, E1β, E2 and E3. E1α, E2 and E3 encode p16^{INK4a} and E1β, E2, and E3 encode p14/p19^{ARF}. Splicing of exon 1β to exon 2 allows translation to continue in

the -1 reading frame relative to $p16^{INK4a}$, resulting in the expression of the $p14/p19^{ARF}$ protein (Figure 1.6).

Both the $p16^{INK4a}$ and $p14/p19^{ARF}$ promoters reside within CpG islands, which can be silenced by DNA methylation (Robertson and Jones, 1998). CpG islands are regions rich in the CpG nucleotide which are often associated with genes and are normally kept un-methylated in cells. CpG islands are frequently associated with tumour-derived promoter silencing events, although the presence of Sp1 sites in both the INK4a and ARF promoters may allow the promoters to be retained in an unmethylated form and hence maintain the expression of both proteins under physiological conditions.

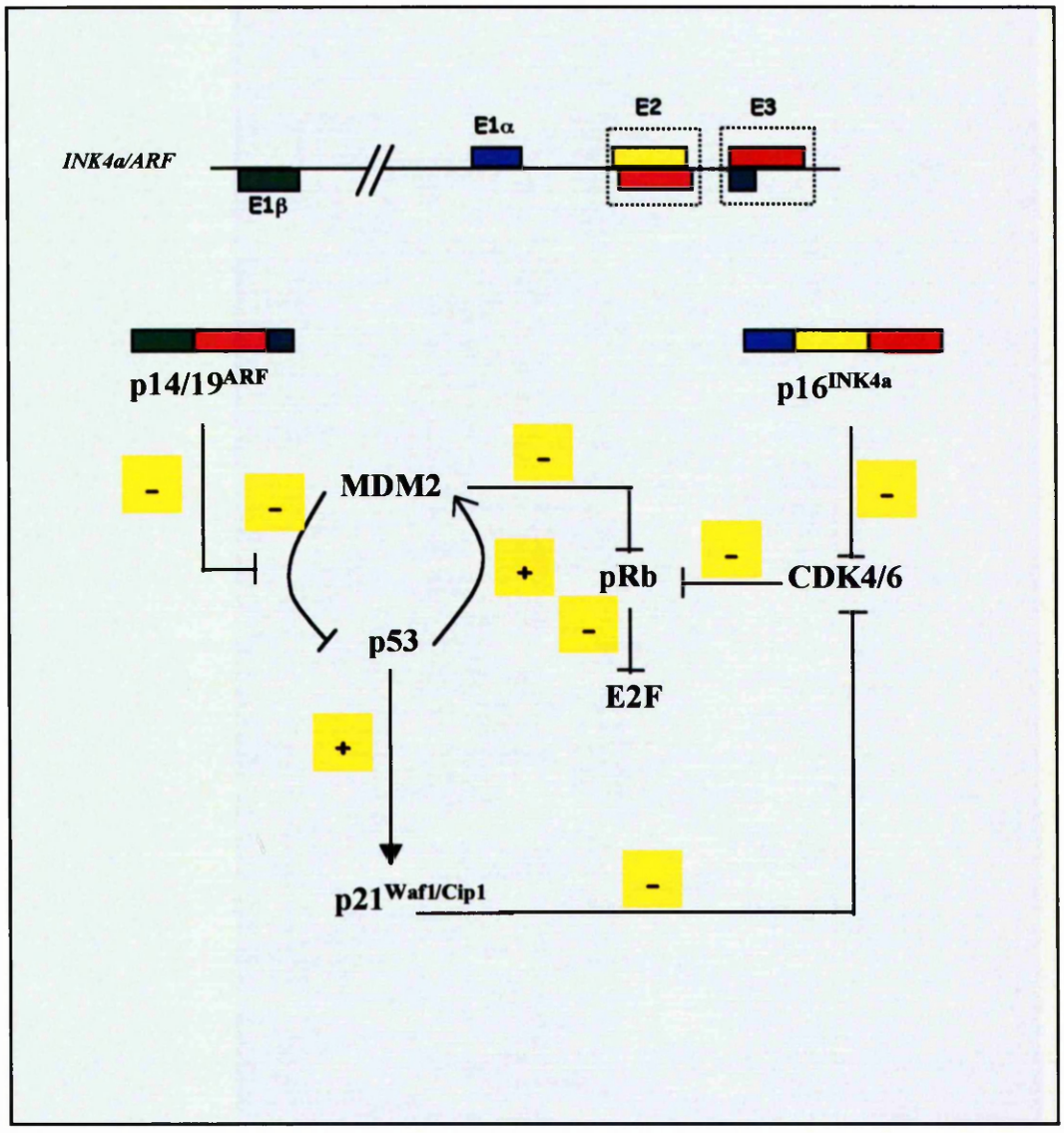
1.3.4 INK4a/ARF Expression

The TATA-less $p14/19^{ARF}$ promoter contains several potential consensus E2F sites, while the $p16^{INK4A}$ promoter contains none. The $p14^{ARF}$ promoter contains at least four potential negative strand E2F binding sites, two of which are high affinity and two are poor matches (Robertson and Jones, 1998). The $p19^{ARF}$ promoter contains two high affinity E2F sites, one coded by the negative strand and one positive strand (Inoue *et al.,* 1999).

E2F is able to induce $p14/p19^{ARF}$ expression, and *trans*activate the $p14^{ARF}$ promoter (Bates *et al.,* 1998; Robertson and Jones., 1998; Inoue *et al.,* 1999). Over-expression of E2F-1 induces $p14^{ARF}$ mRNA expression in a non cell cycle regulated manner

Figure 1.6 The INK4a/ARF locus

Schematic diagram of the INK4a/ARF locus and the pathways its two gene products feed into. $p14/19^{ARF}$ targets p53 through its interaction with MDM2, while $p16^{INK4a}$ inhibits the activity of cdks. Other cellular proteins involved in the INK4a/ARF pathways are shown.


INK4a/ARF
E1α
E2
E3
E1β
p14/19ARF
p16INK4a
MDM2
pRb
CDK4/6
p53
E2F
p21Waf1/Cip1

(Bates *et al.,* 1998; Inoue *et al.,* 1999). Further studies showed that E2F-1 and E2F-2 are able to increase $p19^{ARF}$ mRNA levels, but not E2F-3, 4 or 5 (DeGregori *et al.,* 1997). The induction of $p14^{ARF}$ mRNA expression by E2F is paralleled by a marked increase in the levels of $p14^{ARF}$ protein (Bates *et al.,* 1998).

Neither $p19^{ARF}$ nor $p16^{INK4a}$ are expressed during mouse embryonic development, but upon culture mouse fibroblasts begin to express both $p16^{INK4a}$ and $p19^{ARF}$. The distinct spacial expression pattern of $p16^{INK4a}$ and $p14/p19^{ARF}$ during both human and mouse development and ageing suggests that the transcriptional regulation of the two products differ. $p16^{INK4A}$ is only found to be expressed in a few tissues while $p19^{ARF}$ is more ubiquitously expressed. (Quelle *et al.*, 1995, Zindy *et al.,* 1998).

In transformation assays, $p16^{INK4a}$ and $p19^{ARF}$ displayed both distinct activity profiles, and additive effects, which suggests that these proteins suppress neoplasia through separable but co-operative mechanisms of action. The action of $p19^{ARF}$ in transformation has been shown to be p53 dependent (Pomerantz *et al.,* 1998). Interestingly, data shows that efficient execution of an apoptotic response depends on the entire INK4a locus function. In knock out mouse lens studies, $rb^{-/-}$ animals display apoptosis, while $rb^{-/-}/ink4a^{-/-}$ animals have abrogation of this response. Since $p16^{INK4a}$ is presumed to be without effect when Rb is absent, the doubly null lenses can be taken as the equivalent of $rb^{-/-};p19^{ARF-/-}$, and so these findings can explain how $p19^{ARF}$ functions as a suppressor of neoplasia, through its capacity to enhance the p53-mediated elimination of inappropriately cycling cells *in vivo* (Pomerantz *et al.,* 1998). In this model, as $p16^{INK4a}$ acts upstream of pRb and ARF responds to

deregulated E2F, this effect most likely reflects an ARF, cell-cycle checkpoint function (Sherr and Weber, 2000).

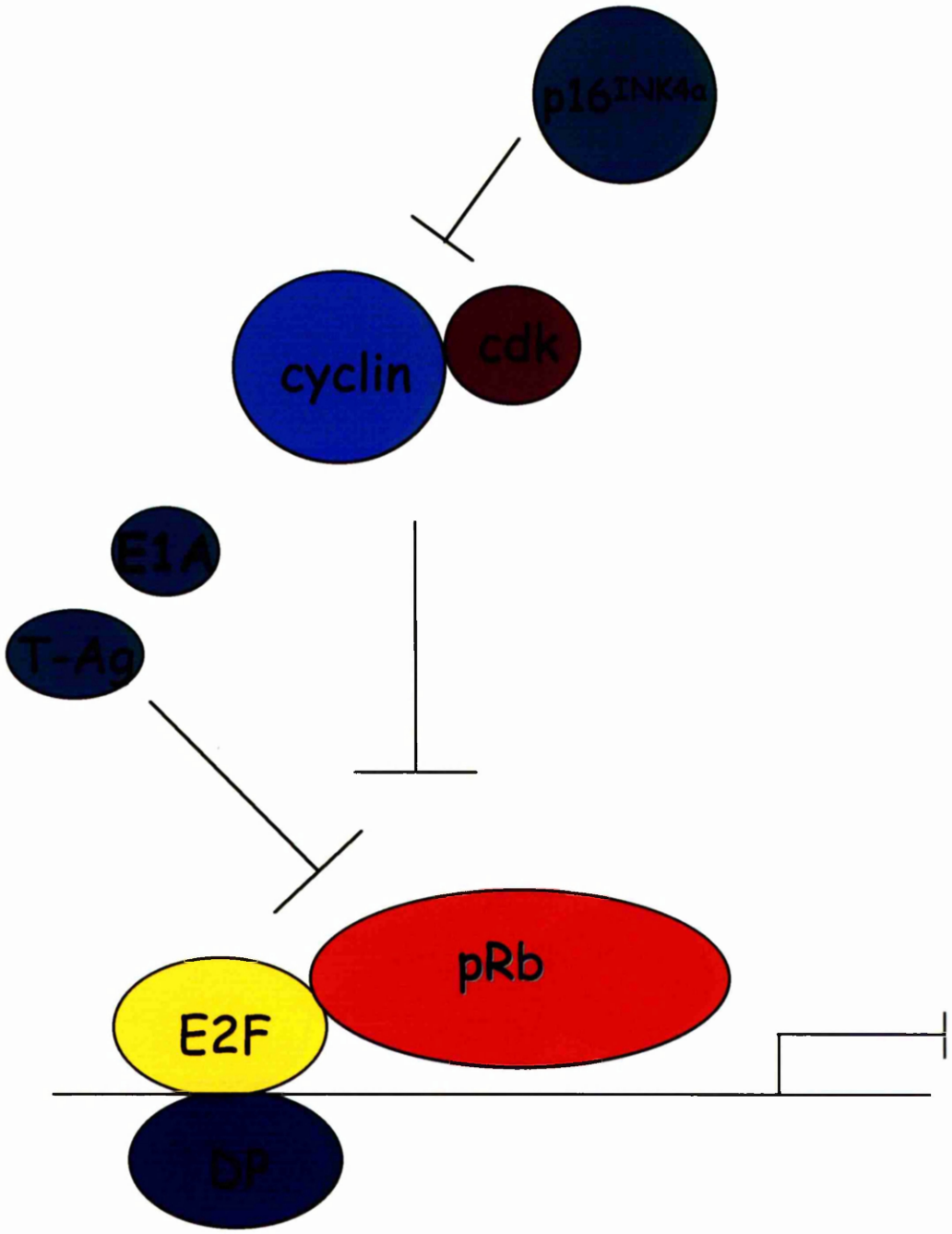
1.3.5 $p16^{INK4a}$

$p16^{INK4a}$ is commonly mutated or deleted in human cancers, particularly in leukemia, melanomas and lymphomas (Ruas and Peters, 1998; Sharpless and DePhino, 1998) $p16^{INK4a}$ was first identified as a CDK associated protein and inhibitor of CDK4 activity (Serrano *et al.,* 1993). Inhibition of CDK4/6 leads to the hypophosphorylation of pRb which in turn represses E2F and blocks G1 progression (Figure. 1.7), and this arrest can be rescued by introduction of exogenous CDK4/6 (Ruas and Peters, 1998). It has recently been shown that $p16^{INK4a}$ cell cycle arrest is dependent on pRb, and either p107 or p130. Mutation of pRb alone is not sufficient for $p16^{INK4a}$-dependent cell cycle arrest (Bruce *et al.,* 2000).

$p16^{INK4a}$ levels do not fluctuate significantly during the cell cycle, and the lack of p16 and pRb mutations found in the same tumour together with the observation that *rb* negative cells display high levels of $p16^{INK4a}$ indicates the lack of a selective advantage in deregulation of two genes in the same pathway (Raus and Peters, 1998). $p16^{INK4a}$ has a role in senescence and its expression profile is up-regulated in senescent cells (Ruas and Peters, 1998). When $p16^{INK4a}$ is induced DNA synthesis is inhibited and cells acquire morphological features of senescence (Dai and Enders, 2000).

Figure 1.7 $p16^{INK4a}$ inhibition of cyclin dependent kinases

Inhibition of cdk activity by p16INK4a leads to E2F transcriptional inhibition through hypophosphorylation of pRb.Viral proteins E1A and SV-40 large T-Antigen can also block the E2F pocket protein interaction.


p16INK4a
cyclin
cdk
E1A
T-Ag
pRb
E2F
DP

1.3.6 p14/p19^{ARF}

p14^{ARF} is a 132 amino acid residue protein in humans, and a 169 amino acid residue protein in mouse, both proteins are highly basic, with no homology to other proteins in the database, have no functional motifs and have only 50% amino acid identity to one another (Quelle *et al.,* 1995; Stott *et al.,* 1998). p14/19^{ARF} induces cell cycle arrest at both the G1 and G2 stages, resulting in a decrease in S phase cells (Quelle *et al.,* 1995; Pomerantz *et al.,* 1998; Zhang *et al.,* 1998; Kurokawa *et al.,* 1999). To date, the major role of p14/19^{ARF} described in the literature is the ability to positively regulate p53 through the oncogene MDM2 (Pomerantz *et al.,* 1998). Although p14/19^{ARF} regulates p53 there is no difference in p19^{ARF} expression when a temperature sensitive p53 cell line is moved to the permissive temperature, suggesting that p53 does not directly regulate p19^{ARF} expression (Kamijo *et al.,* 1997).

Both p14^{ARF} and p19^{ARF} have been shown to bind directly to MDM2 and form a ternary complex with p53 (Pomerantz *et al.,* 1998). Whether or not p14/19^{ARF} is able to bind directly to p53 or not is yet to be elucidated but evidence suggests that p14/p19^{ARF} is unable to bind p53 directly (Stott *et al.,* 1998; Zhang *et al.,* 1998). However, p19^{ARF} has been shown to bind directly to p53 in one study, via the N terminal region of p19^{ARF} (Kamijo *et al.*, 1998). Also, data has been reported in which p19^{ARF} can retard the mobility of a p53-oligonucleotide complex in an electrophoretic mobility shift assay (Sherr, 1998). The interactions between p14^{ARF} and p53 have not yet been fully documented (Sherr and Weber, 2000).

The MDM2-p14/19^{ARF} interaction is mediated by the N terminal region of p14^{ARF} and the C-terminus of MDM2 (Zhang *et al.,* 1998). The p14^{ARF}-MDM2 binding domain has recently been elucidated and is thought to involve a number of regions from p14^{ARF} 1-22, 22-64 and 65-132 (Lohrum *et al.,* 2000) in cells. *In vitro*, however, only a peptide corresponding to the first 20 amino acids of p14^{ARF} binds to MDM2 (Midgley *et al.,* 2000). In p19^{ARF} the two regions from 1-14 and 26-37 are required for binding to the central 210-304 region of MDM2 (Weber *et al.,* 1999).

p14^{ARF} has recently been identified as interacting with topoisomerase I and stimulating its activity (Karayan *et al.*, 2001). Topoisomerase I activity is required for proliferating cells to go through S-phase into mitosis. p14^{ARF} physically associates with topoisomerase I and recombinant p14^{ARF} stimulated the DNA relaxation activity of topo I (Karayan *et al.*, 2001). p14^{ARF} and topoisomerase I were also shown to co-localise in the nucleolus (Karayan *et al.*, 2001). These data suggests that the growth suppression induced by p14^{ARF} may be partially due to its effect on topoisomerase I.

1.3.7 p14/19^{ARF} nucleolar localisation

p14/p19^{ARF} has an interesting cellular localisation in that the protein localises to the nucleolus (Zhang *et al.,* 1998; Lindstrom *et al.,* 2000; Rizos *et al.,* 2000.). This localisation is poorly understood, although nucleolar structures are known sites of rRNA localisation, rRNA synthesis and ribosomal assembly (Scheer and Weisenburger, 1994). Much controversy surrounds the subject of p14^{ARF} nucleolar

localisation. While it has been shown that two arginine rich domains mediate this localisation in $p14^{ARF}$, one in each of the exon1β and exon 2 encoded regions (Quelle *et al.*, 1995; Stott *et al.*, 1998; Lohrum *et al.*, 2000; Rizos *et al.*, 2000), other studies have shown that only the C terminus of $p14^{ARF}$ mediates nucleolar localisation, and that a region from amino acid 83-101 is required for this (Zhang *et al.*, 1999). Evidence which backs up this experimental data is the fact that tumour associated C terminal mutants in $p14/p19^{ARF}$ lose their ability to localise to the nucleolus (Zhang *et al.*,1999).

1.3.8 Role of $p14/19^{ARF}$ in p53 Stability

Overexpression of $p14^{ARF}$ activates a p53 response, and results in a cell cycle arrest (Stott *et al.*, 1998). This effect may be partially due to the ability of $p14/p19^{ARF}$ to promote the degradation of MDM2 (Zhang *et al.*, 1998). Recent experiments have shown that although (in the context of $p14^{ARF}$), most MDM2 in the cell is bound to $p14^{ARF}$, only a small fraction of $p14^{ARF}$ is bound to MDM2, implicating a wider role for $p14^{ARF}$ in cell cycle control (Llanos *et al.*, 2001).

MDM2 normally shuttles between the nucleus and the cytoplasm in order to target p53 for degradation (Roth *et al.*, 1998). Honda *et al.*,(1999) demonstrated that association of $p19^{ARF}$ with MDM2 inhibits the ubiquitin ligase activity of MDM2 for p53, and that the activity of a $p19^{ARF}$-MDM2 complex in a ubiquitination assay is lower than the activity of free MDM2. These data highlight the importance of $p19^{ARF}$ mediated p53 stabilisation.

Co-expression of $p19^{ARF}$ and MDM2 prevents nuclear-cytoplasmic shuttling of MDM2, and as time increases, MDM2 can move to the nucleolus (Tao *et al.,* 1999). Much data supports this MDM2 nucleolar localisation and this relocalisation also occurs following Myc activation and replicative senescence (Weber *et al.,* 1999). MDM2, p53 and $p14/19^{ARF}$ are able to form nuclear bodies, ARF can block nuclear export of p53 and MDM2, or else retain them in the nucleolus. Formation of these nuclear bodies requires both the N and C terminus of $p14^{ARF}$ (Zhang *et al.*, 1999). Studies have suggested that $p19^{ARF}$ contains two nucleolar localisation signals (NrLS) which both contribute to nucleolar localisation, however, MDM2 also contains a NrLS and so the nucleolar co-localisation of $p19^{ARF}$ and MDM2 can be viewed as a bi-directional interaction (Weber *et al.*, 1999). Data imply that $p19^{ARF}$ binding to MDM2 may induce a conformational change that facilitates nucleolar import of the ARF-MDM2 complex (Weber *et al.,* 1999).

It has been shown that the regions in $p14^{ARF}$ which interact with MDM2 are also able to relocalise MDM2 to the nucleolus and inhibit MDM2-dependent p53 degradation (Lohrum *et al*, 2000). However, recently, a more physiological approach has suggested that nucleolar localisation of $p14^{ARF}$ is not essential for its function (Llanos *et al.,* 2001). The experiments showed that stabilisation of MDM2 and p53 occurs without relocation of MDM2 to the nucleolus and that forms of $p14^{ARF}$ which are not nucleolar retain the ability to stabilise p53 and MDM2 (Llanos *et al.,* 2001). Further work is required in order to elucidate the importance and function of the observed ARF-MDM2 nucleolar bodies.

1.3.9 Importance of the p14/19^{ARF} -p53 pathway

Established MEF cell lines that lack ARF preserve p53 function, while those that retain ARF have sustained p53 mutations (Sherr and Weber, 2000). Cells lacking a functional p53 gene are resistant to p19^{ARF}-induced cell cycle arrest, implying that p53 acts downstream of ARF (Kamijo *et al.,* 1997). However, ARF-null cells exhibit an intact p53 checkpoint following ionising or UV irradiation, so p19^{ARF} does not relay signals to p53 in response to DNA damage. Loss of p53 can occur in cancer cells that arise in ARF-null mice, again indicating that ARF plays a more specialised role in tumour suppression than p53, and that selection against p53 can further contribute to malignancy (Kamijo *et al.,* 1997; Sherr 1998).

A murine tumour progression model for pancreatic cancer further highlights the importance of the p14/19^{ARF}-p53 pathway (Wagner *et al.,* 2000). A transgenic mouse overexpressing TGFα, when crossed with a *p53*$^{-/-}$ mouse displayed greatly accelerated tumour progression, and it was observed that one in three tumours developing in the *TGFα/p53*$^{-/-}$ mice sustained biallelic deletion of the INK4a/ARF locus (Wagner *et al.,* 2000). These data identify the p53 protein as a rate limiting step in tumourigenesis and indicate that the INK4a/ARF locus is synergistic to p53 in tumour progression (Wagner *et al.,* 2000).

In order to investigate the role of MDM2 in the p53-ARF pathway, triple knock out mice were generated (TKO) lacking p19^{ARF}, MDM2 and p53 (Weber *et al.,* 2000).

TKO mice developed more tumours that *p53*$^{-/-}$*/mdm2*$^{-/-}$, or *p53*$^{-/-}$ mice and the reintroduction of ARF into TKO cells resulted in a G1 arrest (Weber *et al.*, 2000). Interestingly, re-introduction of ARF into *p53*$^{-/-}$*/arf*$^{-/-}$ cells did not result in G1 arrest. As cells lacking p53 or p53 and p19^{ARF} are resistant to ARF induced arrest, these data showed that when present, MDM2 antagonises the ability of ARF to induce cell cycle arrest through targets other than p53 (Weber *et al.*, 2000). MDM2 antagonises some activity of ARF on targets other than p53, and in the absence of MDM2 this other ARF activity is revealed. These data suggest that ARF and p53 loss contribute independently to aspects of tumour progression (Weber *et al.*, 2000).

1.3.10 Activation of p14/19^{ARF}

p14/19^{ARF} is activated by hyperproliferative signals from cellular oncogenes such as Myc (Zindy *et al.*, 1998), E1A (De Stanchina *et al.*, 1998), E2F-1 (Bates *et al.*, 1998), mutated Ras (Palmero *et al.*, 1998; Lin and Lowe, 2001) and v-Abl (Radfar *et al.*, 1998), and also by cellular transcription factors such as DMP1 (Inoue *et al.*, 2000).

The activation of p14/p19^{ARF} is highly important for cell cycle regulation and so it is important to understand the cellular mechanisms by which it occurs. Moreover, the induction of ARF by oncoproteins such as Myc, E1A, Ras and v-Abl highlights its role in sensing hyperproliferative signals in cancer cells (Sherr and Weber, 2000).

Myc selectively induces $p19^{ARF}$ and not $p16^{INK4a}$ and rapidly activates p53 dependent apoptosis (Zindy *et al.,* 1998). An Eμ-Myc mouse model system was used to elucidate the effect of Myc on the $p19^{ARF}$ tumour suppressor (Eischen *et al.,* 1999; Schmitt *et al.,* 1999). Myc activation strongly selects for spontaneous inactivation of the ARF-MDM2-p53 pathway.

In the Eμ-Myc mouse model, initially Myc activation induces p53 dependent apoptosis and then gradually selects for cells mutated in either p53 or $p19^{ARF}$ therefore cancelling its protective checkpoint function and accelerating progression to mailignancy. Eμ-Myc mice develop B cell lymphoma through loss of p53 or $p19^{ARF}$ (Eischen *et al.,* 1999). In addition to these INK4A/ARF mutations, Eμ-Myc mice can accelerate tumour progression and impair apoptosis by compromising p53 function (Schmitt *et al.,* 1999). These INK4A/ARF null lymphomas form rapidly, are highly invasive and interestingly, are highly resistant to chemotherapy. Previous studies have suggested that $p16^{INK4A}$ is not involved in this process and that tumourigenesis is dependent on $p19^{ARF}$ mutation (Schmitt *et al.,* 1999). It is thought that disruption of Myc-p53 induced apoptosis in these cells leads to selection of chemoresistant cells and that these mutations can have a negative impact on the outcome of cancer therapy (Schmitt *et al.,* 1999). Cytotoxic drugs can kill cells via DNA damage induced apoptosis and this again highlights synergy between the ARF and DNA damage induced pathways leading to p53 activation (Sherr and Weber 2000). Hence, a better understanding of $p19^{ARF}$ mutations may lead to more effective cancer therapies.

DAP kinase, a pro-apoptotic, calcium regulated serine/threonine kinase activates a p53/p19^{ARF} mediated apoptotic response (Ravel *et al.,* 2001). DAP kinase expression is frequently lost in human tumours and interestingly, both E2F-1 and Myc are able to increase DAP kinase levels, indicating a role for p53 induced apoptosis in the E2F and Myc apoptotic pathways. Induction of this apoptotic response probably underlies the anti-oncogenic activity of DAP kinase (Ravel *et al.,* 2001).

Adenovirus E1A proteins function in cells to inactivate check-point controls by the pRb pathway through binding to pRb, and activation of p53 transcription (de Stanchina *et al.,* 1998). Experiments in p19$^{ARF-/-}$ cells showed that the induction of p53, and hence, p53 mediated apoptosis is compromised in knock out cells, and that reintroduction of p19^{ARF} can restore these functions (de Stanchina *et al.,* 1998).

DMP1 is a cyclin D binding, b-Myb like protein that binds to a single recognition site in the p19^{ARF} promoter to upregulate p19^{ARF} transcription. DMP1 is able to activate transcription in conjunction with the transcription factor E2F-1 that also activates the ARF promoter (Inoue *et al.,* 1999; Bates *et al.,* 1998). Expression of DMP1 induces growth arrest in wild-type MEFs but not in *ARF*$^{-/-}$ MEFs (Inoue *et al.,* 1999), and disruption of *dmp1* facilitates cell immortalisation, Ras transformation and tumourigenesis (Inoue *et al.,* 2000). DMP1$^{-/-}$ MEFs have low levels of p53, MDM2 and p19^{ARF}, and are unable to senesce. Also these DMP1 cells are transformed by activated Ras similarly to p53 or p19$^{ARF-/-}$ MEFs indicating a de-regulation of the p53-p19^{ARF} pathway in *dmp1*$^{-/-}$ MEFs (Inoue *et al.*, 2000).

BRCA1 is a tumour suppressor gene, which functions as a transcriptional co-activator (Scully *et al.,* 1997) and has been shown to activate p53 expression (Somasundaram *et al.,* 1999). Intriguingly, SAOS2 cells infected with adenovirus expressing BRCA1, showed a two fold increase in $p14^{ARF}$ mRNA, suggesting a role for BRCA1 in $p14^{ARF}$ activation, and hence a role in p53 stabilisation (Somasundaram *et al.,* 1999).

v-Abl is a viral protein, expressed in Abelson murine leukemia virus mediated pre-B cell transformation, and is able to activate the oncogenes Myc and Ras (Radfar *et al.,* 1998). Normally, v-Abl infected cells go through a 'crisis point' that characterises the transition from primary transformant to fully malignant cell line. However deletion of the INK4a/ARF locus allows cells to bypass this crisis point (Radfar *et al.,* 1998). Overexpression of $p19^{ARF}$ but not $p16^{INK4a}$ induced apoptosis in v-Abl transformed B cells, indicating that $p19^{ARF}$ is responsible for the bypass of apoptotic crisis (Radfar *et al.,* 1998). This study identifies $p19^{ARF}$ as one of the components of the cellular defence mounted against v-abl mediated transformation.

Knock-out *c-myc* $^{-/-}$ mice die at E9.5, and myc itself is deregulated in many human cancers (Jacobs *et al.,* 1999b). Bmi-1 collaborates with c-myc in tumorigenesis through its ability to downregulate the INK4a/ARF locus and inhibit c-myc induced apoptosis (Jacobs *et al.,* 1999b). *bmi-1* $^{-/-}$ cells proliferate slowly and prematurely senesce while increased apoptosis is observed, a phenomenon that can be rescued by the deletion of the INK4a/ARF locus (Jacobs *et al.,* 1999a).

Activation of oncogenes increases susceptibility to apoptosis so tumourigenesis must depend in part on compensating mutations that protect from apoptosis. *twist*, is a potential oncogene, which inhibits p53 dependent apoptosis, and bypasses p53 mediated growth arrest (Maestro *et al.,* 1999). The effect of *twist* on ARF is interesting in that it is able to impair p53 transcription. However, twist over-expressing cells showed a huge decrease in ARF expression, which is unexpected, given that loss of p53 usually leads to ARF up-regulation (Maestro *et al.,* 1999).

1.3.11 Roles of p14/p19^{ARF} and p16^{INK4A} in Senescence

Both p16^{INK4A} and p14/19^{ARF} have been implicated in cellular senescence, the process by which cells lose the ability to proliferate after the completion of a finite number of cellular divisions (Campisi, 1996). Senescence is characterised by a growth arrest, apoptotic resistance and an altered spectrum of differentiation types, and often comes about as a result of telomere shortening (Campisi, 1996). Cultured primary cells express increasing amounts of p16^{INK4a} as they approach the limit of their *in vitro* life span, and forced expresssion of activated ha-Ras in primary cells induces senescence that is relieved following inactivation of either p16^{INK4a} or p53 (Haber, 1997)

E2F-1 is able to induce a senescence phenotype in normal human cells as a result of its ability to upregulate p14^{ARF} and as this phenomenon is p53 independent it can be assumed that p14^{ARF} is the critical component in the induction of senescence (Dimri *et al.,* 2000).

Antisense vectors were employed in an interesting study, which showed that both $p19^{ARF}$ and $p16^{INK4A}$ are involved in senescence, and that they work in overlapping pathways (Carnero *et al.*, 2000). Experiments showed that while $p16^{INK4A}$ induced growth arrest can be overcome by compromising the function of its upstream regulator pRb, to overcome $p19^{ARF}$ growth arrest requires the inactivation of both the pRb, and p53 pathways. Interestingly, loss of either one of $p16^{INK4A}$ or $p19^{ARF}$ contributes to a bypass of senescence. However loss of $p19^{ARF}$ bypasses senescence more efficiently than loss of $p16^{INK4a}$ (Lloyd *et al.*, 2000). The importance of this observation is highlighted by the fact that $p19^{ARF-/-}$ fibroblasts are able to proliferate indefinitely, so are immortal (Lloyd *et al.*, 2000).

The SV40 large T antigen has at least three domains which participate in cellular transformation through targeting the cell-cycle regulatory proteins pRb and p53 (Chao *et al.*, 2000). Further evidence to suggest that $p19^{ARF}$ may be involved in pRb regulation is provided by Chao *et al.*, 2000. who found that loss of $p19^{ARF}$ eliminates the requirment for the pRb binding motif in SV40 virus Large T transformation, and that this regulation is through the LXCXE motif required for pRb binding to large T. In $rb^{-/-}$ MEFS all three transforming domains were required for full transformation, however in $ARF^{-/-}$ MEFs anchorage-independent growth was acquired (Chao *et al.*, 2000).

A new regulator of $p19^{ARF}$ was recently identified using a senescence bypass screen, *TBX2*, which is a T box transcription factor which represses the $p19^{ARF}$ promoter by binding to the initiator -19-54 region of the promoter (Jacobs *et al*, 2000). *TBX2* is

found to be amplified in a subset of human breast cancers and prevents E2F-1, Myc and H-Ras induction of $p19^{ARF}$ (Jacobs *et al.*, 2000).

1.3.12 Therapeutic Strategies.

As the p53 pathway is a member of an integral DNA damage response in cells (Levine, 1997), it is of interest to ask how $p14/19^{ARF}$ affects the p53 response to DNA damage? One such study has shown that $p19^{ARF}$ is required to induce p53 responses to DNA damage and microtubule disruption, but not other cellular stresses such as ribonucleotide reduction or RNA synthesis, which are both independent of $p19^{ARF}$ (Khan *et al.*, 2000). A situation, which emphasises the complexity of the biochemical and cell cycle responses to diverse stresses that activate the p53 pathway.

The mutated adenovirus dl1520 (ONYX-015) does not express the E1B protein which binds and inactivates p53 and so is able to selectively replicate only in tumour cells with mutant p53 (Ries *et al.*, 2000). This therapeutic virus has shown promising responses in patients with solid tumours, with a high percentage of full regression. Loss of $p14^{ARF}$ has also been identified as a mechanism which allows replication of this virus in tumour cells that retain wild type p53, and reintroduction of $p14^{ARF}$ into these cells is able to suppress dl1520 replication in a p53 dependent manner (Ries *et al.*, 2000)

ATM induces cellular responses to DNA damage and mutations in the *atm* gene cause *ataxia telengiectasia*. This is a progressive, degenerative disease characterised

by cerebella degeneration, immunodeficiency and a pre-disposition to cancer. *ATM* encodes a protein kinase activity specific for serine and theronine residues and has been shown to phosphorylate p53 (Kamijo *et al.,* 1999). ATM null fibroblasts undergo premature replicative arrest, which is relieved by loss of p53. In these cells, loss of ARF extends the life span of the ATM fibroblasts but does not alter their sensitivity to ionising radiation or their disposition to development of lymphoma (Kamijo *et al.,* 1997). These data suggest that loss of ARF can modify p53 dependent features of the ATM null phenotype, and that ARF and ATM can likely reinforce the others activities and response to stress (Kamijo *et al.,* 1999). These data furthermore highlight a prospective cross talk between cellular stress pathways (Sherr and Weber, 2000).

1.4 Conclusions and Objectives

1.4.1 Concluding remarks

The de-regulation of many components of the pRb-E2F pathway in human cancer shows how important it is to elucidate the physiological processes within this pathway and develop efficient tumour therapies. The role of E2F-1 as both a tumour suppressor and an oncogene, and its importance in both cellular proliferation and apoptosis highlights it as an essential cell cycle regulator. Recent knockout mouse models have provided us with great insight into the roles of the individual E2F family members, however, much more work is required to fully elucidate their roles in the cell. The identification of post-translational mechanisms such as phosphorylation and acetylation, which affect E2F function are of particular interest and more work is required to elucidate the proteins involved in these processes.

The INK4a/ARF locus and its two gene products $p16^{INK4a}$ and $p14/19^{ARF}$, both play a key role in regulating the cell cycle check point controlling proteins, p53 and pRb respectively. Given the high level of germ-line mutations in the INK4A/ARF locus in cancer cells, the importance of this locus in the maintenance and development of the healthy cell is seemingly unquestionable. The transcription of two unrelated genes from the same genetic locus has undoubtedly come about through a genetic selection event. Given that each protein plays a role in the control of two similar cellular pathways, this genetic locus is of great interest. The reason for such

conservation of genetic information in mammalian cells is unknown but will provide an exciting avenue for future research.

Clearly the ability to fully understand both the p53 and pRb tumour suppressor pathways, and the interplay that exists between them will be of great benefit in the design of drugs for the treatment of cancer.

1.4.2 Objectives

The main function of $p14^{ARF}$ identified to date is its ability to regulate the p53 pathway through sequestration of MDM2. Recent studies have identified p53 independent functions of $p19^{ARF}$ (Carnero *et al.*, 2000; Weber *et al.*, 2000) and indicate that $p14^{ARF}$ may also play a role in p53 independent pathways in cell cycle regulation. Given that E2F-1 function and sub-unit composition is down-regulated by MDM2 (Loughran and La Thangue 2000), and $p14^{ARF}$ is a negative regulator of MDM2 function (Kamijo *et al.*, 1998), MDM2 could also be important in other $p14^{ARF}$ cellular pathways, such as the regulation of E2F.

The objectives of this thesis were to investigate a role for $p14^{ARF}$ in the E2F pathway, primarily using E2F-1 and further to investigate whether $p14^{ARF}$ can interact with E2F-1. Furthermore to look for MDM2 independent roles of $p14^{ARF}$ and to define the mechanism of action of $p14^{ARF}$ upon the E2F pathway. Understanding the role of $p14^{ARF}$ and its interacting proteins should ultimately help us to understand the roles of the tumour suppressor proteins in the cellular pathways they regulate.

Chapter 2. Materials and Methods.

2.1. Plasmids.

The following plasmids have been previously described pRcCMVHA-E2F-1 (Helin *et al.*, 1992), pCMVE2F1Y411C (Helin *et al.*, 1992), CMV-DP1 (Bandara *et al.*, 1993), CMV-p14^{ARF} (Stott *et al.*, 1998), pCHDM2 (Chen et al., 1996), pcMDM2 (Martin *et al.*, 1995), pCMVβgal (Zamanian and La Thangue, 1992), myc-ARF, myc-ARF(N), myc-ARF(C) (Zhang *et al.*, 1998), Tx-ARF-1-132, Tx-ARF-1-22, Tx-ARF-1-34, Tx-ARF-1-64, Tx-ARF-65-132, Tx-ARF-54-64, (Lohrum *et al* 2000), E1β-luciferase (Bates *et al.*, 1998), cyclinE-luciferase (Botz *et al.*, 1996), 3XWT-luciferase, 3XMT-luciferase (Zamanian and La Thangue, 1992), Apaf-1-luciferase promoters (-871/+208), (-396/+208), (+35/+208) (Moroni *et al.*, 2001).

CMV-DP1dl70, CMV-DP1dl171, CMVDP1dl230, CMV-DP1205-310 and HA-DP-1 were a gift from Chang-Woo Lee (University of Glasgow). His-E2F-1, GST-DP1, pRcCMVHA-E2F-1ΔC were a gift from Laurent Delavaine (University of Glasgow). pRcCMVHA-E2F-1ΔC was made by Bgl11/BamH1 digestion of the pRcCMVHA-E2F-1 plasmid, removal of the *trans*activation domain fragment and re-ligation of the vector. HA-E2F-1 181-221 and HA-E2F-1 141-221 were prepared by PCR amplification using the following pairs of primers HA-E2F-1 181-221 5' 'GGGGATCCGCCAAGAAGTCCAAGAACCAC' and 3' 'GGTCTAGACTCCGAAGAGTCCACGGCTTG', HA-E2F-1 141-221 5' 'GGGGATCCGAGCTGCTGAGCCACTCGGCT' and 3' 'GGTCTAGACTCCGAAGAGTCCACGGCTTG' and were a gift from Laurent

Delavaine. PGEX-p14^{ARF} was prepared by digestion of CMVp14^{ARF} with BamH1/EcoR1 (Promega) and ligation of the fragment into the BamH1/EcoR1 site in the pGEX^{KG} vector (Pharmacia). Similarly, pGEXp14^{ARF}-N62 was generated by cutting the myc-ARF(N) construct (Zhang and Xiong 1998) with EcoR1/Xho1 (Promega) and ligation of the fragment into the EcoR1/Xho1 site of pGEX^{KG}

pGEX-p14^{ARF}mycC was cloned by PCR amplification (using *taq pfu*-Promega) of the myc-ARF(C) fragment (Zhang and Xiong 1998) using the primers 5'-3' GGAATTCCGGTCATGATGATGGGCAG and 3'-5' CTCTAGAGTCAGCCAGGTCCACGGGC to introduce restriction sites for EcoR1 and Xba1 and the resulting fragment was gel purified using the Gelclean kit (Quiagen). The gel-purified p14^{ARF} fragment was then TA tailed (with *taq* polymerase-Promega) and cloned into the pGEMEasy vector (Promega). pGEMEasy ligations were transformed into JM109 cells (Promega) and colonies determined as containing the insert were mini-prepped and checked for fragment orientation, the fragment was then digested out with EcoR1 and then non-directionally cloned into the EcoR1 site of pGEX^{KG}

Orientation of all cloning was verified by restriction digest and sequencing of all vectors was carried out at Leicester University.

2.2 Antibodies

Primary

The E2F1 antibody (KH95) (Santa Cruz) was used at at 1: 500 dilution and is a monoclonal antibody which interacts with the Rb binding domain of mouse, rat and

human E2F-1. DP-1 (098) is a rabbit polyclonal anti-serum raised against a DP-1 C terminal peptide (Sorenson *et al.*, 1996), and was used at a 1:200 dilution. p14 ^{ARF} (C-18) (Santa Cruz) was used at a 1:200 dilution and is an affinity purified goat polyclonal antibody raised against a peptide mapped to the C terminus of human $p14^{ARF}$. $p14^{ARF}$PO1 (Stratech Scientific) is a monoclonal antibody raised against recombinant $p14^{ARF}$ and was used at a 1:200 dilution. The HA-11 antibody (Cambridge Bioscience) was used at a 1:1000 dilution and is a monoclonal antibody to the influenza HA tag. HA (F-7) (Santa Cruz) was used at a 1:200 dilution and is a rabbit polyclonal antibody raised against the HA tag. c-Myc (9E10) (Santa Cruz) was used at a 1: 500 dilution and is a mouse monoclonal antibody.

Secondary

Anti-Ig-TRITC rhodamine conjugated (Southern Biotechnology Association inc.) and Anti-Ig-FITC fluorescein conjugated (Southern Biotechnology Association inc.) were used for immunostaining. Anti-Ig-AP alkaline phosphatase conjugated antibodies were used for western blotting (Promega).

2.3 Transformations

25µl of cells (XL1 Blue for DNA preparations and BL21 (DE3) pLysS for GST fusion and His-tagged protein preparations) were thawed on ice. After thawing, 0.2µg of the appropriate DNA was added and the mixture was placed on ice for 30 minutes. The mixtures were heat-shocked at 42°C for 45 seconds and then cooled on ice for two minutes. 900µl of LB was added to the cells, which were then incubated

at 37°C for 30 minutes. After incubation cells were spread on a LB plate (with either ampicillin or kanamycin at 100μg/ml) and incubated at 37°C overnight.

2.4 DNA Preparation

Plasmid minipreps (small scale DNA preparation) were prepared using the Wizard *Plus* SV Miniprep DNA Purification System (Promega) according to the manufacturers instructions and DNA maxipreps (large scale DNA preparation) were prepared using the Quiagen Maxiprep kit, according to the manufacturers instructions.

2.5 Restriction Digests and DNA Analysis.

Restriction digests were performed as previously described. 20μl reactions contained 1μg DNA, 1μl enzyme, 2μl enzyme buffer and were made up to volume with H_2O. All enzymes were supplied by Promega or Roche. Digests were analysed on 2% agarose gels containing ethidium bromide (Sigma) in 6x Agarose Gel DNA Loading Buffer (0.25% Bromophenol Blue, 0.25% Xylene Cyanol, 30% Glycerol) and visualised on a ultraviolet light box

The concentrations of nucleic acid solutions were determined spectrophotometrically using a quartz cuvette.

1 A_{260} = 50μg/ml dsDNA

2.6 Tissue Culture

SAOS2 cells were purchased from the American Type Culture Collection and cultured in DMEM (Gibco) supplemented with 10% FCS, 100U/ml penicillin and streptomycin (Gibco). Cells were passaged 1:3 when confluent *p53*$^{-/-}$*mdm2*$^{-/-}$ MEFs were a kind gift from David Lane (Ninewells hospital, Dundee) and were cultured in DMEM, 10% FCS and 100U/ml P/S and split 1:6 upon reaching confluence. SAOS2 cells and *p53*$^{-/-}$*/mdm2*$^{-/-}$ MEFs were kept at 37°C in an atmosphere of 5% CO_2.

2.7 Transfection

Transfections were carried out in SAOS2 cells or early passage MEFs grown in DMEM/FCS. Cells were plated at $5x10^5$ per 6cm dish or $2x10^6$ per 10cm dish (SAOS2) or $1x10^5$ per 6cm dish or $5x10^5$ per 10cm dish (MEFs). Cells were transfected by the calcium phosphate method as described in (Sambrook *et al.*,). DNA/H_2O mixtures were prepared and then mixed with 10% volume 2M $CaCl_2$ before being added dropwise to an equal volume of 2xHBS (280mM NaCl, 50mM Hepes acid, 1.5mM Na_2HPO_4). Calcium phosphate precipitate was allowed to form at room temperature for 15 minutes before being added to cell monolayers. After 6-20 hours cells were washed twice in PBS, re-grown in DMEM/FCS and then harvested 24-48 hours post transfection.

2.8 Reporter Assays

Reporter gene assays were performed by transfection of the indicated amounts of plasmids together with 500ng CMV-βgal as an internal control per 6cm plate. Quantities of DNA per plate were kept constant by the addition of equivalent amounts of pcDNA3 or pSG5 control vectors, in order to control for promoter specificity. Assays were performed in duplicate. Transfected cells were washed twice in PBS, regrown in DMEM/FCS and harvested in 300μl 1x RLB (Promega (25mM Tris-H_3PO_4 pH7.8, 2mM 1,2-diaminocyclohexane tetra acetic acid, 2mM DTT, 10% glycerol and 1% Triton X-100)), micro-centrifuged at 10,000 for 5 minutes to remove cell debris and the lysates analysed for reporter gene activity.

For the luciferase assay: 60μl cell lysate was mixed with 300 μl luciferase reagent (Diluted 1:4 (Promega)) and luciferase light units measured in a luminometer (Berthold Lumat).

For the βgalactosidase assays: 100μl cell lysate was incubated with 100μl 2xβgal reagent (200mM sodium phosphate buffer pH 7.2, 2mM magnesium chloride, 100mM B-mercaptoethanol, 1.3μg/ml ONPG) and incubated at 37°C until a yellow colour developed. Reactions were stopped by the addition of 600 μl H_2O and the activity was measured in a spectrophotometer (Pharmacia Biotech Ultraspec 1000) at 420nm.

2.9 GST Protein Purification

1μg of plasmid was transformed into BL21 DE3 competent cells (Stratagene). A single colony was inoculated into 50ml LB^{AMP} and allowed to grow to saturation. This culture was diluted 1:10 and grown at 37°C until log phase, at which point the culture was induced with 0.4mM IPTG. Induced cultures were allowed to grow for 3 hours, with the addition of 1mM PMSF every 20 minutes and then bacteria harvested by spinning at 5000 rpm. Pellets were re-suspended in 10ml PBS/1%Triton/1mM PMSF. The bacteria were then lysed by sonication (2x 20 seconds) and spun to collect cell debris. The supernatant was added to 500 μl pre-equilibrated GST beads (Amersham) and incubated at 4°C for 30 minutes with shaking. The beads were then washed 2 times in PBS/Triton/PMSF and 2 times in PBS. GST proteins were stored at –20°C in GST storage buffer (20mM Tris pH 8.0, 100mM $NaCl_2$, 20%Glycerol).

When required proteins were eluted from the GST beads in an equal volume of elution buffer (10mM Glutathione, 50mM Tris pH 8.0). Dialysis was carried out overnight at 4°C in Slyde-a-lyser mini dialysis units (Peirce) in dialysis buffer (25mM sodium phosphate buffer pH 7.8, 1mM EDTA, 5mM mercaptoethanol, 10% glycerol)

2.10 HIS tagged protein purification

BL21 cells were transformed, grown and induced as described above. Bacteria were pelleted by centrifugation at 5000G, the pellet resuspended in 10ml sonication buffer (10mM Tris pH 8.0, 0.5M $NaCl_2$, 10% Glycerol, 0.1% NP-40, 5mM DTT, 1mM

PMSF, 1x Protease Inhibitor Cocktail (Sigma)) and sonicated for 3x15 seconds on ice. Following centrifugation the supernatant was added to 500µl previously equilibrated (10mM Tris pH 8.0, 0.5M $NaCl_2$, 10% Glycerol, 0.1% NP-40) nickel agarose beads (Promega) and incubated at 4^0C for 1 hour with rotation. Beads were washed 3x in BC100 buffer (20% Glycerol, 20mM Tris pH 8.0, 100mM KCl, 5mM DTT, 20mM Imidazole, 1mM PMSF) and, if required, eluted from the beads with 200mM imidazole. Dialysis was carried out as described in 2.9.

2.11 Cell Extracts

Nuclear extracts were prepared from SAOS2 cells or early passage MEFs by scraping monolayers and swelling in Buffer A (20mM Hepes, 20% Glycerol, 250mM $NaCL_2$, 1.5 mM $MgCl_2$, 1mM EDTA, 0.1 % TritonX) for 30 minutes. Followed by centrifugation at 2000 rpm for 10 minutes before lysis in TNE buffer (50mM Tris pH 8.0, 150 mM $NaCl_2$, 5mM EDTA, 0.5%NP-40) for 30 minutes on ice. Supernatants were collected by centrifugation at 13000 rpm for 30 minutes.

Whole cell extracts were prepared by lysis in TNN buffer (50mM Tris 7.4, 120mM $NaCl_2$, 5mM EDTA, 0.5 % NP-40, 50mM NaF, 1mM DTT, 1mM PMSF, 1x Protease Inhibitor Cocktail) on ice for 30 minutes. Cell debris was collected by micro-centrifugation at 13000 rpm.

2.12 Determination of Protein Concentration and Electrophoresis

The protein concentration of cell extracts for biochemical assays was determined by Bradfords assay as described in (Sambrook *et al.*). All biochemical assays were

analysed by SDS-PAGE using Mini-protein 2 (Biorad) kits and western blotting using Mini-protein 2 blotting kits (Biorad).

2.13 Biochemical Assays

For GST-pull down assays nuclear extract (200 μg) was incubated with 1μg GST or GST-$p14^{ARF}$ protein overnight at 4°C. The reactions were carried out in 200μl TNE buffer containing 1mM DTT, 1mM PMSF and 1x protease inhibitors (Sigma). Beads were washed 3 times in TNE buffer and re-suspended in 3xSDS buffer for gel analysis.

Alternatively proteins were *in vitro* translated using the T7 TNT coupled system (Promega) as described by the manufacturers. An equal amount of *in vitro* translated protein was added to 1μg GST protein in 200μl TNE buffer. Reactions were incubated at 4°C for 1 hour and then beads washed 3 times in binding buffer and re-suspended in SDS buffer for gel analysis. Gels were dried and exposed overnight by autoradiography (Biorad).

For the *in vitro* binding assay, 0.5 μg of each eluted purified GST protein was incubated with an equimolar amount of His-E2F-1 for 30 minutes at 4°C in 200μl TNE. Reactions were washed three times in binding buffer and subjected to SDS-PAGE analysis and Western blotting.

2.14 Immunoprecipitations.

For the endogenous immunoprecipitation HeLa cell nuclear extract (Computer Cell Culture) (500μg) was incubated in 100μl TNE with anti-E2F-1, anti-$p14^{ARF}$, anti-MDM2 or anti-HA antibody overnight at 4°C with rotation. Protein A agarose was then added to samples for 3 hours at 4°C with rotation. Immunoprecipitates were washed 3 times in binding buffer and resuspended in 3x SDS loading buffer, analysed by SDS-page and western blotting.

For the over-expression immunoprecipitation experiments SAOS2 cells (2×10^6) were transfected with equal amounts (25μg) of the indicated plasmids. Cells were washed 8 hours post transfection and harvested 24 hours post transfection. Extracts were prepared by lysis of cell pellets in 100μl IP buffer (150mM $NaCl_2$, 1% NP-40, 50 mM Tris pH 8.0) containing protease inhibitors. Extracts were incubated with anti-E2F1 (KH95 Santa Cruz) overnight at 4°C. Protein A agarose was then added to samples for 3 hours at 4°C and the samples washed 3 times in IP buffer. Beads were re-suspended in 20μl SDS buffer for analysis on SDS gels. Western blots were probed with anti-myc (Santa Cruz) and anti-HA-11 (Cambridge Bioscience) respectively.

2.15 Immunostaining

Cells were plated at $3x10^5$ on coverslips in 35mm dishes and transfected 24 hours later with the indicated plasmids. Transfections were washed after 20 hours and harvested 36 hours post transfection. Cells were fixed in 4% paraformaldehyde for

10 minutes and then permealised in 0.1% Triton, 0.1% sodium citrate in PBS for 10 minutes. Immunostaining was performed using HA-11, c-myc or HA rabbit polyclonal antibodies in % FCS as indicated, followed by washing 40 times in 10%FCS/PBS and staining with FITC or TRITC conjugated secondary antibodies (Europath) in 10%FCS. Coverslips were mounted on Citiflour (Agar Scientific) and examined using an Olympus fluorescence microscope BX-60. Photographs were taken using an Olympus PM-30 automatic micrographic system.

2.16 TUNEL Assay

SAOS2 cells were plated $3x10^5$ on coverslips and transfected by with 6μg of each indicated plasmid. Following transfection, cells were washed twice in PBS and serum starved overnight in DMEM 0.2% FCS. Post-transfection, cells were fixed for 15 minutes (4% paraformaldehyde) and permeablised for 10 minutes (0.1%Triton, 0.1% Sodium Citrate in PBS). TUNEL staining was performed, using the TUNEL kit (Roche) as described by the manufacturer. Coverslips were mounted in Citriflour (Agar Scientific) and examined using an Olympus fluorescence microscope. Positively stained cells were counted a minimum of 3 times, under low and high power fluorescence microscopy. Counts were normalised for transfection efficiency (Determined by double staining for HA-E2F-1).

2.17 BrdU assay

Cells were plated at $3x10^5$ (SAOS2) or $1x10^5$ (*mdm2*$^{-/-}$/*p53*$^{-/-}$ MEFs) on coverslips and transfected with 5μg of each indicated plasmid. Following transfection, cells were

washed twice in PBS and left overnight in DMEM 10% FCS. Labelling was carried out for 15 minutes at 37^{0}C and cells were then fixed (ethanol,glycine buffer pH 2) for a minimum of 30 minutes at -20^{0}C, and stained with the 5-Bromo-2'-deoxy-uridine Labelling and Detection Kit 1 (Roche) according to the manufacturers instructions. Cells going through S-phase incorporate BrDU into newly synthesised DNA. BrDU incorporation was assayed by detection with anti-BrDU antibody, followed by staining with a FITC-conjugated secondary antibody. Cells were mounted and examined as described for the TUNEL assay. Positively staining cells were counted as described for the TUNEL assay.

2.18 Band Shifts

Oligonucleotides (oligos) E2F wildtype 19 'AGCTAGTTTTCGCGCTTAAATT', 20 'AGCTAATTTAAGCGCGAAAACT' and E2F mutant M3 'TCGATAGTTATCTGAGTAAACTAGTG' and M5 'TCGACACTAGTTTACTCAGATAACTA' derived from the adenovirus E2A promoter sequence were synthesised by Genosys. Single stranded oligos were annealed by heating at 100°C for 10 minutes and then allowed to cool slowly before storage at –20°C.

^{32}P-labelling was carried out by incubation of 1µl DNA probe with 1µl kinase buffer, 1 µl T4 kinase enzyme (Promega) and 1 µl 32P γ ATP (Amersham). The reaction was incubated at 37^{0}C for 30 minutes and then purified on a Quickspin column (Roche) in TE buffer (10mM Tris pH 7.4, 1mM EDTA).

Bandshifts were performed in 20μl reactions containing 150ng un-labelled mutant oligo, 2 μg salmon sperm DNA, 4x reaction buffer (60% glycerol, 4mM DTT, 0.8mM EDTA, 200mM Tris pH 8.0, 24mM $MgCl_2$) and the indicated *in vitro* translated or purified proteins. Proteins were *in vitro* translated using the Promega T7 coupled TNT kit to the manufacturers instructions, or bacterially purified and eluted as previously described (2.9). Where complexes were identified by antibody shifts 1μl of the indicated antibody was added to the reaction. Reactions were incubated at room temperature for 10 minutes, then 1μl radio-labelled probe was added to each reaction and incubated for a further 20 minutes at room temperature. A non-denaturing polyacrylamide gel was pre-run for 30 minutes before loading the samples and running for 100 minutes at 230V. Reactions were visualised by autoradiography.

2.19 Colony Forming assay

SAOS2 cells were plated at $1x10^6$ and transfected by the calcium phosphate method with the indicated plasmids. 16 hours post-transfection, cells were washed and 30 hours post-transfection were split $2x10^5$ and $8x10^5$ into a further $5x10^5$ untransfected SAOS2 cells. Cells were placed under G418 selection (0.8μg/ml) for approximately 17 days until colonies were clearly observed. Colonies were stained with crystal violet and independently counted at least 3 times.

2.20 Degradation Assay

SAOS2 cells or *p53*$^{-/-}$/*mdm2*$^{-/-}$ MEFs were plated at 1x10^6 or 0.5x10^6 cells in 10cm plates respectively and transfected 16 hours later with the indicated plasmids and 1μg CMV-βgal as an internal control. 16 hours post-transfection cells were washed in PBS. Cells were harvested 48 hours post-transfection and whole cell extracts were prepared as described (2.11). Transfection efficiency was determined by β-galactosidase assay (2.8) and then lysates were analysed by SDS-PAGE and western blotting.

2.21 Far Western Blot

Purified GST proteins were subjected to SDS-PAGE and transfer to nitrocellulose membrane (Inverclyde Biologicals). Membranes were washed twice in PBS and then blocked in 2% milk/PBS for 1 hour at 4°C. Proteins were prepared by *in vitro* translation (Promega) as described (2.13). After blocking, membranes were washed twice for 5 minutes in AC buffer (10% glycerol, 100mM NaCl, 20mM Tris, 0.5mM EDTA, 0.1% Tween-20). *In vitro* translated proteins were diluted in 10ml AC buffer/1% milk overnight at 4°C. Membranes were dried and binding of ^{35}S-labelled proteins was determined by autoradiography.

Chapter 3. p14^{ARF} Regulates the activity of the E2F-1/DP-1 Heterodimer.

3.1 Introduction

The E2F-1/DP-1 heterodimeric transcription factor has been shown to induce S-phase progression, and apoptosis in mammalian cells (Johnson *et al.*, 1993; Wu and Levine, 1994). E2F-1 *trans*activates a number of target genes required for S-phase progression and apoptosis, including *cyclinE* (Ontani *et al.*, 1995), *arf* (DeGregori *et al.*, 1997; Bates *et al.*, 1998), *apaf-1* (Moroni *et al.*, 2001) and *dhfr* (Helin, 1998). p14/19^{ARF} is an important regulator of the p53-MDM2 pathway. p14/19^{ARF} stabilises p53 by binding to MDM2, sequestering MDM2 away from p53 and preventing MDM2 mediated p53 degradation (Honda *et al.*, 1999).

It has been shown previously that MDM2 can target E2F-1 and DP-1 for degradation (Loughran and La Thangue, 2000). MDM2 also overcomes E2F-1 mediated apoptosis (Loughran and La Thangue, 2000). It was of interest to further study these cellular pathways in order to define new mechanisms of control for E2F.

E2F-1 and p14^{ARF} are both important cell cycle regulatory proteins, E2F-1 *trans*activates the *arf* promoter and leads to an accumulation of p14^{ARF} (Bates *et al.*, 1998). MDM2 is involved in the regulation of both the E2F pathway (Loughran and La Thangue, 2000) and the p53 pathway, through p14^{ARF} (Honda *et al.*, 1999). This led us to ask the question; is p14^{ARF} involved in the regulation of E2F-1.

Results

3.2 $p14^{ARF}$ regulates E2F-1/DP-1 mediated apoptosis.

In addition to its role in cell cycle progression, E2F-1 has also been shown to induce apoptosis (Wu and Levine, 1994). E2F-1 and DP-1 co-operate to induce higher levels of apoptosis than E2F-1 alone, and this apoptosis is overcome by the MDM2 oncoprotein (Loughran and La Thangue, 2000). As $p14^{ARF}$ acts as a negative regulator of MDM2, causing p53 stabilisation and increased apoptosis (Honda *et al.*, 1999), it was of interest to examine whether $p14^{ARF}$ had an effect on MDM2 activity in E2F-1 mediated apoptosis.

In order to study the ability of $p14^{ARF}$ to regulate the E2F-1 apoptotic response, $p53^{-/-}$ $/rb^{-/-}$ SAOS2 cells were assayed for the DNA fragmentation phenotype characteristic of apoptosis. Fluorescent-labelled nucleotide incorporation into fragmented DNA was visualised directly by immuno-fluorescence using the TUNEL assay. As expected the transient transfection of E2F-1 or DP-1 alone under low serum conditions induced apoptosis (Figure 3.1A) and together E2F-1 and DP-1 co-operate to increase the number of apoptosing cells (Figure 3.1A). Populations of cells over-expressing E2F-1 and DP-1, as determined by immunostaining, showed a 15% increase in the level of apoptosis relative to those non-transfected. As expected the over-expression of MDM2 in E2F-1 and DP-1 transfected cells was able to reduce the level of heterodimer induced apoptosis by approximately 50% (Figure 3.1A).

More interestingly, over-expression of $p14^{ARF}$ in SAOS2 cells produced only a small increase in the level of apoptosis over mock transfected cells (Figure 3.1B). However, over-expression of $p14^{ARF}$, together with the E2F-1 and DP-1 heterodimer resulted in a decrease of 85% in the level of induced apoptosis, to levels observed with $p14^{ARF}$ alone. As $p14^{ARF}$ is able to increase the p53 apoptotic response by sequestering MDM2 away from p53, it was of interest to examine the dual effect of $p14^{ARF}$ and MDM2 on E2F induced apoptosis. In this experiment, the over-expression of E2F-1, DP-1, $p14^{ARF}$ and MDM2 together resulted in the re-establishment of an E2F-1, DP-1 apoptotic response (Figure 3.1C).

The initial objective of this experiment was to examine whether $p14^{ARF}$ could regulate the effect of MDM2 on E2F in a manner analogous to its effect on p53. The fact that $p14^{ARF}$ is able to overcome MDM2 mediated reduction of the E2F apoptotic response implies that this may indeed be the case. However, $p14^{ARF}$ is also able to overcome E2F mediated apoptosis and these data suggest that $p14^{ARF}$ may be able to directly down-regulate E2F mediated apoptosis in a mechanistic pathway separate to that of MDM2s regulation of E2F.

3.3 $p14^{ARF}$ down-regulates E2F dependant transcription.

It has been demonstrated that the $p14/19^{ARF}$ promoter is sensitive to E2F-1 over-expression (Bates *et al.*, 1998; Robertson and Jones, 1998; Inoue *et al.*, 1999). The $p14^{ARF}$ luciferase reporter construct consists of the Exon1β promoter region, from –805 to +59 which contains four potential anti-sense strand E2F consensus sites, fused

to a luciferase reporter gene (Figure 3.2A) (Bates et al., 1998; Robertson and Jones, 1998). It is consistent with these results that the over-expression of E2F-1 efficiently activated the $p14^{ARF}$ promoter in a p53 and pRb negative SAOS2 background (Figure 3.2B).

Given that $p14^{ARF}$ was able to down-regulate E2F-1 induced apoptosis it was of interest to examine the effect of $p14^{ARF}$ on E2F-1 mediated transcription. SAOS2 cells transfected with E2F-1, or DP-1 and the $p14^{ARF}$ E1β-luciferase reporter gene showed activation of the reporter gene and, as expected E2F-1 and DP-1 transfected together showed a co-operative effect (Figure 3.2B). $p14^{ARF}$ transfected with the E1β-luciferase reporter showed no significant effect (similar luc/βgal readings). However, interestingly $p14^{ARF}$ was able to down-regulate the transcription activation by E2F-1-DP-1 in a titratable manner (Figure 3.2B). These data suggest that $p14^{ARF}$ is able to act to down-regulate E2F transcription in a dose-dependent, p53-independent manner.

3.4 $p14^{ARF}$ is a general down-regulator of E2F dependent transcription

Members of the E2F family of transcription factors are able to activate a number of cellular genes including; *dhfr*, *cyclinE* and *arf* (Helin et al., 1998). As $p14^{ARF}$ can down-regulate E2F mediated transcription on the $p14^{ARF}$ E1β-luciferase reporter gene, it was of interest to ask if this effect was specific for the $p14^{ARF}$ promoter or whether $p14^{ARF}$ could down-regulate the transcription of other E2F responsive genes.

And, is this down-regulation specific for E2F or is $p14^{ARF}$ a general down-regulator of activated transcription.

In order to address the issue of E2F specificity, synthetic promoter constructs were used: 3Xwild-type-luciferase, which has three E2F sites engineered into the promoter of the luciferase reporter gene, and 3Xmutant-luciferase which has three mutated E2F sites engineered into the promoter (Zamanian and La Thangue, 1992). These promoter constructs were transfected into SAOS2 cells together with E2F-1, $p14^{ARF}$ or both E2F-1 and $p14^{ARF}$.

As expected, on the wild-type luciferase reporter, E2F-1 caused activation (2 fold), while $p14^{ARF}$ slightly increased reporter gene activity (0.3 fold increase over mock). $p14^{ARF}$ was able to down-regulate E2F-1 mediated transcription to a level comparable to that of $p14^{ARF}$ alone (Figure 3.3A). The same experiment was carried out concurrently on the mutant luciferase reporter gene. As expected E2F-1 was unable to significantly activate the mutant promoter (0.3 fold increase over mock, compare to the 2 fold increase in figure 3.3A), due to the inability of E2F to bind to the mutated E2F binding sites (Figure 3.3B). However, the addition of $p14^{ARF}$ had no effect on the promoter, as did the expression of E2F-1 and $p14^{ARF}$ together. These data suggest that the observed down-regulation of E2F dependent transcription by $p14^{ARF}$ is specific for E2F, and that $p14^{ARF}$ is probably not affecting other components of the basal transcriptional complex.

In order to determine whether $p14^{ARF}$ was able to affect transcription from other cellular E2F target genes, a similar experiment was carried out on the cyclinE-luciferase reporter gene. The *cyclinE* promoter is a well-defined physiological target of E2F (Ohtani *et al.*, 1995). As expected, E2F-1, or DP-1 transfected together with the *cyclinE*-luciferase reporter gene caused an increase in the level of transcription, and $p14^{ARF}$ had no significant effect on the *cyclinE*-luciferase gene activity (Figure 3.3C). Together E2F-1 and DP-1 co-operated to increase the amount of reporter activity relative to the level seen with either E2F-1 or DP-1 alone (Figure 3.3C). When $p14^{ARF}$ was transfected together with E2F-1, DP-1 and *cyclinE*-luciferase a down-regulation of E2F mediated transcription was again observed (Figure 3.3C).

3.5 $p14^{ARF}$ may down-regulate E2F mediated apoptosis through down-regulation of *apaf-1* transcription.

Recent studies have identified the gene for the apoptotic factor *apaf-1* as a transcriptional target for E2F-1 (Moroni *et al.*, 2001). It was therefore of interest to investigate the effects of $p14^{ARF}$ on *trans*activation of this promoter by E2F-1. Firstly, the reporter gene constructs (Moroni *et al.*, 2001) were checked for their ability to be activated by both E2F-1 and p53.

As expected (Moroni *et al.*, 2001) the Apaf-1 –871/+208 reporter gene was activated by both E2F-1 and p53 (Figure 3.4A). The Apaf-1 –396/+208 reporter gene was activated by E2F-1 but not by p53 (Figure 3.4B) and the +35/+208 reporter construct

was not activated by E2F-1 (Figure 3.4C). However, unexpectedly, this reporter did respond to p53, an effect not reported by the authors (Figure 3.4C).

In order to investigate the effect of $p14^{ARF}$ on E2F and p53 activity on the Apaf-1 promoter, reporter assays were performed. Firstly, the reporter constructs were transfected into SAOS2 cells along with E2F-1 or p53. All reporter activities were consistent with what was observed above (Figure 3.4) and described by Moroni *et al.* (2001).

Additionally, cells were transfected with $p14^{ARF}$ both alone, and in combination with E2F-1 or p53 in order to determine the effect of $p14^{ARF}$ on the activity of these reporter genes. Firstly the –396 construct was investigated and as expected, this reporter gene was activated by E2F-1 but not by p53 (Figures 3.4 and 3.5A). Additionally, $p14^{ARF}$ had no significant effect on this promoter, however when transfected together with E2F-1, $p14^{ARF}$ caused a decrease in the reporter activity observed when compared with E2F-1 alone. This decrease was dose-dependent and consistent with previous results obtained from E2F-1 responsive promoters (Figures 3.2B and 3.3) $p14^{ARF}$ and p53 transfected together in this experiment showed no difference compared to p53 alone, indicating that $p14^{ARF}$ is not affecting p53 activity (Figure 3.4A).

From these combined experiments it is possible to conclude that $p14^{ARF}$ can specifically down-regulate E2F-1, and consequently E2F-1/DP-1 mediated transcription. This effect may have physiological significance in that $p14^{ARF}$ may be

able to down-regulate the expression of cellular proteins, such as cyclinE, Apaf-1 and $p14^{ARF}$, which are regulated by E2F-1.

3.6 $p14^{ARF}$ can activate transcription and co-operate with E2F-1 to *trans*activate reporter gene constructs.

The Apaf-1-871/+208 reporter construct was also investigated in SAOS2 cells, this reporter gene responded to both E2F-1 and p53 as previously described (Figure 3.4A and 3.5B). $p14^{ARF}$ was transfected alone and in combination with both E2F-1 and p53. Unexpectedly, $p14^{ARF}$ caused an increase in reporter gene activity when compared to mock (Figure 3.5B, 2 fold increase). In contrast to previous observations, $p14^{ARF}$ co-operated with E2F-1 to increase reporter gene activity (Figure 3.5B). When p53 and $p14^{ARF}$ were transfected together with the Apaf-1 – 871/+208 reporter gene an increase in reporter gene activity was observed when compared to p53 alone (Figure 3.5B).

As a negative control for these experiments, the Apaf-1 +35/+208 reporter gene construct was used. This construct lacks both the p53 and E2F-1 binding sites, however has been shown previously (Figure 3.4C) to be responsive to p53. As expected, E2F-1 was unable to activate reporter gene expression, and p53 caused a slight activation of this promoter construct (Figure 3.4C). When $p14^{ARF}$ was transfected with the +35/+208 Apaf-1 reporter gene construct, activation of the reporter gene was observed (Figure 3.5C), and when $p14^{ARF}$ and E2F-1 were transfected together then an increase in activation was observed (Figure 3.5C).

$p14^{ARF}$ also caused an activation of this promoter when transfected together with p53 (Figure 3.5C).

These results suggest a role for $p14^{ARF}$ in transcriptional activation, and that it can positively regulate the transcription of certain reporter genes in combination with site-specific DNA binding transcription factors.

3.7 $p14^{ARF}$ can overcome E2F mediated S-phase entry.

Given that E2F is a transcription factor that plays a role in the regulation of S-phase entry, through expression of genes required for S-phase progression, and that $p14^{ARF}$ is able to down-regulate E2F dependent transcription it was of interest to ask if $p14^{ARF}$ had any effect on cell cycle progression. In order to investigate the role of E2F-1 and $p14^{ARF}$ in cell cycle progression, a BrDU assay was employed. Cells incorporating BrDU were counted and normalised for transfection efficiency.

E2F-1 over-expression in p53 and pRb negative SAOS2 cells led to an increase in the number of cells undergoing S-phase, over the mock transfected population (Figure 3.6 A, B). DP-1 and $p14^{ARF}$ over-expression also caused an increase in the number of cells in S-phase relative to mock transfected cells (Figure 3.6 A, B). As expected, when E2F-1 and DP-1 were expressed together an increase in the number of S-phase cells over either E2F-1 or DP-1 alone was observed (Figure 3.6 A, B) and when $p14^{ARF}$ was over-expressed together with E2F-1 and DP-1 the number of cells in S-phase reverted back to that observed with $p14^{ARF}$ alone.

These data support the observations made from the reporter assay experiments. As $p14^{ARF}$ is able to down-regulate E2F mediated transcription, then it is not surprising that this also leads to a cell cycle arrest. Also, the data support a physiological consequence of $p14^{ARF}$ having an effect on E2F, namely a block of cell cycle progression.

3.8 $p14^{ARF}$ down-regulates E2F mediated cell growth

It has previously been shown that expression of E2F-1 and DP-1 together in a colony forming assay resulted in a much decreased level of colonies than with empty vector alone (Loughran and La Thangue., 2000), suggesting that the E2F-1 and DP-1 heterodimer has tumour suppressor properties. As $p14^{ARF}$ overcomes E2F mediated cellular proliferation (Figure 3.6A) I reasoned that $p14^{ARF}$ may also co-operate with E2F in cell viability.

As expected from previous experiments E2F-1 and E2F-1/DP-1 together caused a significant decrease in the number of colonies observed from the empty vector control, however DP-1 alone had no significant effect over the pcDNA control population (Figure 3.7B). $p14^{ARF}$ alone reduced colony formation, and when expressed together with E2F-1 and DP-1 caused a decrease in viability compared to E2F-1 and DP-1 alone (Figure 3.7 A,B). This effect of $p14^{ARF}$ on E2F-1-DP-1 was suggestive of a role for $p14^{ARF}$ and E2F-1/DP-1 in the suppression of cell growth.

3.9 Conclusions

The experiments performed in this chapter show that $p14^{ARF}$ can down-regulate E2F transcription, and overcome E2F-1/DP-1 mediated apoptosis, cell cycle progression and growth. These data show that $p14^{ARF}$ is able to down-regulate E2F-1 activity and that the observed effects on apoptosis and cell cycle progression may be linked to down-regulation of E2F target genes. In some situations, $p14^{ARF}$ may be able to act as a transcriptional activator, and increase transcription mediated by other cellular transcription factors. $p14^{ARF}$ has been shown to induce apoptosis and this could be a result of its ability to activate Apaf-1 reporter gene constructs. Further work is required in order to elucidate the mechanisms by which $p14^{ARF}$ regulates E2F function.

3.10 Discussion

The data presented establish the previously described role for $p14^{ARF}$ as a growth suppressor and also suggest a role for $p14^{ARF}$ in the E2F pathway. $p14^{ARF}$ has previously been shown to induce a p53 dependent and independent cell cycle arrest (Quelle *et al.,* 1995; Pomerantz *et al.,* 1998; Zhang *et al.,* 1998; Kurokawa *et al.,* 1999), and these experiments imply that this may be through the targeting of E2F activity. $p14^{ARF}$ is able to down-regulate E2F-1 activity in a number of independent transfection assays, suggesting that these effects may occur *in vivo*.

The fact that E2F-1 is able to activate $p14^{ARF}$ expression, and $p14^{ARF}$ itself can down-regulate E2F-1 mediated expression of the $p14^{ARF}$ promoter suggests the presence of an auto-regulatory feedback loop, where $p14^{ARF}$ is able to regulate its own expression as well as that of other E2F target genes. Many genes can control their own regulation in this manner, an example of such being the p53-MDM2 auto-regulatory feedback loop (Prives, 1998).

Recent data which shows that E2F-1 can *trans*activate the Apaf-1 gene (Moroni *et al.*, 2001), could explain the ability of $p14^{ARF}$ to down-regulate E2F mediated apoptosis, through its transcriptional inhibition of E2F-1 responsive genes. However, the results presented suggest that $p14^{ARF}$ may act as both an activator and repressor of transcription under certain circumstances.

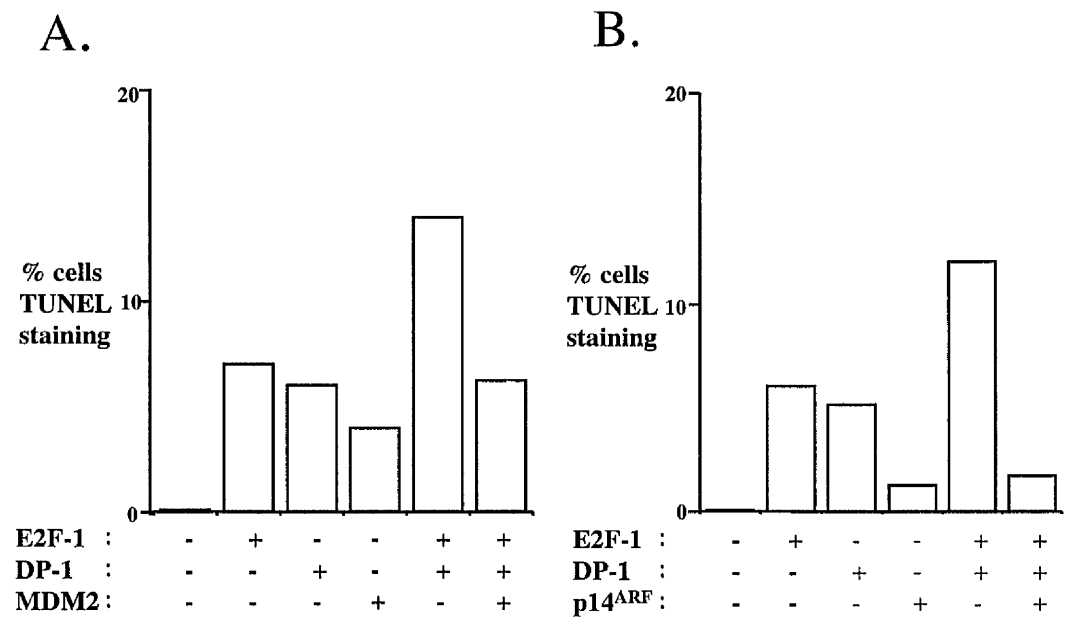
Consistently, $p14^{ARF}$ has been described as a regulator of p53 activity (Pomerantz *et al.*, 1998), these data show that $p14^{ARF}$ may also regulate the E2F pathway in cells and further investigation of the mechanisms involved is required.

Figure 3.1 $p14^{ARF}$ regulates E2F-1/DP-1 mediated apoptosis.

(A) SAOS2 cells were transfected as described with 6μg of each indicated plasmid: pcHDM2, CMV-DP-1, and pRcHA-E2F-1. After transfection cells were washed and further grown overnight in 0.2% fetal calf serum in DMEM. Apoptosis was assayed approx 18 hours post transfection and data is expressed as % apoptosis relative to the total number of transfected cells. The data shown is representative of at least three independent experiments.

(B) SAOS2 cells were transfected as described with 6μg of each $p14^{ARF}$, CMV-DP-1, and pRcHA-E2F-1. After transfection cells were washed and further grown overnight in 0.2% fetal calf serum in DMEM. Apoptosis was assayed 18 hours post transfection and data is expressed as % apoptosis relative to the total number of transfected cells. The data shown is representative of at least three independent experiments.

(C) SAOS2 cells were assayed as described for (A) and (B).


A.
B.
20
10
0
% cells
TUNEL
staining
E2F-1 : - + - - + +
DP-1 : - - + - + +
MDM2 : - - - + - +
E2F-1 : - + - - + +
DP-1 : - - + - + +
p14ARF : - - - + - +

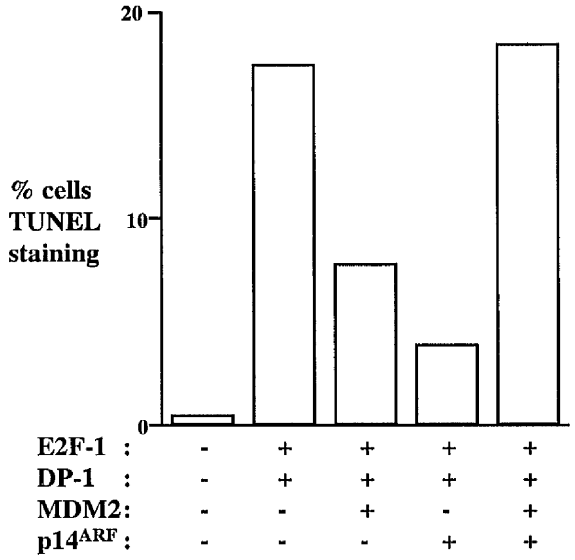
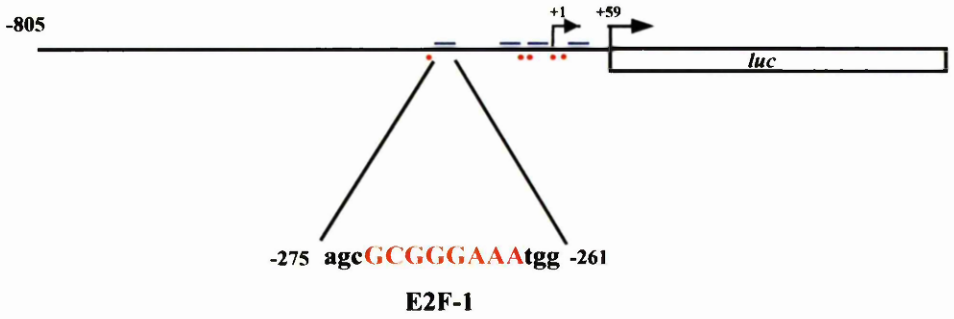

C.
20
10
0
% cells
TUNEL
staining
E2F-1 : - + + + +
DP-1 : - + + + +
MDM2 : - - + - +
p14ARF : - - - + +

Figure 3.2. $p14^{ARF}$ effect on E2F-1 mediated transcriptional activation of the E1β-luciferase promoter.

(A) Schematic representation of the Exon1B-luciferase promoter construct. The four potential anti-sense E2F sites are shown as blue lines and SP1 sites are shown as red spots. The E2F sites at –265 and +27 are good matches to the E2F consensus site (TTTCCCGCCA/TA/T) whereas the sites at –249 and –69 are poor matches (Robertson and Jones, 1998). The indicated initiation codon for luciferase expression is located at +59 and the previously mapped transcriptional start site within the $p14^{ARF}$ promoter is defined as position +1 (Mao et al., 1995). The previously described E2F-1 responsive site at –275 to –261 is highlighted (Bates et al., 1998).

(B) SAOS2 cells were transfected with the following plasmids per 6cm plate; pRcHA-E2F-1 (50ng), CMV-DP1 (500ng), $p14^{ARF}$ (250ng,500ng,750ng,1μg respectively), E1β-luciferase (4μg), CMV-βgal (500ng). Lysates were assayed 40 hours post-transfection and each assay was performed in duplicate. Data is shown as luciferase/βgal value. The data shown is representative of at least three typical experiments.

A.


-805
+1
+59
luc
-275 agcGCGGGAAAtgg -261
E2F-1

B.

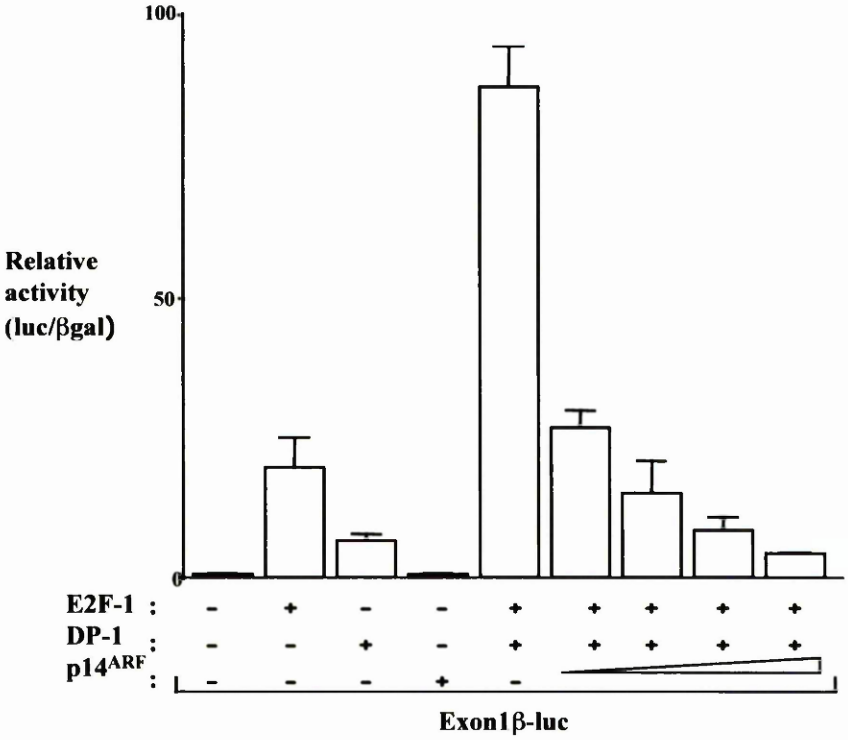

100
50
0
Relative activity (luc/βgal)
E2F-1 :
DP-1 :
p14ARF :
Exon1β-luc

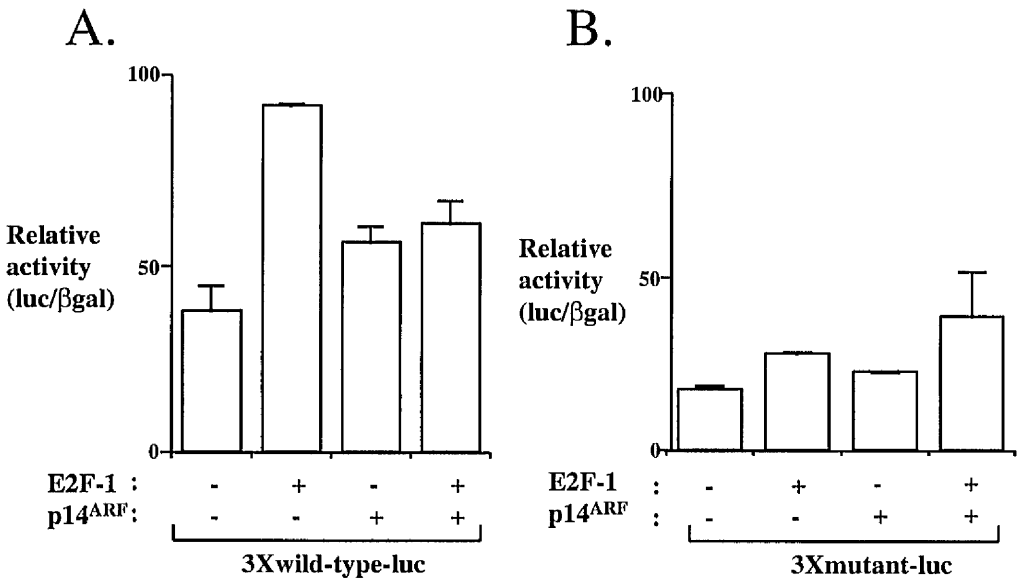
Figure 3.3. $p14^{ARF}$ effect on E2F1 mediated transcriptional activation of E2F-1 responsive genes.

(A) SAOS2 cells were transfected with the following amounts of plasmids per 6cm plate; 3X wild-type-luciferase (1μg), CMV-βgal (500ng), pRcHA-E2F-1 (500ng) $p14^{ARF}$ (500ng).

(B) SAOS2 cells were transfected as described in (A) except 3X mutant-luciferase (1μg) was used in place of 3X wild-type-luciferase.

(C) SAOS2 cells were transfected with the following amounts of the indicated plasmids per 6cm plate: CyclinE-luciferase (200ng), CMV–βgal (500ng), pRcHA-E2F-1 (200ng), CMV-DP1 (1μg), $p14^{ARF}$(500ng).

(A), (B) and (C). Lysates were harvested 40 hours post-transfection and each plate was performed in duplicate. Data are shown as luciferase/βgal value. The data shown is representative of at least three experiments.


A.
B.
100
50
0
Relative activity (luc/βgal)
E2F-1 :
p14ARF:
- + - +
- - + +
3Xwild-type-luc
3Xmutant-luc

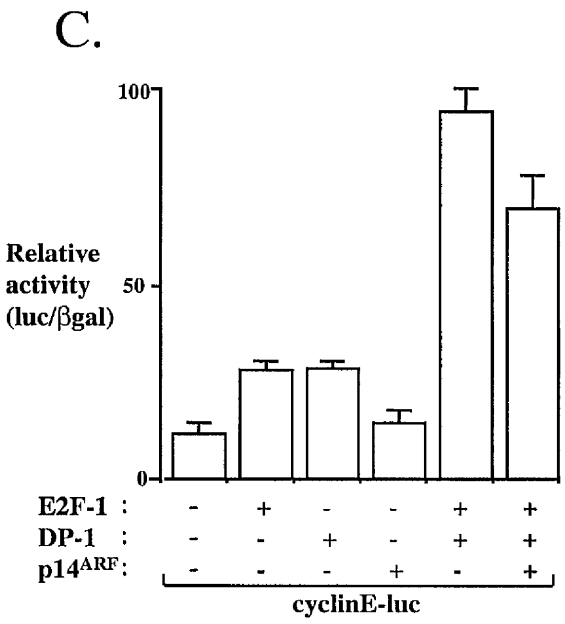

C.
100
50
0
Relative activity (luc/βgal)
E2F-1 :
DP-1 :
p14ARF:
- + - - + +
- - + - + +
- - - + - +
cyclinE-luc

Figure 3.4. Characterisation of the *Apaf-1* reporter genes.

SAOS2 cells were transfected with the following plasmids per 6cm plate; pRcHA-E2F-1 (500ng, 1μg), p53 (1μg, 2μg), Apaf-1 reporter gene (2μg), CMV-βgal (500ng). Lysates were assayed 40 hours post-transfection and each assay was performed in duplicate. Data are shown as luciferase/βgal value. The data shown are representative of at least three typical experiments.

(A) The Apaf-1 –871/+208 reporter gene was used.
(B) The Apaf-1 –396/+208 reporter gene was used.
(C) The Apaf-1 +35/+208 reporter gene was used.

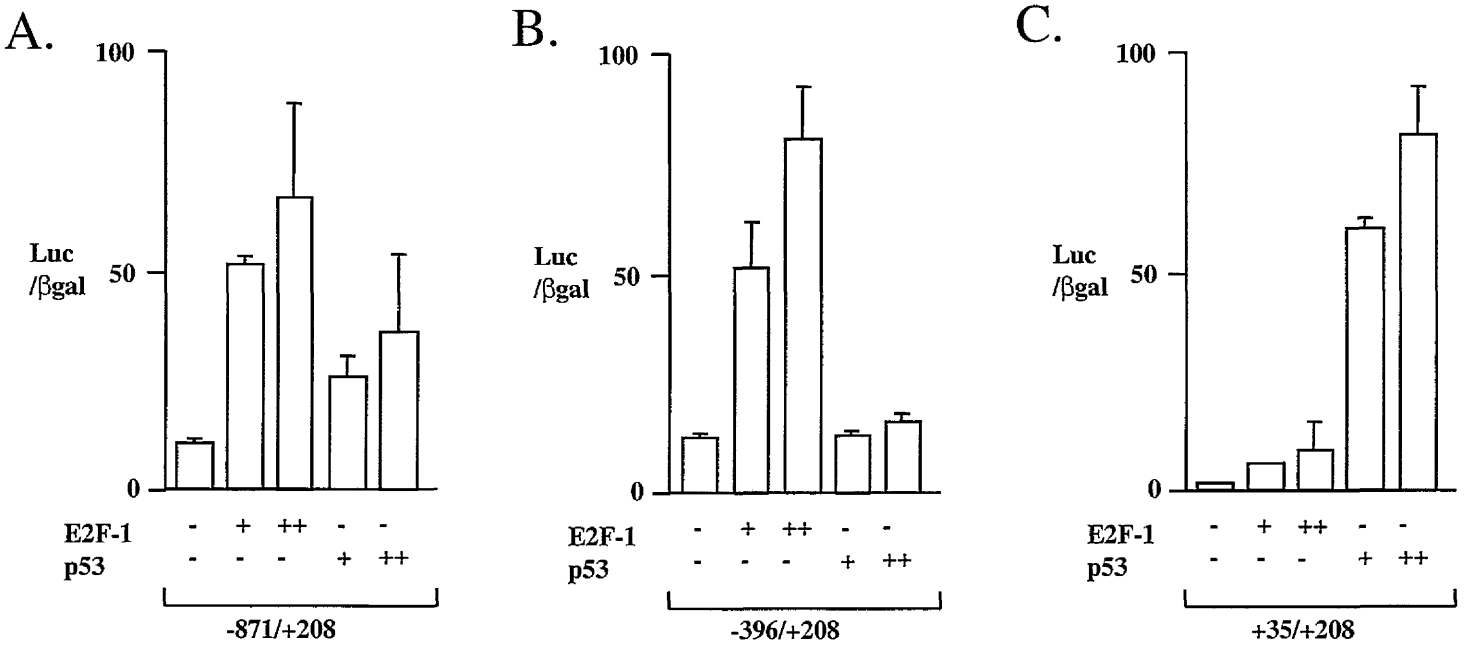
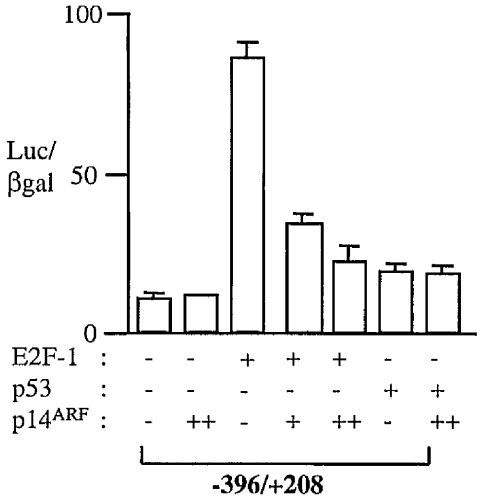

A.
100
Luc
/βgal
50
0
E2F-1 - + ++ - -
p53 - - - + ++
-871/+208
B.
100
Luc
/βgal
50
0
E2F-1 - + ++ - -
p53 - - - + ++
-396/+208
C.
100
Luc
/βgal
50
0
E2F-1 - + ++ - -
p53 - - - + ++
+35/+208

Figure 3.5 $p14^{ARF}$ can regulate the *apaf-1* reporter gene constructs.

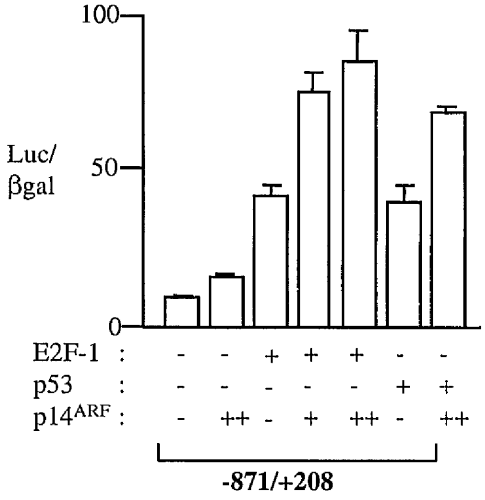
SAOS2 cells were transfected with the following plasmids per 6cm plate; pRcHA-E2F-1 (500ng), p53 (2μg) $p14^{ARF}$ (+3μg, ++ 6μg), Apaf-1 reporter gene (2μg), CMV-βgal (500ng). Lysates were assayed 40 hours post-transfection and each assay was performed in duplicate. Data is shown as luciferase/βgal value. The data shown is representative of at least three typical experiments.

(A) The Apaf-1 –396/+208 reporter gene was used.
(B) The Apaf-1 –871/+208 reporter gene was used.
(C) The Apaf-1 +35/+208 reporter gene was used.

A.


100
Luc/
βgal
50
0
E2F-1 : - - + + + - -
p53 : - - - - - + +
p14ARF : - ++ - + ++ - ++
-396/+208

B.


100
Luc/
βgal
50
0
E2F-1 : - - + + + - -
p53 : - - - - - + +
p14ARF : - ++ - + ++ - ++
-871/+208

C.

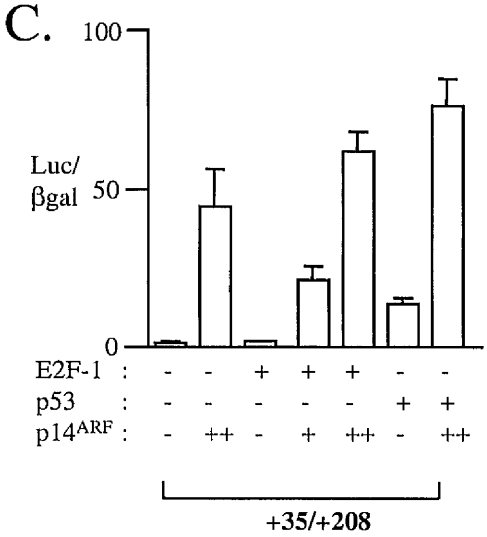
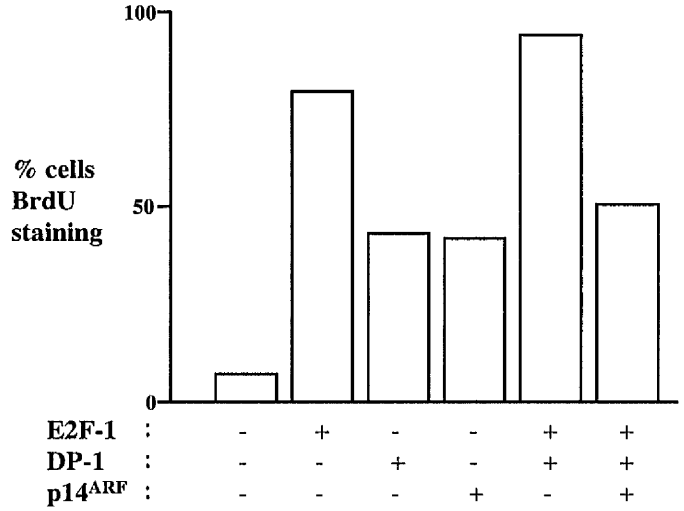

100
Luc/
βgal
50
0
E2F-1 : - - + + + - -
p53 : - - - - - + +
p14ARF : - ++ - + ++ - ++
+35/+208

Figure 3.6 $p14^{ARF}$ down-regulates E2F-1/DP-1 mediated S phase progression.

(A) SAOS2 cells were transfected as described with 5μg of each pRcHA-E2F-1, CMV-DP-1, $p14^{ARF}$. After transfection cells were washed and further grown overnight. BrDU incorporation was assayed 18 hours post-transfection and data is presented as %BrDU incorporated relative to number of transfected cells/β-galactosidase value. The data shown is representative of at least three independent experiments.

(B) BrDU data are presented as number of cells counted. Data shown is from a single experiment. BrDU incorporation was calculated by dividing the number of BrDU positive cells by the total number of cells counted and then normalised for transfection efficiency (determined by βgal activity).

A.



B.

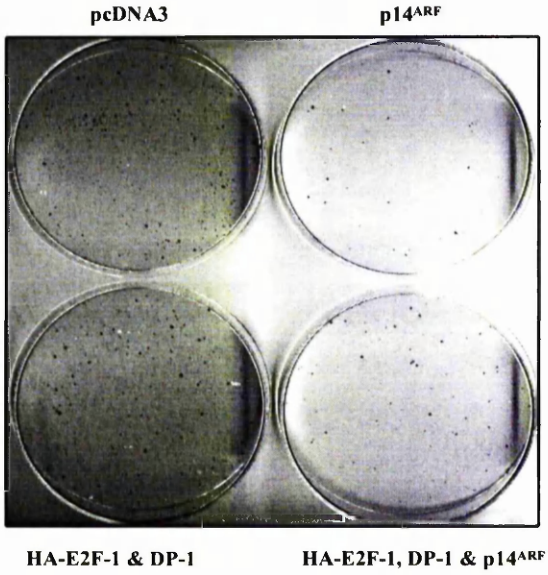
	Total cells counted	β-gal Activity	BrDU +ve	BrDU incorporation relative to β-gal.
Mock	144	39.5	4	8
E2F-1	179	44.6	63	80
DP-1	138	40.4	22	40
$p14^{ARF}$	259	32.4	32	39
E2F-1 & DP-1	279	47.3	125	96
E2F-1, DP-1 &$p14^{ARF}$	199	40.0	36	46

Figure 3.7 $p14^{ARF}$ overcomes E2F mediated colony forming activity.

(A) SAOS2 cells were transfected with 10μg each of pcRHA-E2F-1, CMV-DP-1 and $p14^{ARF}$. Total DNA transfected was normalised with pcDNA3. After 3 days, cells were split 1:5 with non-transfected SAOS2 cells. Transfected cells were selected in G418 antibiotic (Sigma) for 2 weeks and colonies were stained with crystal violet solution.

(B) Graphical representation of colony forming data. Experiments were performed as described in (A). The average number of colonies from the counts was plotted against the pcDNA3 control plate. The data shown is representative of at least three independent experiments.

A.


pcDNA3
p14ARF
HA-E2F-1 & DP-1
HA-E2F-1, DP-1 & p14ARF

B.

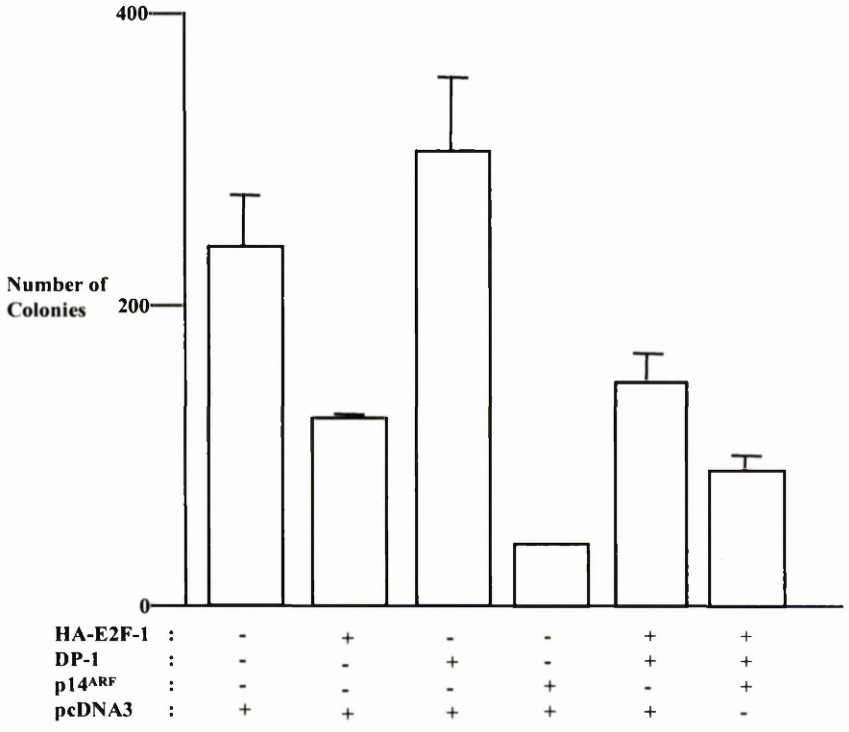
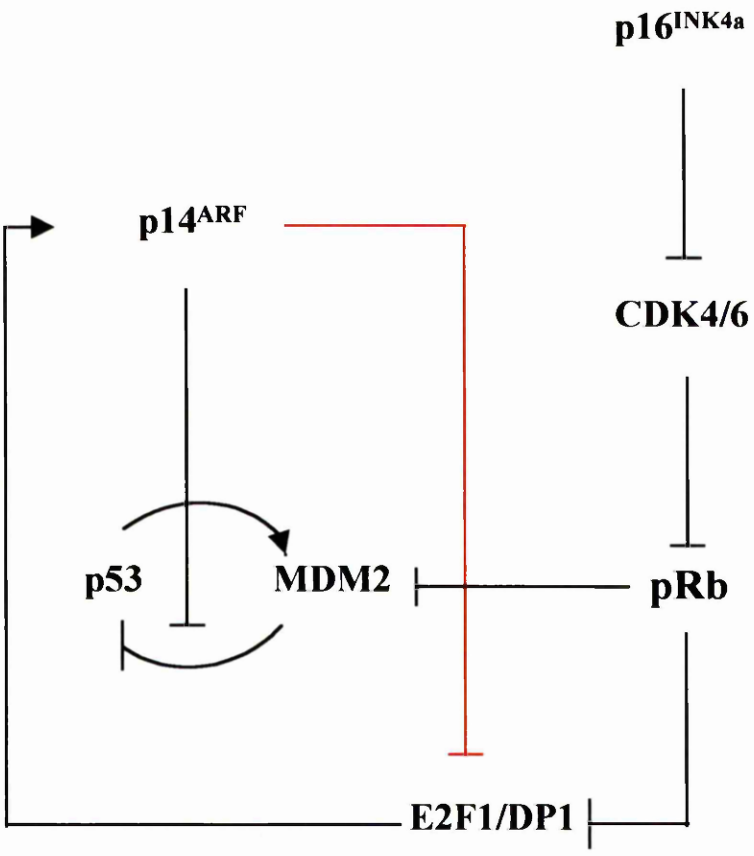

400
200
0
Number of Colonies
HA-E2F-1 : - + - - + +
DP-1 : - - + - + +
p14ARF : - - - + - +
pcDNA3 : + + + + + -

Figure 3.8 Model for $p14^{ARF}$ down-regulation of E2F.

(A) Model for the $p14^{ARF}$-E2F-1 auto-regulatory feed-back loop. Black lines indicate previously identified pathways. The red line shows the $p14^{ARF}$ mediated down-regulation of E2Factivity.


p16INK4a
p14ARF
CDK4/6
p53
MDM2
pRb
E2F1/DP1

Chapter 4. $p14^{ARF}$ and E2F-1 biochemical analysis.

4.1 Introduction

The interactions between cellular proteins are commonly required for them to regulate the activity of one another. E2F-1 binds to, and is regulated by a large number of cellular proteins including; pRb (Helin *et al.*, 1993), cyclin A (Krek *et al.*, 1993) and MDM2 (Martin *et al.*, 1995).

Both $p14^{ARF}$ and $p19^{ARF}$ have been shown to bind directly to MDM2 and form a ternary complex with p53 (Pomerantz *et al.*, 1998). Whether or not $p14/19^{ARF}$ is able to bind directly to p53 or not is yet to be elucidated but evidence suggests that it is unable to bind p53 directly (Stott *et al.*, 1998; Zhang *et al.*, 1998). Recently $p14^{ARF}$ has been shown to bind to topoisomerase I, a protein required for S-phase progression (Karayan *et al.*, 2001).

Given that $p14^{ARF}$ can regulate the activity of E2F, it is of interest to investigate whether E2F-1 and DP-1 can bind to $p14^{ARF}$, and if this binding occurs in cells. Additionally, it is also important to attempt to elucidate the domains in E2F-1 and DP-1 which $p14^{ARF}$ can interact with in order to provide us with further clues as to the method of regulation of E2F by $p14^{ARF}$.

Results

4.2 $p14^{ARF}$ interacts with E2F-1 and DP-1

The ability of $p14^{ARF}$ to interact with MDM2 and regulate p53 has been well documented (Zhang *et al.,* 1998; Weber *et al.,* 1999; Zhang and Xiong, 1999; Lohrum *et al.,* 2000). Given that $p14^{ARF}$ can regulate E2F activity it was of interest to ask whether $p14^{ARF}$ is able to interact with either the E2F or DP components of the heterodimer. In order to address this question, a GST binding assay in a cell free system was utilised, and the ability of *in vitro* translated MDM2, E2F-1 and DP-1 to interact with a bacterially purified GST-$p14^{ARF}$ protein was examined (Figure 4.1A and 4.1B). E2F-1, DP-1 and a equimolar mixture of the two proteins were all able to bind to bacterially expressed GST-$p14^{ARF}$. The specificity of each binding reaction was confirmed by the inability of a non-specific luciferase control protein to bind. In all cases GST alone failed to bind to any of the *in vitro* translated proteins (Figure 4.1B).

In order to confirm this reaction with endogenous E2F-1 the same GST and GST-$p14^{ARF}$ proteins (Figure 4.1Aii) were used in a pull-down assay from cell extracts. p53 null SAOS2 cells were used in order to rule out the involvement of p53 in any interaction. Endogenous E2F-1 binds efficiently to GST-$p14^{ARF}$, but not to the GST protein (Figure 4.1C) suggesting that this interaction may indeed exist *in vivo* and so may be physiologically significant.

4.3 Components of the E2F complex interact with p14^{ARF} *in vivo*

In order to investigate whether E2F-1 and DP-1 could bind *in vivo to* p14^{ARF}, an endogenous immunoprecipitations was performed. HeLa cells, which have the p53 pathway inactivated were used for the endogenous immunoprecipitations due to their high levels of p14^{ARF}. As expected p14^{ARF} immunoprecipitated efficiently with MDM2 (Figure 4.2) and failed to immunoprecipitate with a non-specific antibody (Figure 4.2). When immunoprecipitations were performed with an E2F-1 antibody, p14^{ARF} was detected in the immunoprecipitates (Figure 4.2) and in reverse, when a p14^{ARF} antibody was used to immunoprecipitate, E2F-1 was detected in the immunoprecipitations (Figure 4.2). Additionally, MDM2 was present in the E2F-1 and p14^{ARF} immunoprecipitations but E2F-1 was not present in the MDM2 immunoprecipitate (Figure 4.2).

4.4 p14^{ARF} binds to a region in the heterodimerisation domain of DP-1

A GST binding assay approach was used in SAOS2 cells to confirm the DP-1 interaction in cell extracts. Cell extracts were prepared from *p53*$^{-/-}$ *rb*$^{-/-}$ SAOS2 cells and endogenous DP-1 was found to interact specifically with GST-p14^{ARF}, but not with GST protein alone (Figure 4.3A). In order to gain further insight into the DP-1-p14^{ARF} interaction a cell free pull down system was utilised to investigate the binding properties of *in vitro* translated DP-1 mutants (Figure 4.3B). The N-terminal deleted mutants in DP-1 delta 97, delta171 and an internal fragment of DP-1, encompassing the heterodimerisation domain 205-310 were all

able to bind efficiently to GST-p14^{ARF}. In contrast an N-terminal mutant deleted up to amino acid 242, encompassing the C terminal region of DP-1 was unable to bind to GST-p14^{ARF}. In all cases, GST protein alone failed to bind to any of the *in vitro* translated proteins (Figure 4.3C). These data suggest that p14^{ARF} binds to DP-1 in a region within DP-1 205 to DP-1 242. As this is the region in DP-1 which binds to E2F, it is possible that p14^{ARF} may affect heterodimer formation and hence explain its ability to compromise the function of E2F in transcription and apoptosis.

4.5. p14^{ARF} binds to a number of distinct domains in E2F-1.

Given that p14^{ARF} binds to the heterodimerisation domain in DP-1, and that E2F-1 has a similar domain, it was of interest to determine whether p14^{ARF} also bound to the heterodimerisation region in E2F-1, and if not, then which region of E2F-1 does p14^{ARF} bind to.

A number of mutants in E2F-1 (Figure 4.4A) were *in vitro* translated and tested for binding to GST-p14^{ARF} in the pull-down assay system. The E2F-1 mutants E2F-1-Y411C, a point mutant compromised in pRb binding (Helin *et al.*, 1992) and E2F-1 delta C, lacking the *trans*activation domain were able to bind p14^{ARF} with equal affinity to wild-type E2F-1 (Figure 4.4B).

In order to further identify binding domains for p14^{ARF} in E2F-1, E2F-1 delta 24, compromised in cyclin A binding, and E2F-1 constructs encompassing the regions from amino acid 141-261 and 181-261 were *in vitro* translated and tested for

binding to GST-p14^{ARF}. Both of these central regions of E2F-1 were able to bind to p14^{ARF} (Figure 4.4C), suggesting a role for the DNA binding domain in the ARF-E2F-1 interaction. These data suggest that p14^{ARF} may bind to the DNA binding domain in E2F-1 and prevent E2F-1 from contacting the DNA and activating transcription. In addition to this, the delta 24 E2F-1 mutant could also bind to GST-p14^{ARF} (Figure 4.4C), indicating that the inability to bind to cyclin A does not affect ARF-E2F-1 binding.

4.6 Conclusions

These experiments show that p14^{ARF} and E2F-1, and p14^{ARF} and DP-1 can interact with one another both *in vivo* and *in vitro*. Mapping data indicates that a number of binding domains exist for p14^{ARF} in E2F-1 and that the binding domain for p14^{ARF} in DP-1 is in the heterodimerisation domain.

4.7 Discussion

Experiments performed both *in vivo* and *in vitro* indicate that p14^{ARF} can bind to both components of the E2F heterodimer, E2F-1 and DP-1. The interaction between p14^{ARF} and E2F-1 occurs in cells and so may be physiologically significant for the regulation of E2F activity by p14^{ARF}. This binding of E2F-1 to p14^{ARF} could be direct or could be mediated by a cellular protein, and further work is required to investigate these possibilities. The fact that MDM2 is detected in p14^{ARF} and E2F-1 immunoprecipitates also indicates that the three proteins may exist in a trimeric complex, and that p14^{ARF} regulation of E2F-1 could be mediated through MDM2.

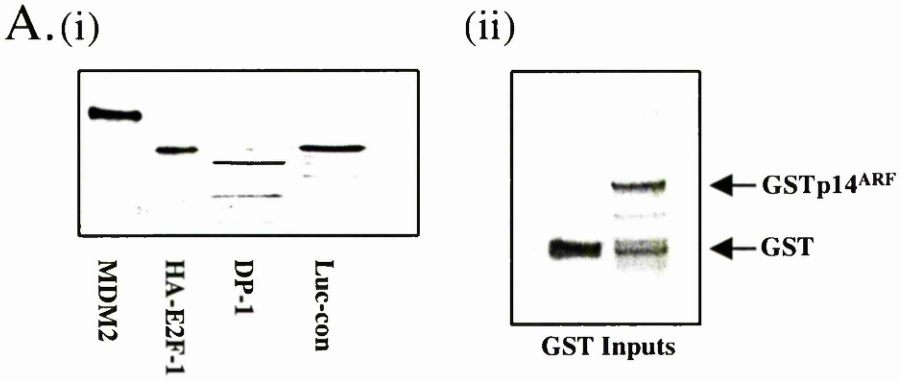
The mapping data presented suggest that $p14^{ARF}$ may be able to disrupt the E2F-1-DP-1 interaction by binding to the heterodimerisation domain in DP-1, however further mapping of the E2F-1 interaction domain is required to confirm this observation. It is possible that $p14^{ARF}$ may act to prevent E2F-DP heterodimerisation, and prevent efficient heterodimer activities, although both proteins are able to bind to $p14^{ARF}$ at the same time (Figure 4.1B). $p14^{ARF}$ is able to bind to a number of domains in E2F-1 (Figure 4.4B, C). More work is required to identify the exact binding domains in E2F and their function in cells in order to fully understand the E2F-$p14^{ARF}$ interaction and its physiological consequences.

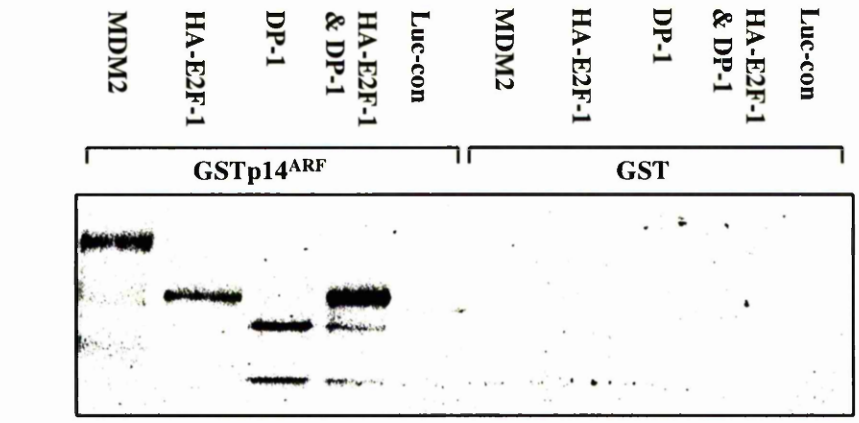
Figure 4.1 $p14^{ARF}$ binds to E2F-1 and its heterodimeric partner DP-1

(A) (i) *in vitro* translated inputs used for pull-down assay. The indicated plasmids were *in vitro* translated and 10% of each input ran on an SDS-PAGE gel. (ii) GST proteins were purified from bacteria and expression verified by SDS-gel elecrophoresis.

(B) Equal amounts of pcMDM2, pRcHA-E2F-1, CMV-DP-1, and firefly luciferase *in vitro* translates were incubated with approx. 1 μg GST or GST-$p14^{ARF}$protein (determined by electrophoresis) for 1 hour at 4°C. Reactions were washed 3 times before SDS-PAGE analysis and exposure to film overnight.

(C) Nuclear extracts were prepared from SAOS2 cells. Equal amounts of extract (200μg) were incubated with GST or GST-$p14^{ARF}$ overnight at 4°C. Samples were washed 3 times before SDS-PAGE analysis and western blotting with an E2F-1 specific antibody KH95 (Santa Cruz).


A.
(i)
(ii)
MDM2
HA-E2F-1
DP-1
Luc-con
GSTp14ARF
GST
GST Inputs


B.
MDM2
HA-E2F-1
DP-1
HA-E2F-1 & DP-1
Luc-con
GSTp14ARF
GST

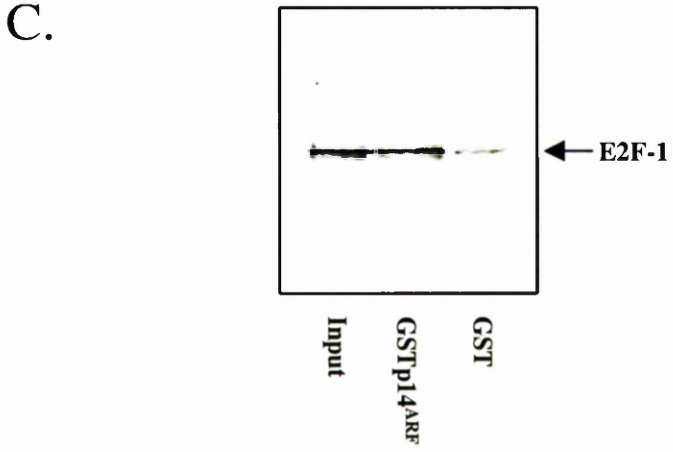

C.
E2F-1
Input
GSTp14ARF
GST

Figure 4.2 $p14^{ARF}$ binds to E2F-1in cells.

HeLa cell nuclear extract (Computer Cell Culture) (500μg) was incubated in 100μl TNE with anti-E2F-1, anti-$p14^{ARF}$, anti-MDM2 or anti-HA antibody overnight at 4°C with rotation. Protein A agarose was then added to samples for 3 hours at 4°C with rotation. Immunoprecipitates were washed 3 times in binding buffer and resuspended in 3x SDS loading buffer, analysed by SDS-page and western blotting.

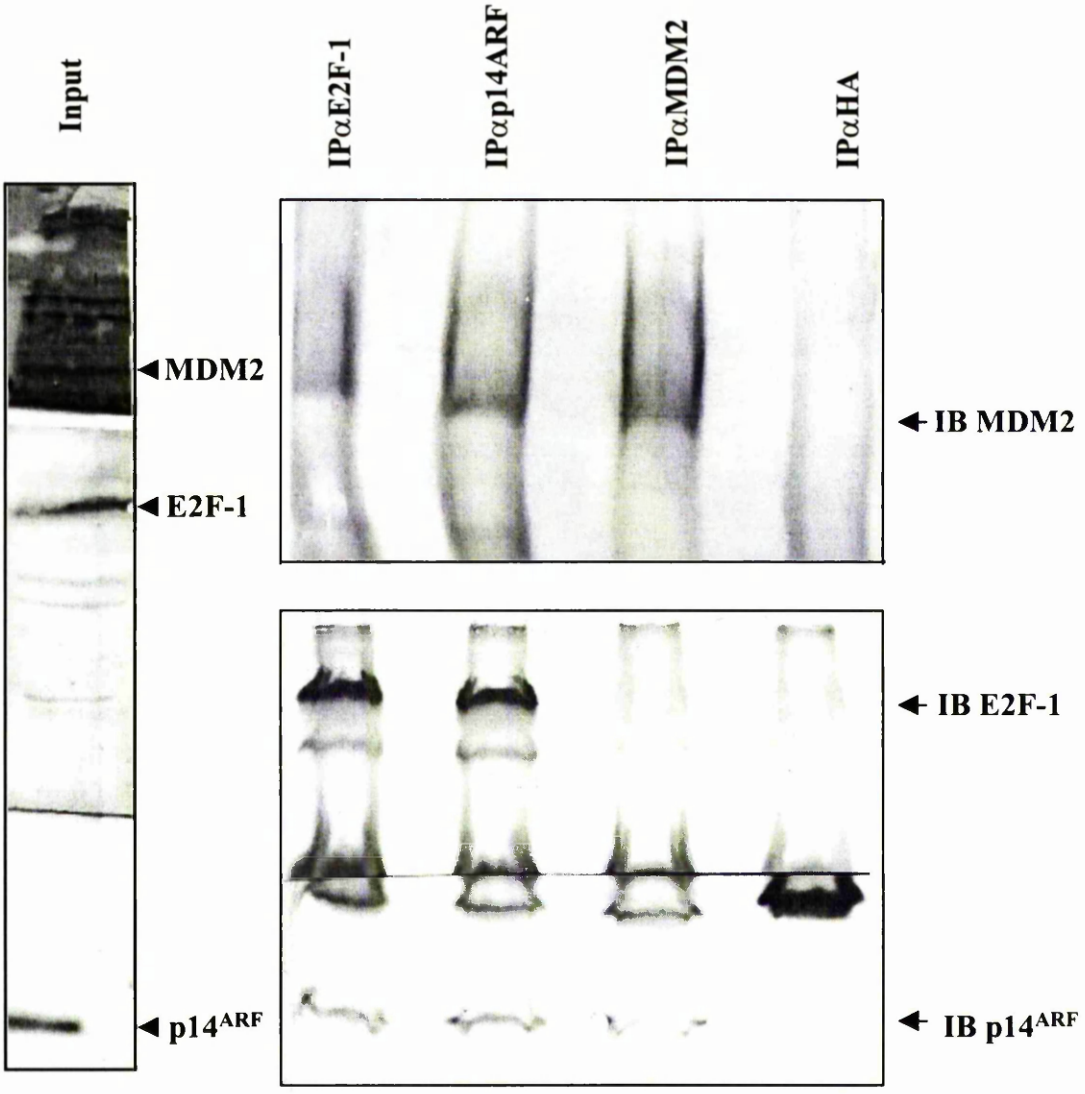

Input
IPαE2F-1
IPαp14ARF
IPαMDM2
IPαHA
MDM2
E2F-1
p14ARF
IB MDM2
IB E2F-1
IB p14ARF

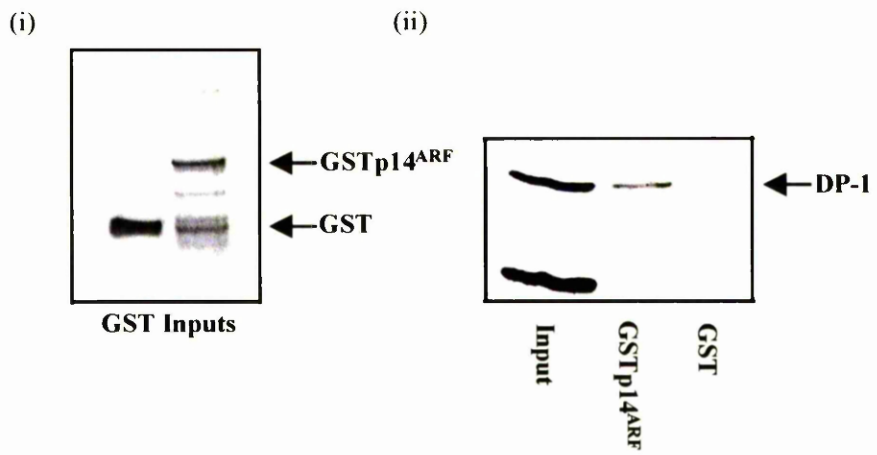
Figure 4.3 $p14^{ARF}$ binds to DP-1

(A) (i) Nuclear extracts prepared from SAOS2 cells were incubated with approx. 1μg purified GST protein overnight at 4°C. Samples were washed and resuspended in SDS loading buffer prior to SDS-PAGE analysis. (ii)Western blotting was performed with the DP-1 specific anti-sera 098 ().

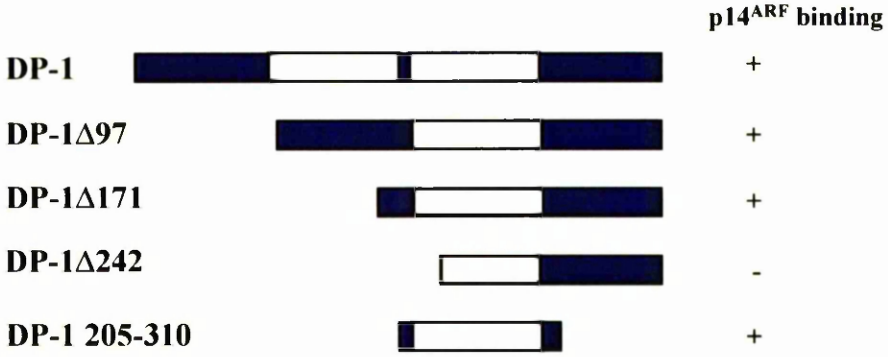
(B) Schematic of mutant constructs used in the *in vitro* binding assay, and their binding to E2F-1 and $p14^{ARF}$.

(C) The indicated DP-1 constructs (Figure 3.6B) were *in vit*ro translated and an equimolar amount of each protein was incubated with approx. 1μg of purified GST protein for 1 hour at 4°C. Samples were washed before re-suspension in SDS loading buffer and SDS-PAGE analysis. Gels were dried and exposed on film overnight.

A.


(i)
(ii)
GSTp14ARF
GST
GST Inputs
DP-1
Input
GSTp14ARF
GST

B.


p14ARF binding
DP-1
+
DP-1Δ97
+
DP-1Δ171
+
DP-1Δ242
-
DP-1 205-310
+

C.

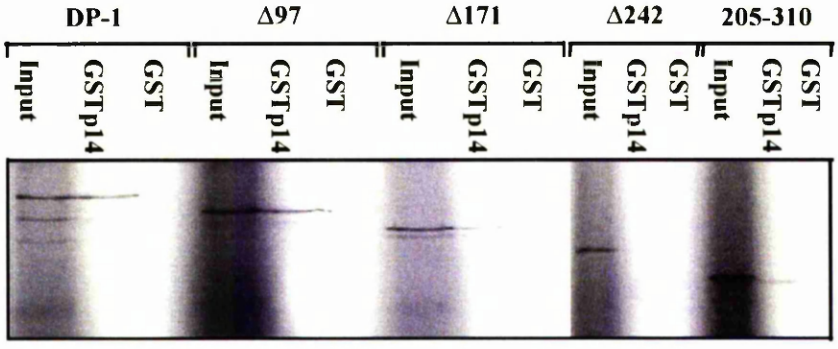
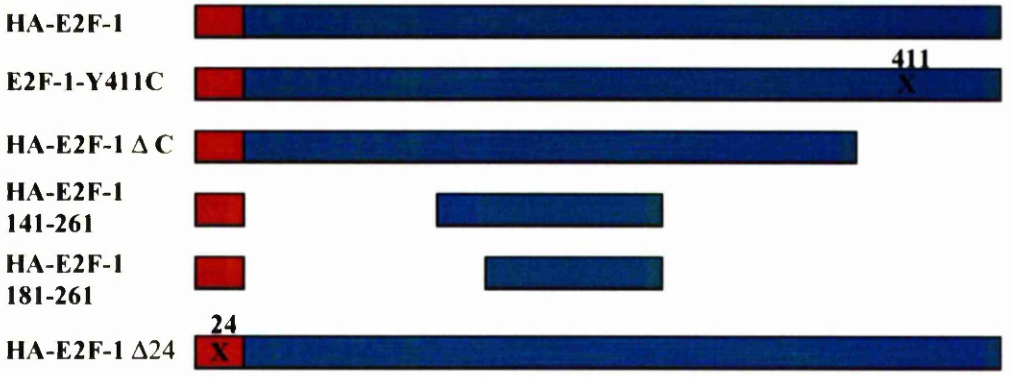

DP-1
Δ97
Δ171
Δ242
205-310
Input
GSTp14
GST

Figure 4.4. $p14^{ARF}$ binds to a number of domains in E2F-1.

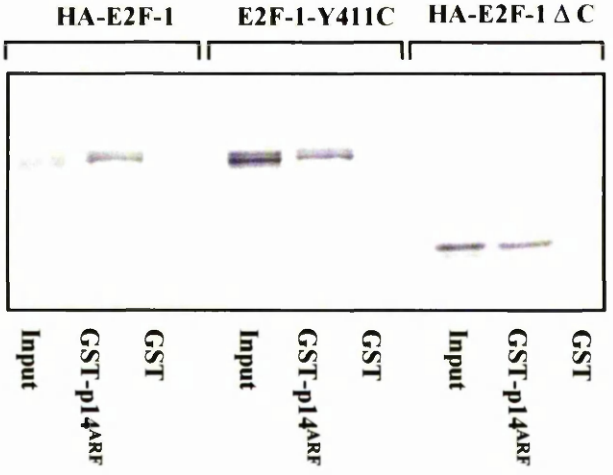
(A) The pRc-HA-E2F-1, YC11C and pRcHAE2F-1deltaC plasmids were *in vitro* translated and an equimolar amount of each *in vitro* translate was incubated with 1μg of GST or GST-$p14^{ARF}$ protein for 1 hour at 4°C. Reactions were washed extensively before being subjected to SDS-page analysis.

(B) The plasmids HAE2F-1, HA-E2F-1 delta 24, 141-261 and HAE2F-1 181-261 were *in vitro* translated and analysed for $p14^{ARF}$ binding as described in (A).

A.


HA-E2F-1
E2F-1-Y411C
411
X
HA-E2F-1 Δ C
HA-E2F-1
141-261
HA-E2F-1
181-261
24
X
HA-E2F-1 Δ24

B.


HA-E2F-1
E2F-1-Y411C
HA-E2F-1 Δ C
Input
GST-p14ARF
GST
Input
GST-p14ARF
GST
Input
GST-p14ARF
GST

C.

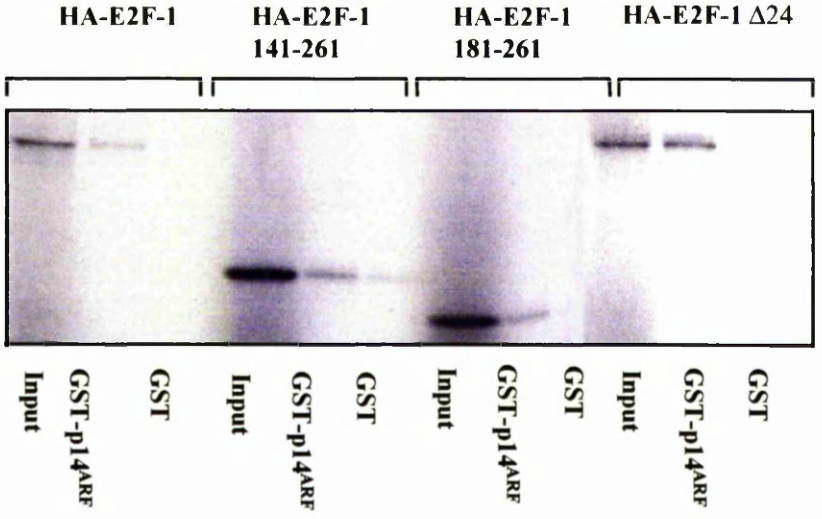
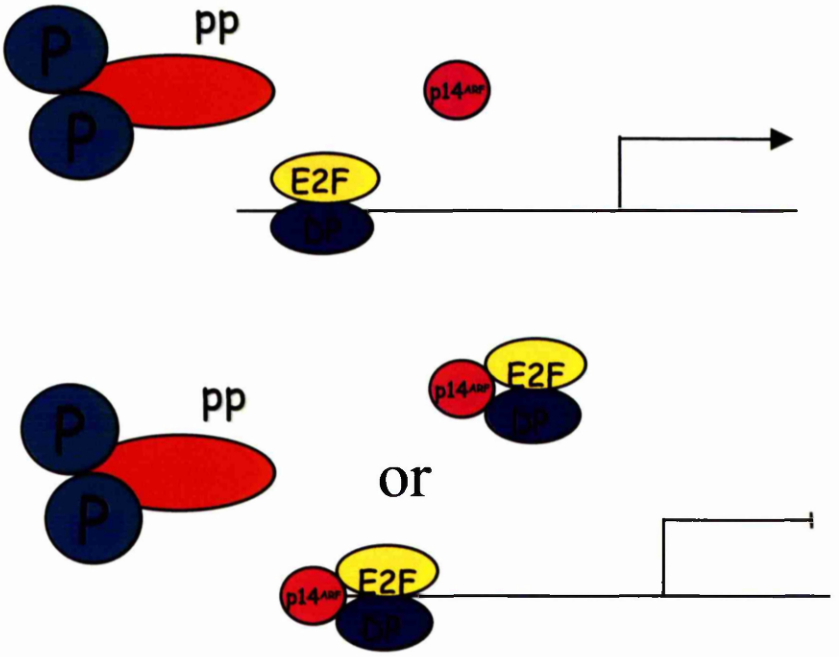

HA-E2F-1
HA-E2F-1
141-261
HA-E2F-1
181-261
HA-E2F-1 Δ24
Input
GST-p14ARF
GST
Input
GST-p14ARF
GST
Input
GST-p14ARF
GST
Input
GST-p14ARF
GST

Figure 4.5 Model for E2F regulation by $p14^{ARF}$.

Diagram showing possible mechanism for regulation of E2F activity through binding to $p14^{ARF}$. Red ovals indicate pocket proteins (pp) and green circles (P) phosphorylation.


pp
P
P
p14ARF
E2F
DP
pp
P
P
p14ARF
E2F
DP
or
p14ARF
E2F
DP

Chapter 5. $p14^{ARF}$ regulation of E2F is MDM2 independent.

5.1 Introduction

MDM2 was first identified as an amplified gene in a transformed mouse 3T3 cell line (Cahilly-Snyder *et al.,* 1987). Since then it has been shown that the human form of MDM2 is over-expressed in a number of human tumours, particularly sarcomas, leukaemias, breast carcinomas and malignant gliomas (Cordon-Cardo *et al.,* 1994, Ladanyi, *et al.,* 1993, Leach *et al.,* 1993, Sheikh *et al.,* 1993).

MDM2 is involved in a negative feedback loop with its transcriptional activator, p53 (Wu *et al.,* 1993). p53 up-regulates MDM2 expression, and in turn MDM2 can export p53 from the nucleus, and target it for ubiquitin mediated degradation (Haupt, *et al.,* 1997; Honda et al. 1997). MDM2 can also bind directly to the p53 *trans*activation domain and down-regulate p53 dependent transcription and apoptosis (Haines *et al.,* 1994). The functional relationship between MDM2 and p53 is dramatically illustrated by the finding that a p53-null state in mice can completely rescue the early embryonic-lethal phenotype of MDM2 deficiency (Jones *et al.*, 1995), demonstrating that the p53-MDM2 pathway is essential in development, and that the regulation of p53 by MDM2 in development is crucial.

Both $p14^{ARF}$ and $p19^{ARF}$ have been shown to bind directly to MDM2 and form a ternary complex with p53 (Pomerantz *et al.,* 1998). MDM2 normally shuttles between the nucleus and the cytoplasm in order to target p53 for degradation (Roth *et al.*, 1998). Honda *et al.* (1999) demonstrated that association of $p19^{ARF}$

with MDM2 inhibits the ubiquitin ligase activity of MDM2 for p53, and that the activity of a $p19^{ARF}$-MDM2 complex in a ubiquitination assay is lower than the activity of free MDM2 (Honda *et al.,* 1999). These data highlight the importance of $p19^{ARF}$ mediated p53 stabilisation.

Co-expression of $p19^{ARF}$ and MDM2 prevents nuclear-cytoplasmic shuttling of MDM2, and as time increases, MDM2 can move to the nucleolus (Tao *et al.,* 1999). Much data supports this MDM2 nucleolar localisation, and re-localisation of MDM2 also occurs following Myc activation and replicative senescence (Weber *et al.,* 1999). Data imply that $p19^{ARF}$ binding to MDM2 may induce a conformational change that facilitates nucleolar import of the ARF-MDM2 complex (Weber *et al.,* 2000)

In order to investigate the role of MDM2 in the p53-ARF pathway, triple knock out (TKO) mice were generated lacking $p19^{ARF}$, MDM2 and p53 (Weber *et al.,* 2000). TKO mice developed more tumours than $p53^{-/-}/mdm2^{-/-}$, or $p53^{-/-}$ mice and the reintroduction of ARF into TKO cells resulted in a G1 arrest (Weber *et al.,* 2000). Interestingly, re-introduction of ARF into $p53^{-/-}/arf^{-/-}$ cells did not result in G1 arrest, implying that $p19^{ARF}$ interacts with targets other than MDM2 to inhibit cell proliferation. MDM2 antagonises some activity of ARF on targets other than p53, and in the absence of MDM2 this other ARF activity is revealed. These data suggest that ARF and p53 loss contributes independently to some aspects of tumour progression (Weber *et al.,* 2000). $p19^{ARF}$ is also able to suppress colony formation in $p53^{-/-}$ cells by targeting the Rb pathway (Carnero *et al.*, 2000), providing further evidence for p53 independent targets for $p14/19^{ARF}$.

Given that MDM2 can regulate E2F activity (Martin et al., 1995; Loughran and La Thangue 2000), and that $p14^{ARF}$ is a known regulator of MDM2 function (Pomerantz et al., 1998), it was of interest to investigate whether $p14^{ARF}$ was controlling E2F activity through MDM2 or an independent mechanism.

5.2 $p14^{ARF}$ can down-regulate E2F mediated transcription in *p53*$^{-/-}$/*mdm2*$^{-/-}$ cells

In order to investigate the role of MDM2 in $p14^{ARF}$ mediated down-regulation of E2F-1 transcription, *p53*$^{-/-}$/*mdm2*$^{-/-}$ mouse embryonic fibroblasts were used for transfection experiments. As these cells lack both the p53 and MDM2 proteins, any effects observed in these cells must occur independently of MDM2. We have previously shown that E2F-1 can activate the activity of two reporter genes, Exon1β-luciferase (Figure 3.2), and 3X wild-type (Figure 3.3A) in $p53^{-/-}$ SAOS2 cells.

In *p53*$^{-/-}$/*mdm2*$^{-/-}$ cells transfected with the 3X wild-type luciferase reporter gene, E2F-1 caused activation, while $p14^{ARF}$ had no significant effect on reporter gene activity (Figure 5.1A). When $p14^{ARF}$ was co-expressed with E2F-1, $p14^{ARF}$ was able to down-regulate E2F-1 mediated transcription to a level comparable to that of $p14^{ARF}$ alone (Figure 5.1A). The same experiment was carried out concurrently on the mutant luciferase promoter. As expected E2F-1 was unable to significantly activate the mutant promoter, due to the inability of E2F to bind to the mutated

E2F binding sites (Figure 5.1B). However, the addition of $p14^{ARF}$ had no effect on the promoter, as did the expression of E2F-1 and $p14^{ARF}$ together.

These data suggest that the observed down-regulation of E2F dependant transcription by $p14^{ARF}$ is specific for E2F, and that $p14^{ARF}$ is not affecting other components of the basal transcriptional complex, in cells lacking MDM2 and p53 as well as in SAOS2 cells. In addition to this, MDM2 is not required for $p14^{ARF}$ to down-regulate E2F-1 mediated transcription.

In order to further investigate the role of MDM2 in the ARF-E2F pathway, and to verify that the observed effects were not promoter specific, the Exon1β-luciferase promoter was also used in the *p53*$^{-/-}$*/mdm2*$^{-/-}$ cells.

p53$^{-/-}$*/mdm2*$^{-/-}$ cells transfected with E2F-1, DP-1 and the $p14^{ARF}$ E1β-luciferase reporter gene showed activation of the reporter (Figure 5.1C). $p14^{ARF}$ transfected with the E1β-luciferase reporter had a slight effect on reporter gene activity (Figure 5.1C), however, $p14^{ARF}$ was able to down-regulate the transcription activation by E2F-1/DP-1 to a similar level as observed in SAOS2 cells (Figure 5.1C). These data suggest that $p14^{ARF}$ is able to act to down-regulate E2F transcription in an MDM2 independent manner, and as a result of this down-regulation may consequently act to regulate its own expression and steady state levels via a negative feedback loop which does not involve MDM2.

5.3 $p14^{ARF}$ can down-regulate E2F mediated S-phase progression in *$p53^{-/-}$ /$mdm2^{-/-}$* cells

Given that we have previously shown that $p14^{ARF}$ can down-regulate E2F mediated S-phase progression in a p53 independent manner, and that $p14^{ARF}$ is able to down-regulate E2F dependant transcription in the absence of MDM2 it was of interest to ask if $p14^{ARF}$ can still prevent E2F mediated S-phase progression in the absence of MDM2. In order to investigate the role of E2F-1 and $p14^{ARF}$ in cell cycle progression in *$p53^{-/-}$/$mdm2^{-/-}$* cells, a BrDU assay was employed. Cells incorporating BrDU were counted and normalised for transfection efficiency.

E2F-1 over-expression in *$p53^{-/-}$/$mdm2^{-/-}$* cells led to an increase in the number of cells undergoing S-phase, over the mock transfected population (Figure 5.2), and DP-1 over-expression had no effect on the number of cells in S-phase relative to mock-transfected cells (Figure 5.2). As expected, when E2F-1 and DP-1 were expressed together, an increase in the number of S-phase cells over either E2F-1 or DP-1 alone was observed (Figure 5.2).

Interestingly, in these cells $p14^{ARF}$ caused a marked decrease in the number of cells undergoing S-phase when expressed alone, and when $p14^{ARF}$ was over-expressed together with E2F-1 and DP-1 the number of cells in S-phase decreased to the levels observed with mock-transfected cells (Figure 5.2). These data further supports the observations made from the transcription assay data. $p14^{ARF}$ is able to down-regulate E2F-1 mediated transcription in the absence of MDM2, and can

also prevent E2F-1 mediated cell cycle progression. Interestingly, in the *p53*$^{-/-}$ */mdm2*$^{-/-}$ cells p14^{ARF} has a significant effect on S-phase progression when over-expressed alone, indicating a p53/MDM2 independent function for p14^{ARF} in cell cycle regulation.

5.4 p14^{ARF} can bind to E2F-1 in the absence of MDM2

The ability of p14^{ARF} to interact with MDM2 and regulate p53 has been well documented (Zhang *et al.,* 1998; Weber *et al.,* 1999; Zhang and Xiong, 1999; Lohrum *et al.,* 2000). We have previously shown that bacterially expressed p14^{ARF} can interact with exogenous E2F-1 from SAOS2 cells (Figure 4.1Aii). p14^{ARF} can down-regulate E2F-1 mediated transcription and S-phase progression in *p53*$^{-/-}$*/mdm2*$^{-/-}$ cells and so we decided to investigate whether p14^{ARF} could still bind to E2F-1 in these cells.

In order to test this interaction with endogenous E2F-1 the same GST and GST-p14^{ARF} proteins (Figure 4.1Aii) were used to do a pull-down assay from *p53*$^{-/-}$ */mdm2*$^{-/-}$ cell extracts. Endogenous E2F-1 bound efficiently to GST-p14^{ARF}, but not to the GST protein (Figure 5.3A) indicating that MDM2 is not required for this interaction to occur. We further confirmed the p14^{ARF}-E2F-1 interaction to be independent of MDM2 through the use of a direct binding assay. Bacterially expressed His-E2F-1 was purified and equimolar amounts of His-E2F-1 were incubated with equal amounts of the indicated bacterially purified GST proteins, direct binding was analysed by SDS-page. As expected GST-DP-1 bound to His-E2F-1 and p14^{ARF} also bound to His-E2F-1 (Figure 5.3B). In contrast a minimal

amount of $p14^{ARF}$ background binding was observed with or GST beads (Figure 5.3B), indicating that the interaction of E2F-1 and $p14^{ARF}$ is direct, specific and as efficient as the interaction between E2F-1 and DP-1. These data further support an MDM2 independent and direct E2F/$p14^{ARF}$effect.

5.5 Conclusions

Together these data imply that $p14^{ARF}$ is able to regulate E2F activity independently of MDM2. $p14^{ARF}$ can bind independently of MDM2 and directly to E2F1. In addition to this, $p14^{ARF}$ can down-regulate E2F mediated transcription and E2F mediated E2F S-phase progression independently of MDM2.

5.6 Discussion

MDM2 is an important mediator of many cell cycle regulatory pathways. Its importance as a cell cycle regulator is highlighted by the embryonic-lethal phenotype observed in MDM2 knock out mice (Jones *et al.*, 1995). MDM2 can regulate the p53 pathway through a variety of mechanisms. Firstly, through a negative feedback loop, in which p53 activates MDM2 expression and in turn MDM2 down-regulates p53 activity (Wu *et al.*, 1993) and also through its interactions with the p300 co-activator protein (Grossman *et al.*, 1998). MDM2 binds to p300 and has a role in p53 degradation (Grossman *et al.*, 1998). p300 also acts in an MDM2 negative feedback loop to regulate p53, and is required for *mdm2* induction by p53 and the subsequent inhibition of p53 stabilisation (Thomas and White, 1998).

MDM2 has also been shown to bind to and regulate the activity of E2F-1 and DP-1 (Martin *et al.*, 1995; Loughran and La Thangue 2000). MDM2 can bind to E2F-1 and DP-1 *in vitro* and can increase the activity of the heterodimer in reporter assay experiments (Martin *et al.*, 1995). In addition to this, MDM2 co-operates with E2F-1 and DP-1 to promote growth and S-phase progression, while additionally overcoming E2F dependent apoptosis and down-regulating the cellular levels of the E2F sub-units (Loughran and La Thangue, 2000).

We have previously shown that $p14^{ARF}$ can regulate E2F-1 activity in a p53 independent manner. This data led us to further investigate the role of MDM2 in this interaction. Previous studies have implied targets other than p53 for $p14/19^{ARF}$ (Carnero *et al.*, 2000; Weber *et al.*, 2000). One study in particular directly implicated that $p19^{ARF}$ can regulate the Rb pathway independently of MDM2 (Carnero *et al.*, 2000). The evidence presented here indicate an MDM2 independent, E2F-1-$p14^{ARF}$ interaction. In addition to this, $p14^{ARF}$ can overcome both E2F mediated transcription and S-phase progression in $p53^{-/-}/mdm2^{-/-}$ cells (Figure 5.1 and 5.2), indicating that $p14^{ARF}$ is able to regulate E2F activity independently of MDM2.

Interestingly, in $p53^{-/-}/mdm2^{-/-}$ cells $p14^{ARF}$ causes a reduction in the number of cells undergoing S-phase compared to mock-transfected cells (Figure 5.2). This phenomenon has been observed by others (Weber et al., 2000) and further highlights a cell cycle regulatory role, independent of the p53-MDM2 pathway for $p14^{ARF}$ in cell cycle regulation.

Various studies have centred on the role of MDM2 in cell cycle regulation and although most MDM2 in the cell is bound to $p14^{ARF}$, most $p14^{ARF}$ is not bound to MDM2 (Llanos *et al.*, 2001), (Figure 5.4)). The majority of MDM2 has also been shown to bind to p300 (Grossman *et al.*, 1998). Whether this binding of MDM2 is cell type or status dependent is yet to be elucidated. However, it could be that $p14^{ARF}$, MDM2 and p300 exist in a multi-protein complex which can regulate both p53 and E2F activity, depending on the cellular signals received; or that MDM2 can bind preferentially to different cell-cycle regulatory proteins depending on yet unknown cellular conditions (Figure 5.4). In addition to this $p14^{ARF}$ can regulate E2F activity independently of MDM2, suggesting that while some $p14^{ARF}$ may be bound to MDM2, some must also be free to bind and regulate the activity of other cellular proteins.

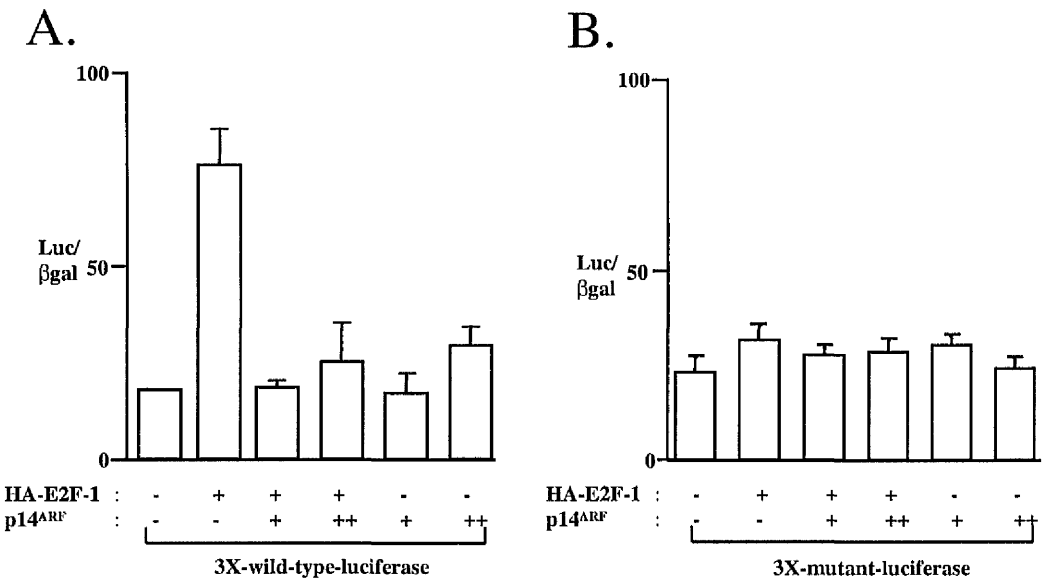
Further work is required to elucidate the mechanisms by which MDM2 governs cell-cycle progression, and to investigate whether MDM2 can also be involved in E2F regulation by $p14^{ARF}$, and whether the p300 co-activator can affect this pathway.

Figure 5.1. $p14^{ARF}$ transcriptional repression of E2F-1 is MDM2 independent

(A) *$p53^{-/-}$/$mdm2^{-/-}$* MEFs were transfected with the following amounts of the indicated plasmids per 6cm plate; 3xWT-luciferase (1μg), CMV-βgal (500ng), HA-E2F-1 (500ng), $p14^{ARF}$ (5μg). Lysates were assayed 40 hours post-transfection and each plate was performed in duplicate. Data are shown as luciferase/βgal value. The data shown are representative of at least three typical experiments.

(B)Cells were transfected and assayed as described in (A) except 3XMutant-luciferase was used in place of 3Xwild-type-luciferase.

(C)Cells were transfected and assayed as described in (A) except E1β-luciferase (4μg per 6cm plate) was used as the reporter.


A.
100
Luc/
βgal
50
0
HA-E2F-1 : - + + + - -
p14ARF : - - + ++ + ++
3X-wild-type-luciferase
B.
100
Luc/
βgal
50
0
HA-E2F-1 : - + + + - -
p14ARF : - - + ++ + ++
3X-mutant-luciferase

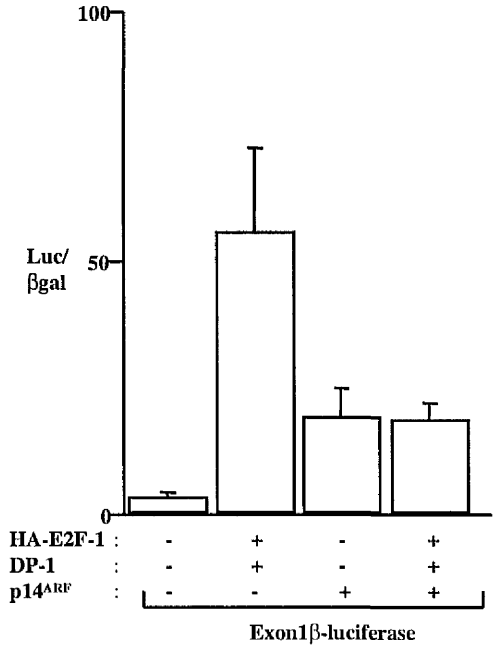

C.
100
Luc/
βgal
50
0
HA-E2F-1 : - + - +
DP-1 : - + - +
p14ARF : - - + +
Exon1β-luciferase

Figure 5.2. $p14^{ARF}$ down-regulation of E2F mediated S-phase progression is MDM2 independent

(A) *$p53^{-/-}$/$mdm2^{-/-}$* cells were transfected as described with 5μg of each pRcHA-E2F-1, CMV-DP-1, $p14^{ARF}$. After transfection cells were washed and further grown overnight for 15 hours. BrdU incorporation was assayed 18 hours post-transfection and data is presented as %BrdU incorporated relative to number of transfected cells/β-glactosidase value. The data shown is representative of at least three independent experiments.

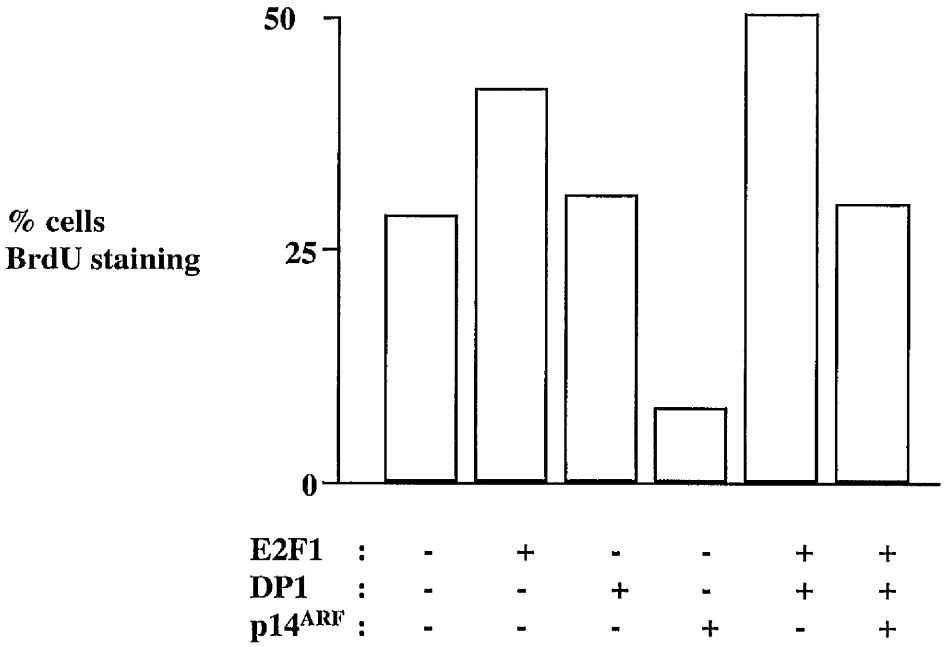
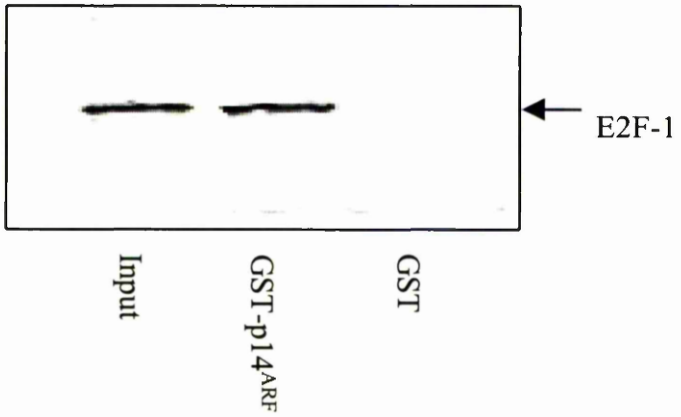

% cells
BrdU staining
50
25
0
E2F1 : - + - - + +
DP1 : - - + - + +
p14ARF : - - - + - +

Figure 5.3. E2F-1 binding to $p14^{ARF}$ is MDM2 independent.

(A) Nuclear extracts were prepared from *$p53^{-/-}$/$mdm2^{-/-}$* cells. Equal amounts of extract (200μg) were incubated with GST or GST-$p14^{ARF}$ overnight at 4°C. Samples were washed 3 times before SDS-PAGE analysis and western blotting with an E2F-1 specific antibody KH95 (Santa Cruz).

(B) Equal amounts of GST-proteins (0.5μg), DP-1, $p14^{ARF}$ and GST were incubated with an equimolar amount of eluted His-E2F-1. Binding was carried out for 30 minutes at 4°C. Samples were washed 3 times before SDS analysis and western blotting with an E2F-1 specific antibody KH95 (Santa Cruz).

A.


E2F-1
Input
GST-p14ARF
GST

B.

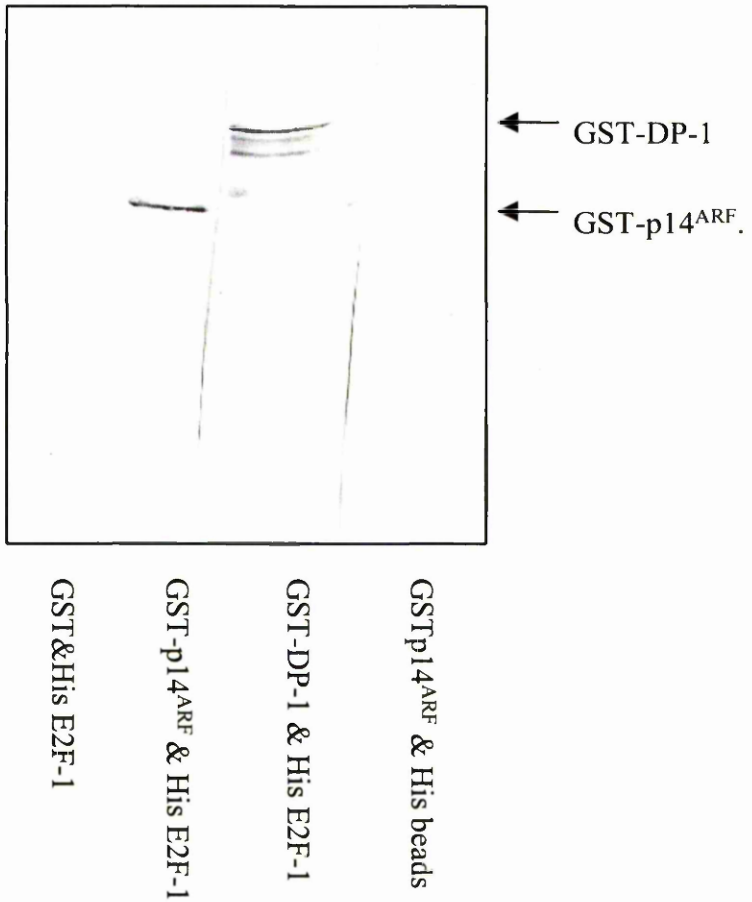
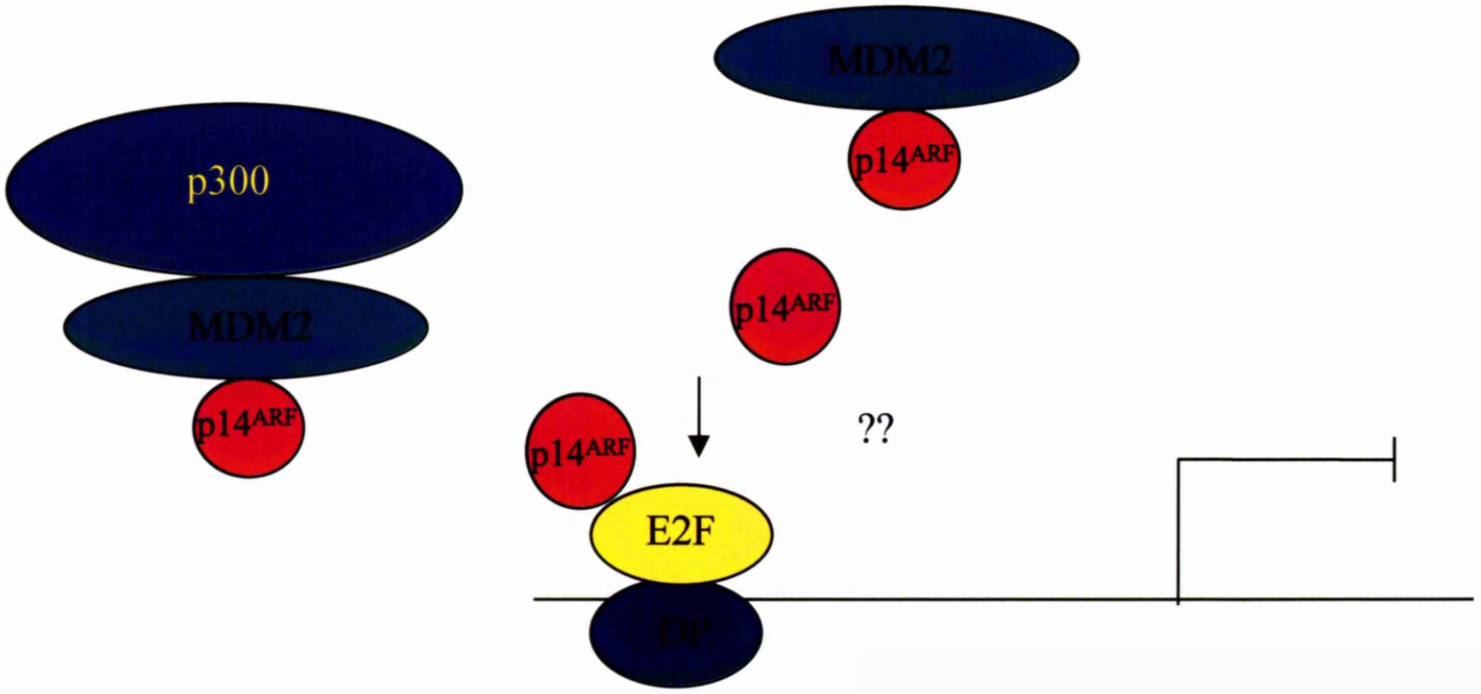

GST-DP-1
GST-p14ARF.
GSTp14ARF & His beads
GST-DP-1 & His E2F-1
GST-p14ARF & His E2F-1
GST&His E2F-1

Figure 5.4 Model for MDM2 independent $p14^{ARF}$ regulation of E2F.

$p14^{ARF}$ can regulate E2F independently of MDM2. p14 can bind to both E2F-1 and MDM2 independently of one another. However, the possibility that E2F-1, $p14^{ARF}$and MDM2 may form a ternary complex under certain conditions still exists.

The correlation between $p14^{ARF}$ binding to E2F-1 and E2F-1 transcriptional regulation suggests that binding of $p14^{ARF}$ to E2F-1 may be required for $p14^{ARF}$ regulation of E2F-1. However, $p14^{ARF}$ may also be able to indirectly affect E2F-1 activity.

p300 is bound to MDM2 in cells and p300 also transcriptionally co-activates E2F-1 activity. It is possible that p300, MDM2 and $p14^{ARF}$ can exist in a multiprotein complex in cells.


p300
MDM2
p14ARF
MDM2
p14ARF
p14ARF
??
p14ARF
E2F
DP

Chapter 6. Mutational analysis of the $p14^{ARF}$-E2F function

6.1 Introduction

The main role of $p14/19^{ARF}$ so far elucidated is its ability to regulate p53 through binding and sequestering the negative regulator MDM2 (Zhang and Xiong, 1998). The N-terminal region of $p19^{ARF}$ and the C-terminus of MDM2 mediate the $p19^{ARF}$-MDM2 interaction. The two regions identified so far in $p19^{ARF}$, which bind to MDM2, are from amino acids 1-14 and 26-37 (Weber *et al.*, 2000) and binding of $p19^{ARF}$ to MDM2 may induce a conformational change that facilitates nucleolar import of the ARF-MDM2 complex (Weber et al., 2000).

A number of domains have been identified in $p14^{ARF,}$ which are important for its targeting of, and interactions with MDM2 (Lohrum *et al.*, 2000). Small fragments of $p14^{ARF}$, tagged with a Tx-Myc tag were expressed in cells and immunoprecipitated with MDM2. The Tx tag is the active site loop of thioredoxin (Lohrum *et al.*, 2000). In these experiments the regions of $p14^{ARF}$ identified, which are able to bind to MDM2 in cells, were from amino acid residues 1-22, and 65-132 (Lohrum *et al.*, 2000). Interestingly however, *in vitro*, only a peptide corresponding to the first 20 amino acids of $p14^{ARF}$ could bind to MDM2 (Midgley *et al.*, 2000). These differing results could imply that, although $p14^{ARF}$ can interact with MDM2 directly *in vitro,* other cellular factors may be involved in the $p14^{ARF}$-MDM2 complex in cells.

All of the Tx-mutants of $p14^{ARF}$ that interact with MDM2 can also relocalise MDM2 to the nucleolus and inhibit MDM2-dependent p53 degradation (Llanos *et al.*, 2000). Recently however, a more physiological approach to looking at $p14^{ARF}$-MDM2 nucleolar localisation has suggested that $p14^{ARF}$ nucleolar localisation is not essential for its ability to regulate MDM2 dependent p53 degradation (Llanos *et al.*, 2000).

Given that I had shown that $p14^{ARF}$ could interact with E2F-1 and regulate its activity in cells, it was of interest to identify the regions in $p14^{ARF}$ that are responsible for this binding and regulation of E2F-1. More detailed analysis of this interaction and its function should give us more complete information regarding the importance of the E2F-$p14^{ARF}$ interaction and the effects it has on cell cycle regulation.

Results

6.2 Purification and identification of GST$p14^{ARF}$C65 and GST$p14^{ARF}$N64.

In order to identify the region of $p14^{ARF}$ that binds to E2F, GST fusion proteins expressing the N-terminus or C-terminus of $p14^{ARF}$ were constructed and purified. The purified proteins ran at the expected size and could be discriminated between by a slight difference in migration in SDS-PAGE gel analysis (Figure 6.1A).

To confirm that the constructs being expressed were the N and C terminal regions of the $p14^{ARF}$ protein the recombinant bacterial fusion proteins were subjected to SDS-PAGE analysis and immunoblotting. Purified proteins were first detected

using a $p14^{ARF}$ polyclonal antibody. Where the epitope recognised has been mapped to the C-terminal region and in line with this observation GST-$p14^{ARF}$C65 was exclusively recognised with this antibody (Figure 6.1B). Proteins were also detected using the $p14^{ARF}$ monoclonal antibody (Figure 6.1B), which recognised the N-terminal region of $p14^{ARF}$.

6.3 $p14^{ARF}$ binds to E2F-1 and DP-1 *in vitro* via the $p14^{ARF}$ N-terminus.

In order to determine the region of $p14^{ARF}$ that bound to E2F-1 and DP-1 *in vitro* far western blotting was carried out. Equal amounts of purified GST-$p14^{ARF}$, GST-$p14^{ARF}$N64, GST-$p14^{ARF}$C65 and GST were immobilised on nitro-cellulose and membranes were probed with equal amounts of ^{35}S-labelled *in vitro* translated proteins (Figure 6.2(i)). As previously described, MDM2 bound to both GST-$p14^{ARF}$ and GST-$p14^{ARF}$N64 (Zhang and Xiong, 1998), but not to GST-$p14^{ARF}$C65 or GST (Figure 6.2(ii)A). Both *in vitro* translated E2F-1 and DP-1 also bound to both GST-$p14^{ARF}$ and GST-$p14^{ARF}$N64), but not to GST-$p14^{ARF}$C65 or GST (Figure 6.2(ii)B,C). A luciferase negative control protein showed minimal binding to the GST proteins compared to MDM2, E2F-1 and DP-1 (Figure 6.2(ii)D).

6.4 Down-regulation of E2F transcription is through the N-terminus of $p14^{ARF}$

Given that I had demonstrated that over-expression of $p14^{ARF}$ can overcome E2F-1 *trans*activation in reporter gene assays, it was of interest to identify the region of

$p14^{ARF}$ responsible for the down-regulation. SAOS2 cells transfected with the E1β-luciferase reporter gene, and both E2F-1 and DP-1 showed transcriptional activation and, as previously demonstrated over-expression of $p14^{ARF}$, together with E2F-1 and DP-1, showed a decrease in the level of reporter gene activity relative to that observed with the heterodimer alone (Figure 6.3B).

Increasing amounts of the Myc-tagged N- and C-terminal regions of $p14^{ARF}$, Myc-ARF(N) and Myc-ARF(C) (Figure 6.3A) were transfected, together with the E1β-luciferase promoter and E2F-1 and DP-1 expression plasmids. The N-terminal region of $p14^{ARF}$ was able to down-regulate E2F-1 and DP1 on reporter activity independently of the C-terminus (Figure 6.3B). The N-terminal region of $p14^{ARF}$ was able to down-regulate the transcriptional activation by E2F-1 and DP-1 in a dose-dependent manner (Figure 6.3Cii), whereas the C-terminus had little effect (Figure 6.3B)

In order to check the expression level of the Myc-tagged constructs and rule out that the effect of Myc-ARF(C) was due to a lack of expression, SAOS2 cells were transfected with each construct and immuno-blotted with a Myc antibody (Figure 4.3Ci). As expected all constructs were expressed: Myc$p14^{ARF}$ and Myc-ARF(C) more efficiently than Myc-ARF(N) (Figure 6.3Ci).

These data show that the N terminal region of $p14^{ARF}$ is responsible for the observed down-regulation of E2F transcription, whilst the C-terminal region of $p14^{ARF}$ appeared to be dispensable for this transcriptional down-regulation.

Indeed, the $p14^{ARF}$ N terminus can regulate the transcriptional activity by E2F in a dose-dependent manner analogous to that of wild-type $p14^{ARF}$.

6.5. Further identification of the E2F-$p14^{ARF}$ binding domain: E2F-1 binds to a region in $p14^{ARF}$ distinct to that of MDM2.

In order to further identify the regions in $p14^{ARF}$ that bind to E2F-1 an immuno-precipitation approach was undertaken in SAOS2 cells. A panel of Tx-Myc tagged $p14^{ARF}$ deletion mutants (Figure 6.4A) (Lohrum *et al.*, 2000) were examined for their ability to bind to E2F-1 *in vivo* (Figure 6.4B and Figure 6.5).

Anti-E2F-1 immuno-precipitates from SAOS2 cells over-expressing pRc-HA-E2F-1 all contained an HA-specific polypeptide that corresponded to the expected size of E2F-1 (Figure 6.5). E2F immunoprecipitates from over-expressing $p14^{ARF}$ mutants, Tx-Myc-$p14^{ARF}$ 1-132, Tx-Myc-$p14^{ARF}$ 1-34 and Tx-Myc-$p14^{ARF}$ 65-132 Tx-Myc-$p14^{ARF}$1-64 cells contained the $p14^{ARF}$ mutant derivative in complex with E2F-1 (Figure 4.5, 4.6). In contrast, E2F immuno-complexes from over-expressing $p14^{ARF}$ mutant Tx-Myc-$p14^{ARF}$ 1-22 or Tx-Myc-$p14^{ARF}$ 54-64 contained no traces of the $p14^{ARF}$ mutant derivatives (Figure 6.5, 6.6). These results indicate that firstly, E2F-1 and $p14^{ARF}$ can interact in cells when over-expressed, providing further evidence for the relevance of the E2F-$p14^{ARF}$ interaction, and that E2F-1 can bind to distinct regions of $p14^{ARF}$ including one N (amino acids 22-34 and one C terminal region (amino acids 65-132).

6.6 $p14^{ARF}$ binding to E2F-1 correlates with its ability to down-regulate transcription.

Given that the E2F-1 binding region in $p14^{ARF}$ has been mapped to amino acids 22-34 it was of interest to investigate the relationship between $p14^{ARF}$-E2F-1 binding, and $p14^{ARF}$ mediated down-regulation of E2F transcription.

It was important to check that the addition of a large tag to the $p14^{ARF}$ protein did not affect its ability to down-regulate E2F trancription before using these constructs in functional assays. SAOS2 cells transfected with the E1β-luciferase reporter gene, E2F-1 and DP-1, showed activation of the reporter, and as expected, E2F-1, DP-1 and Tx-Myc-$p14^{ARF}$ 1-132 showed a decrease in the level of reporter gene activity observed with the heterodimer alone, comparable to that observed with wild-type $p14^{ARF}$ (Figure 6.7A). Indicating that despite the Tx-Myc tag, Tx-Myc-$p14^{ARF}$ is able to overcome E2F mediated transcription with the same efficiency as untagged $p14^{ARF}$ (Figure 6.7A).

Tx-Myc-$p14^{ARF}$1-22, Tx-Myc-$p14^{ARF}$1-64 and Tx-Myc-$p14^{ARF}$65-132 (Figure 6.4A) were transfected with the E1β-luciferase promoter, E2F-1 and DP-1 and as previously observed, the N-terminal region of $p14^{ARF}$ was able to down-regulate the effect of E2F-1 and DP-1 on reporter gene activity, as efficiently as wild-type $p14^{ARF}$, while the C-terminal region had no effect on the level of E2F/DP-1 reporter gene activity (Figure 6.7B). In contrast to this effect, the Tx-Myc-$p14^{ARF}$1-22 mutant was unable to overcome E2F mediated transcriptional

activation and an increase in the reporter gene activity, compared to that of the heterodimer was observed (Figure 6.7B).

To further clarify these observations, and correlate the activity of $p14^{ARF}$ mutants in E2F mediated transcription with E2F-1 binding, a similar experiment was performed. In this case, Tx-Myc-$p14^{ARF}$1-22, Tx-Myc-$p14^{ARF}$1-34 and Tx-Myc-$p14^{ARF}$1-132 (Figure 6.4A) were transfected with the E1β-luciferase promoter, and both Tx-Myc-$p14^{ARF}$1-34 and Tx-Myc-$p14^{ARF}$1-132 were able to overcome E2F mediated transcription (Figure 6.7C). As previously demonstrated the Tx-Myc-$p14^{ARF}$1-22 mutant was unable to overcome E2F mediated transcriptional activation, and an increase in the reporter gene activity, compared to that of the E2F heterodimer alone was observed (Figure 6.7C).

These observations imply that N-terminal regions of $p14^{ARF}$ which can bind to E2F-1 are able to regulate E2F-1 activity. In contrast, the region of $p14^{ARF}$ encompassing amino acids 1-22 which is unable to bind to E2F-1 is also unable to down-regulate E2F mediated transcriptional activation (Figure 6.6 B and C).

Consistently, in reporter assay experiments, the N terminal constructs Myc-$p14^{ARF}$N64 and Tx-Myc-$p14^{ARF}$1-64 could decrease the levels of reporter assay activity observed with E2F-1 and DP-1 to a greater degree than wild-type $p14^{ARF}$ (Figure 6.4B and Figure 6.6B). This effect appears not to be due to expression levels, as the C terminal Myc-ARF(C) and Tx-Myc-$p14^{ARF}$ constructs are expressed more efficiently than wild-type $p14^{ARF}$, while less expression of the N

terminal constructs is generally observed compared to wild-type $p14^{ARF}$ (Figure 6.3Bi and data not shown).

6.7 $p14^{ARF}$ binding to E2F-1 correlates with its ability to prevent cell-cycle progression.

As $p14^{ARF}$ binding to E2F-1 correlates with its ability to repress E2F transcription it was of interest to assess whether this interaction influenced S-phase progression, a property ascribed to E2F (Johnson *et al.*, 1993). A BrDU assay was utilised to measure cells in S-phase in cells transfected with E2F-1, DP-1 and mutants of $p14^{ARF}$.

SAOS2 cells transfected with plasmids expressing E2F-1 and DP-1 displayed a detectable increase in BrDU incorporation over mock-transfected cells (Figure 6.8A). In order to confirm that Tx-Myc tagged $p14^{ARF}$ was able to act similarly to wild-type $p14^{ARF}$. I directly compared the activity of the two proteins in combination with E2F-1 and DP-1. It was observed that Tx-Myc-$p14^{ARF}$ was able to overcome E2F mediated S-phase progression to a similar level as wild-type $p14^{ARF}$ (Figure 6.8A).

SAOS2 cells were transfected with E2F-1 and DP-1 either together, or in combination with the $p14^{ARF}$mutants Tx-Myc-$p14^{ARF}$1-132, Tx-Myc-$p14^{ARF}$1-22, Tx-Myc-$p14^{ARF}$1-64 and Tx-Myc-$p14^{ARF}$65-132. As expected Tx-Myc-$p14^{ARF}$1-132 was able to reduce E2F-mediated S-phase progression, and the N-terminal region of $p14^{ARF}$ Tx-Myc-$p14^{ARF}$1-64 also reduced the amount of BrDU

incorporation observed, in combination with E2F-1 and DP-1 together (Figure 6.8B).

Interestingly, Tx-Myc-p14^{ARF}65-132 in combination with E2F-1 and DP-1 also caused a slight decrease in the amount of BrDU incorporation observed compared to that of the heterodimer alone (Figure 6.8B). Although this decrease was slight, it was consistently observed throughout different experiments, indicating that the C-terminus of p14^{ARF} may also be capable of overcoming E2F mediated S-phase progression to a slight extent. This effect of the C-terminal region of p14^{ARF} could be transcriptionally independent, and this would explain the lack of effect in reporter assay experiments. In agreement with the reporter assay data, expression of Tx-Myc-p14^{ARF}1-22 was unable to overcome E2F-mediated S-phase entry, but in fact showed a significant increase in the number of cells incorporating BrDU when co-expressed with E2F-1 and DP-1 than observed when E2F-1 and DP-1 were together transfected (Figure 6.8B).

6.8 Conclusions.

The data presented shows that both the N and C terminus of p14^{ARF} can bind to E2F-1 in cells, and that a region encompassing amino acid residues 1-22 of p14^{ARF} , that is important for ARF-MDM2 binding, is unable to bind to E2F-1.Therefore the binding regions of MDM2 and E2F-1 to p14^{ARF} are distinct. p14^{ARF}s ability to overcome E2F mediated transcriptional repression and S-phase entry is mediated by an N terminal region of p14^{ARF} which can bind to E2F-1,

indicating that regulation of E2F activity by $p14^{ARF}$ is dependent on $p14^{ARF}$ binding.

6.9 Discussion.

Previous studies have centred on a panel of $p14^{ARF}$ mutants and their role in p53 stabilisation mediated through ARF binding to MDM2 (Lohrum *et al.*, 2000). Regions of $p14^{ARF}$ were identified which could bind and regulate MDM2 activity (Lohrum *et al.*, 2000), and these investigations led us to study E2F-1 regulation by $p14^{ARF}$ mutants in order to further understand the E2F-ARF interaction.

Sudies showed that the N-terminal region of $p14^{ARF}$ could bind to E2F-1 in a direct binding assay (Figure 6.2). This led me to investigate the regulation of E2F activity in a reporter assay system using the N and C terminal regions of $p14^{ARF}$. I found that the N-terminus of $p14^{ARF}$ is responsible for the $p14^{ARF}$ mediated transcriptional repression of E2F-1, and that the C-terminus had little effect on E2F activity (Figure 6.3B). Interestingly, this is also the situation reported for MDM2 regulation by $p14^{ARF}$. Although both the N- and C-terminus of $p14^{ARF}$ can bind to MDM2, only the N-terminal region can regulate its activity in the context of p53 stabilisation (Zhang and Xiong, 1998; Stott *et al.*, 1998, Lohrum *et al.*, 2000).

Given that the N-terminal region of $p14^{ARF}$ is responsible for the observed down-regulation of E2F-1 activity, it was of interest to further map the interaction and regulatory domains in ARF for E2F-1. We used a panel of $p14^{ARF}$ mutant

constructs (Lohrum et al., 2000 (Figure 6.4)) in an immuno-precipitation assay in order to identify the binding domain in $p14^{ARF}$ for E2F-1.

Interestingly, we identified the region of $p14^{ARF}$ 1-22 as unable to bind E2F-1 while a larger region from 1-34 can bind to E2F-1 (Figure 6.5). This suggests that the $p14^{ARF}$-E2F-1 binding domain may be between amino acids 22-34. As the 1-22 amino acid region has previously been identified as the $p14^{ARF}$-MDM2 binding region, these results suggest an interesting scenario where E2F-1 and MDM2 may actually both be able to bind to $p14^{ARF}$ at the same time and form a ternary complex. Alternatively, E2F-1 and MDM2 could compete for $p14^{ARF}$ binding or, one could bind to $p14^{ARF}$ in the absence of the other. The binding and possible competition between E2F-1 and MDM2 for $p14^{ARF}$ could be cell, or tissue dependant. Also of interest is the fact that the C-terminus of $p14^{ARF}$ can also bind to E2F-1 in cells, although the results suggested that this interaction did not alter E2F activity. This is also the case with MDM2, where the C-terminus of $p14^{ARF}$ does not influence MDM2 activity, but nevertheless is able to bind MDM2 and form nuclear bodies with p53 (Zhang and Xiong, 1999).

It may be that the C-terminus of $p14^{ARF}$ regulates E2F under certain circumstances, although more work is required to understand the importance of the ARF C-terminus, in the context of both E2F-1 and MDM2 regulation.

Reporter assay experiments were carried out in order to investigate which region of $p14^{ARF}$ is responsible for the observed down-regulation of E2F activity. The 1-34 amino acid region of $p14^{ARF}$ was shown to be able to efficiently down-regulate

E2F mediated reported gene activity while the 1-22 region was not (Figure 6.6C). Additionally, the $p14^{ARF}$ 1-22 mutant was observed to enhance the activity observed with E2F-1 and DP-1 (Figure 6.6B,C). This activity of $p14^{ARF}$1-22 was unexpected and may be due to a dominant-negative effect on endogenous $p14^{ARF}$ or perhaps amino acid residues 1-22, through binding to MDM2, can somehow regulate E2F activity indirectly? (Figure 6.9)

The ability of the $p14^{ARF}$ mutants to regulate E2F activity in a BrDU assay was also examined and the results obtained with the $p14^{ARF}$1-64 and $p14^{ARF}$1-22 constructs were similar to those from the reporter gene assays. The effect of the 1-22 region of $p14^{ARF}$ in these experiments was particularly striking and a large increase in the number of cells incorporating BrDU compared to E2F-1 and DP-1 transfected cells was observed when $p14^{ARF}$1-22 was transfected in the presence of E2F-1 and DP-1 (Figure 6.7B). This effect may occur as a result of the observed transcriptional enhancement, however it is possible that amino acids 1-22 of $p14^{ARF}$ may be responsible for other, E2F independent, cell cycle regulatory mechanisms.

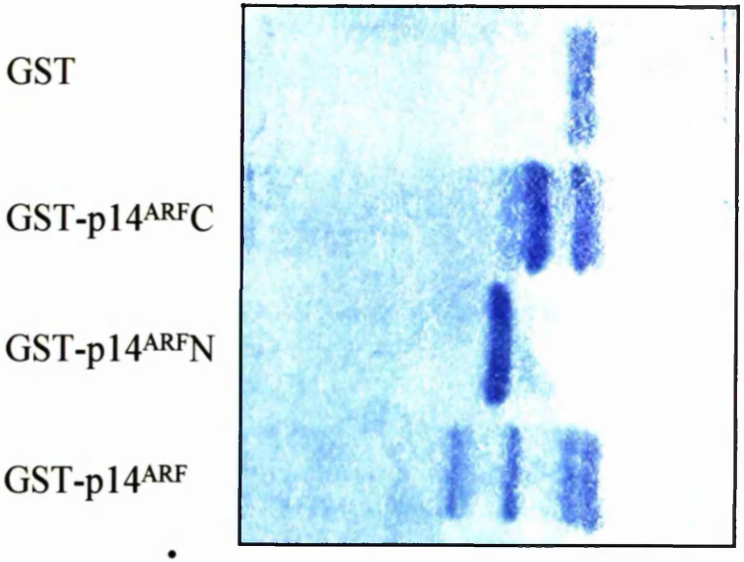
Interestingly, the C-terminus of $p14^{ARF}$ showed a slight down-regulation of the number of S-phase cells in the presence of E2F-1 and DP-1 compared to E2F-1 and DP-1 activity (Figure 6.8B). This effect was unexpected compared to the effect of the C terminal region in reporter assay experiments and may simply be a consequence of over-expression. However, it is possible that C-terminal binding of $p14^{ARF}$ to E2F-1 in cells may have an effect on cell cycle regulation through an as yet unidentified mechanism.

The data presented here provides an important link between $p14^{ARF}$ binding to E2F-1, and the ability of $p14^{ARF}$ to regulate E2F activity. These experiments also show that the domain of $p14^{ARF}$, which is important for MDM2 regulation, does not regulate E2F activity and that E2F-1 and MDM2 bind to different regions of $p14^{ARF}$. These data indicate the possibility that the $p14^{ARF}$ regulation of E2F could be either dependent on, or independent of MDM2. Further investigations are required to fully elucidate the role of $p14^{ARF}$ in E2F regulation, in particular the observed effects of the 1-22 and C-terminal regions of $p14^{ARF}$ are of interest.

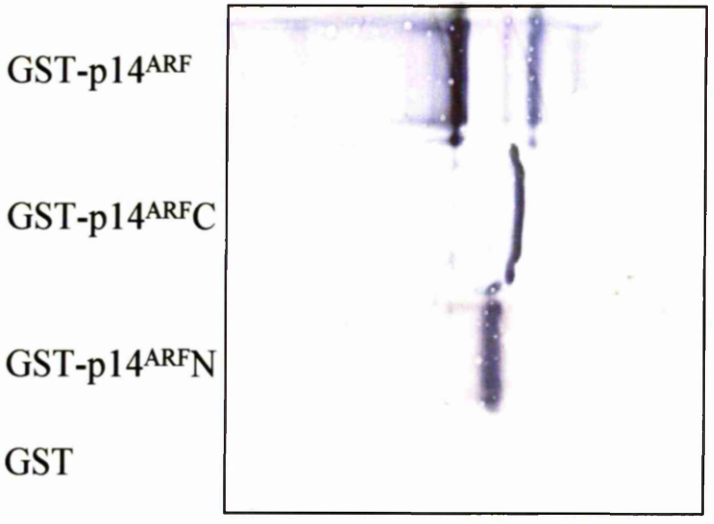
Figure 6.1 Purification and identification of pGEX-p14^{ARF}-N64 and pGEX-p14^{ARF}-C65.

(A) The indicated GST-tagged proteins were purified from bacteria as described (chapter 2). Bead bound proteins were resuspended in 3x SDS loading buffer and subjected to SDS-PAGE analysis, then stained with commassie blue.

(B) Purified proteins were prepared and analysed as described in (A), then transferred to nitrocellulose membrane and immunoblotted with p14^{ARF} specific antibodies.


GST
GST-p14ARFC
GST-p14ARFN
GST-p14ARF

A.


GST-p14ARF
GST-p14ARFC
GST-p14ARFN
GST

B.

Figure 6.2 p14^{ARF} binds to E2F-1 and DP-1 through the N terminal domain

(i) The *in vitro* translated proteins used for input in the far western blot are shown. The indicated plasmids were in vitro translated and 5% was subjected to SDS-PAGE. Labelled proteins were detected by exposure to film overnight.

(ii) The indicated GST-proteins were prepared and immobilised on membrane as previously described. Membranes were incubated with one of each of *in vitro* translated MDM2, HA-E2F-1, DP-1 or luciferase protein in milk/AC buffer. Following incubation, membranes were washed and exposed overnight on film to identify bound proteins.

(i)

Inputs 5%

(ii)

A. MDM2

B. E2F-1

C.

DP-1

D.

Luciferase

Figure 6.3 The $p14^{ARF}$ N terminal region is responsible for $p14^{ARF}$ mediated transcriptional repression.

(A) Schematic of the $p14^{ARF}$ mutant constructs used (Zhang and Xiong., 1998).

(B) Reporter assay. SAOS2 cells were transfected with the following plasmids per 6cm plate; pRcHA-E2F-1 (50ng), CMV-DP1 (500ng), $p14^{ARF}$ (12μg), Myc-ARF(N) (12 μg), Myc-ARF-(C) (12 μg) E1β-luciferase (4μg) and CMV-βgal (500ng). Lysates were assayed 40 hours post-transfection and each plate was performed in duplicate. Data are shown as luciferase values/βgal value. The data shown is representative of at least three typical experiments.

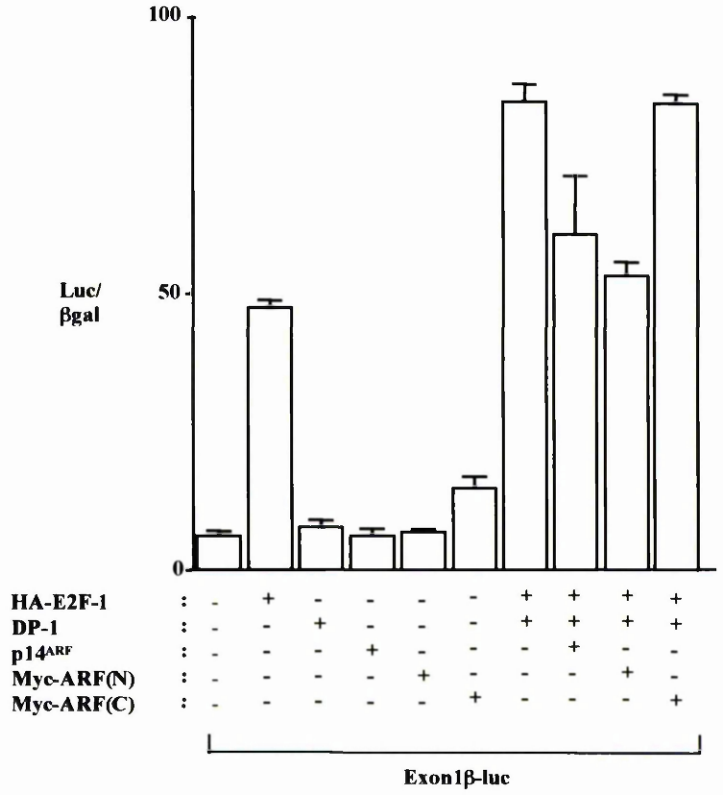
(C) (i)Expression of the Myc-tagged ARF constructs. 10μg each plasmid was transfected into SAOS2 cells. Cells were harvested, run on a 15% SDS gel and blotted with the anti-Myc antibody.

(ii) SAOS2 cells were transfected with the following plasmids per 6cm plate; pRcHA-E2F-1 (50ng), CMV-DP1 (500ng), $p14^{ARF}$ (12μg), Myc-ARF(N) (5, 7.5, 12 μg), E1β-luciferase (4μg) and CMV-βgal (500ng). Experiments were performed and analysed as described in (B)

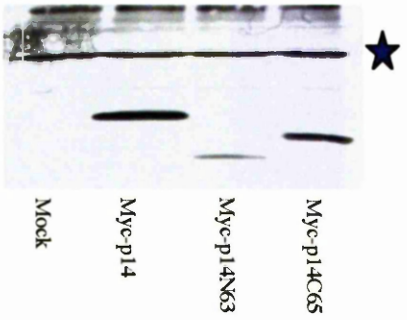
A.


Myc-ARF
Myc-ARF(N)
Myc-ARF(C)

B.


100
50
0
Luc/
βgal
HA-E2F-1 : - + - - - - + + + +
DP-1 : - - + - - - + + + +
p14ARF : - - - + - - - + - -
Myc-ARF(N) : - - - - + - - - + -
Myc-ARF(C) : - - - - - + - - - +
Exon1β-luc

C.(i)


Mock
Myc-p14
Myc-p14N63
Myc-p14C65

(ii)

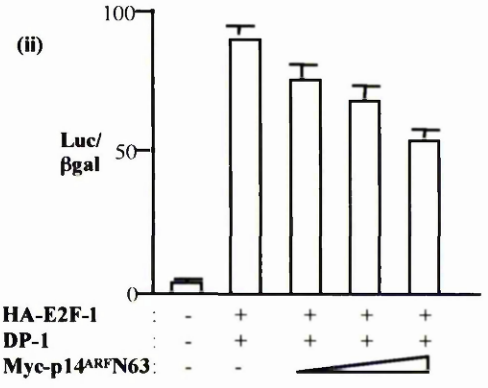
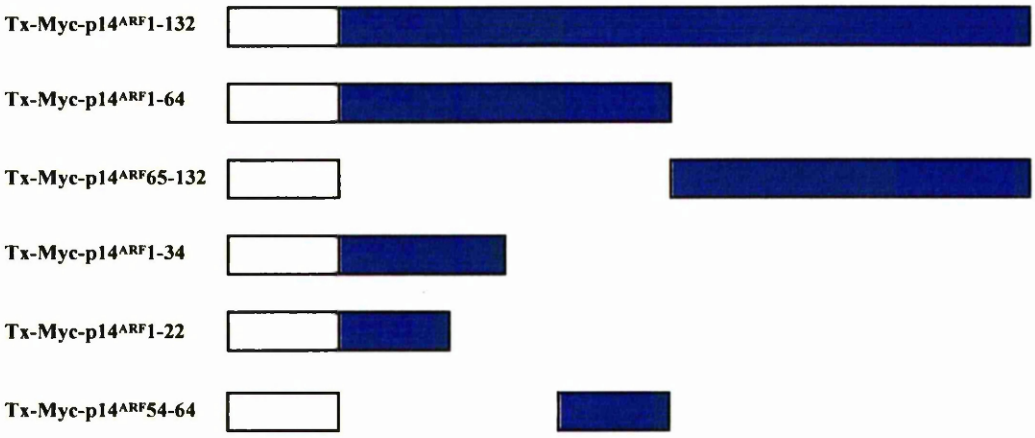

100
50
0
Luc/
βgal
HA-E2F-1 : - + + + +
DP-1 : - + + + +
Myc-p14ARFN63 : - -

Figure 6.4. $p14^{ARF}$ mutant binding properties.

(A) Schematic of Tx-myc tagged constructs used for binding assays (Lohrum *et al.*, 2000).

(B) Binding properties of Tx-tagged $p14^{ARF}$ constructs. Constructs were tested for binding to MDM2 (Lohrum *et al.*, 2000), or E2F-1 by immuno-precipitation (Figure 4.5).

A.



B.

Construct	Binding to MDM2	Binding to E2F-1
Tx-Myc-p14ARF1-132	+	+
Tx-Myc-p14ARF1-64	+	+
Tx-Myc-p14ARF65-132	+	+
Tx-Myc-p14ARF1-34	+	+
Tx-Myc-p14ARF1-22	+	-
Tx-Myc-p14ARF54-64	-	-

Figure 6.5. The p14^{ARF}-E2F binding site maps to the region 22-34.

SAOS2 cells were transfected with 25μg each of pRc-HA-E2F-1 and the indicated Tx-Myc-p14^{ARF} plasmids. Cells were washed 8 hours post-transfection and harvested 24 hours post-transfection. Extracts were prepared and immuno-precipitations performed.

Tx-Myc-p14^{ARF} proteins are indicated by a yellow star.

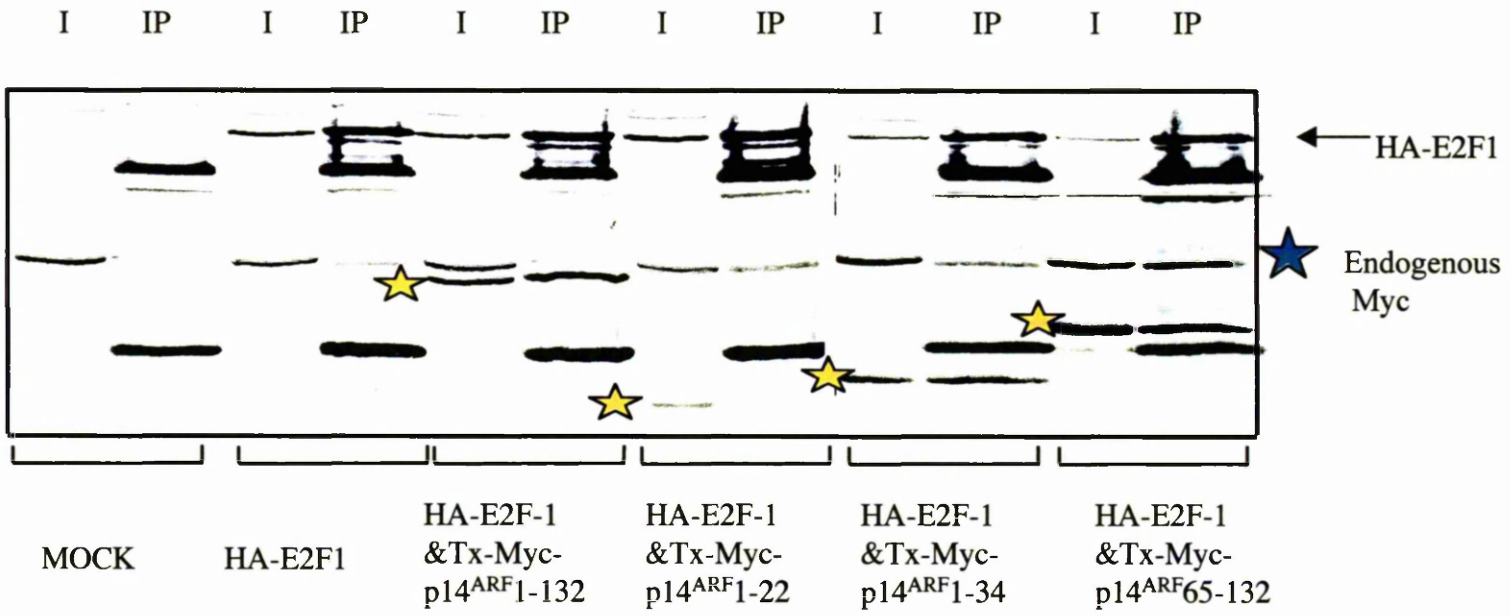

I IP I IP I IP I IP I IP I IP
HA-E2F1
Endogenous Myc
MOCK
HA-E2F1
HA-E2F-1 &Tx-Myc-p14ARF1-132
HA-E2F-1 &Tx-Myc-p14ARF1-22
HA-E2F-1 &Tx-Myc-p14ARF1-34
HA-E2F-1 &Tx-Myc-p14ARF65-132

Figure 6.6. E2F-1 does not bind to $p14^{ARF}$ amino acid residues 56-64

SAOS2 cells were transfected with 25μg each of pRc-HA-E2F-1 and the indicated Tx-Myc-$p14^{ARF}$ plasmids. Cells were washed 8 hours post-transfection and harvested 24 hours post-transfection. Extracts were prepared and immuno-precipitations performed. Tx-Myc-$p14^{ARF}$ proteins are indicated by a yellow star.

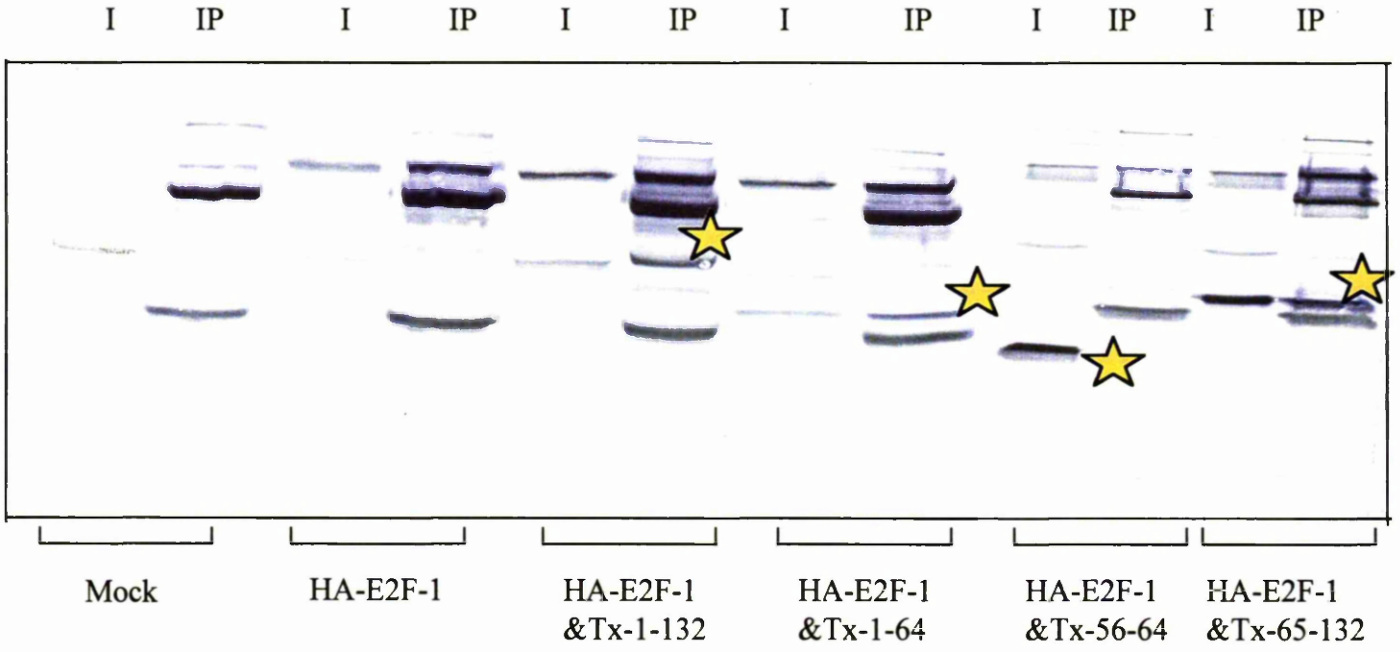

I
IP
I
IP
I
IP
I
IP
I
IP
I
IP
Mock
HA-E2F-1
HA-E2F-1
&Tx-1-132
HA-E2F-1
&Tx-1-64
HA-E2F-1
&Tx-56-64
HA-E2F-1
&Tx-65-132

Figure 6.7 $p14^{ARF}$-E2F-1 binding correlates with transcriptional repression.

(A) SAOS2 cells were transfected with each of the following plasmids per 6cm plate: E1β-luciferase (4μg) CMV-βgal (500ng) pRc-HA-E2F-1 (50ng), CMV-DP1 (500ng), $p14^{ARF}$ (5μg) and Tx-$p14^{ARF}$1-132 (10μg). Lysates were assayed 40 hours post-transfection and each plate was performed in duplicate. Data is shown as luciferase values/βgal value. The data shown is representative of at least three typical experiments.

(B) SAOS2 cells were transfected with each of the following plasmids per 6cm plate: E1β-luciferase (4μg) CMV-βgal (500ng) pRc-HA-E2F-1 (50ng), CMV-DP1 (500ng), Tx-$p14^{ARF}$1-132 (10μg), Tx-$p14^{ARF}$1-22 (10μg),Tx-$p14^{ARF}$1-64 (10μg) and Tx-$p14^{ARF}$65-132 (10μg) Lysates were assayed as described in (A).

(C) SAOS2 cells were transfected with each of the following plasmids per 6cm plate: E1β-luciferase (4μg) CMV-βgal (500ng) pRc-HA-E2F-1 (50ng), CMV-DP1 (500ng), Tx-Myc-$p14^{ARF}$1-132 (10μg), Tx-Myc-$p14^{ARF}$1-22 (10μg),Tx-Myc-$p14^{ARF}$1-64 (10μg) and Tx-Myc-$p14^{ARF}$1-34 (10μg) Lysates were assayed as described in (A).

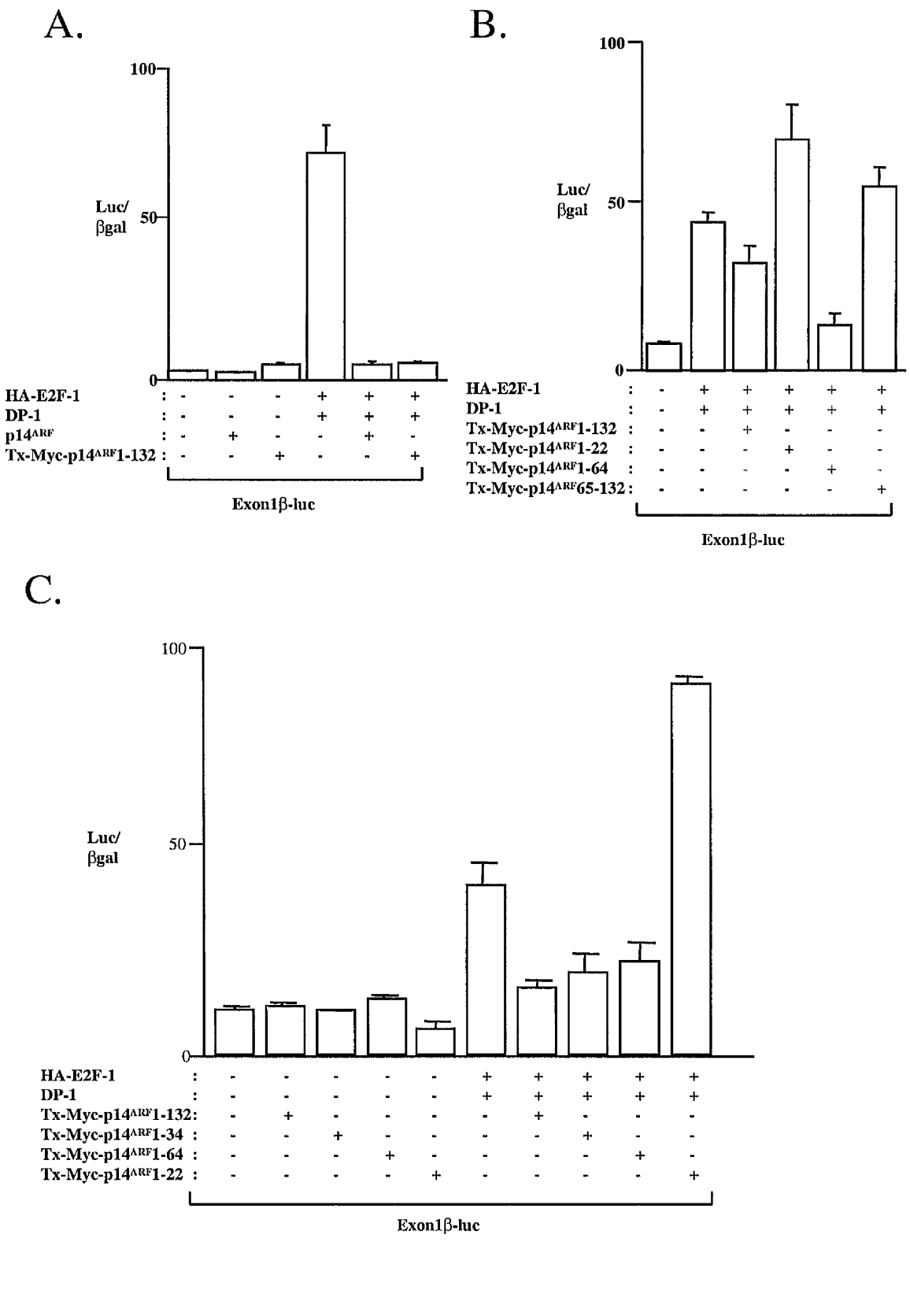
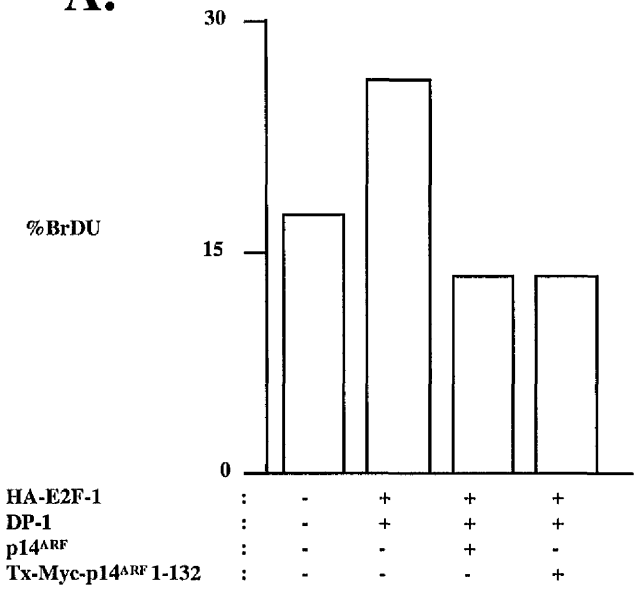

A.
100
50
0
Luc/
βgal
HA-E2F-1 : - - - + + +
DP-1 : - - - + + +
p14ARF : - + - - + -
Tx-Myc-p14ARF1-132 : - - + - - +
Exon1β-luc
B.
100
50
0
Luc/
βgal
HA-E2F-1 : - + + + + +
DP-1 : - + + + + +
Tx-Myc-p14ARF1-132 : - - + - - -
Tx-Myc-p14ARF1-22 : - - - + - -
Tx-Myc-p14ARF1-64 : - - - - + -
Tx-Myc-p14ARF65-132 : - - - - - +
Exon1β-luc
C.
100
50
0
Luc/
βgal
HA-E2F-1 : - - - - - + + + + +
DP-1 : - - - - - + + + + +
Tx-Myc-p14ARF1-132 : - + - - - - + - - -
Tx-Myc-p14ARF1-34 : - - + - - - - + - -
Tx-Myc-p14ARF1-64 : - - - + - - - - + -
Tx-Myc-p14ARF1-22 : - - - - + - - - - +
Exon1β-luc

Figure 6.8. $p14^{ARF}$-E2F-1 binding correlates with S-phase progression.

(A) SAOS2 cells were transfected with 5μg each of the following plasmids: pRc-HA-E2F-1, CMV-DP1, $p14^{ARF}$ and Tx-Myc-$p14^{ARF}$1-132. After transfection cells were washed and further grown overnight. BrdU incorporation was assayed 18 hours post-transfection and data are presented as %BrdU incorporated relative to number of transfected cells/β-galactosidase value. The data shown are representative of at least three independent experiments.

(B) SAOS2 cells were transfected with 5μg of each of the following plasmids: pRc-HA-E2F-1, CMV-DP1, Tx-Myc-$p14^{ARF}$1-132, Tx-Myc-$p14^{ARF}$1-22, Tx-Myc-$p14^{ARF}$1-64 and Tx-Myc-$p14^{ARF}$65-132. Cells were assayed for BrDU incorporation as described in (A).

A.


30
15
0
%BrDU
HA-E2F-1 : - + + +
DP-1 : - + + +
p14ARF : - - + -
Tx-Myc-p14ARF 1-132 : - - - +

B.

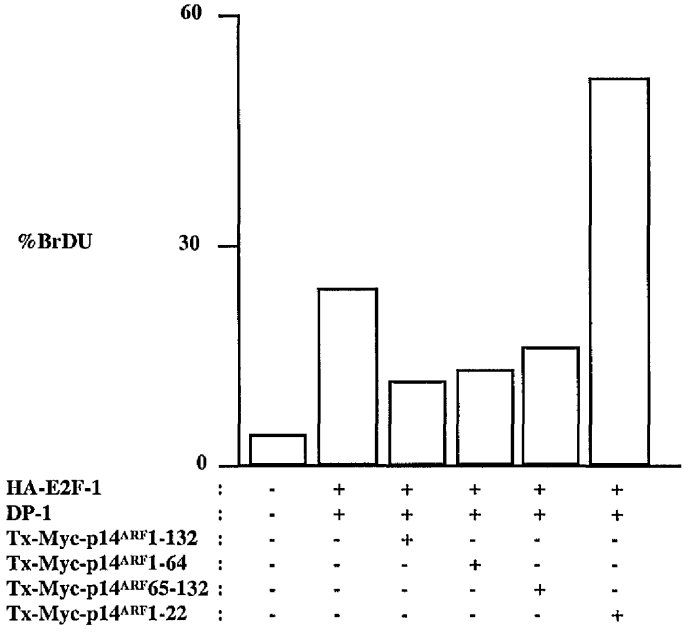
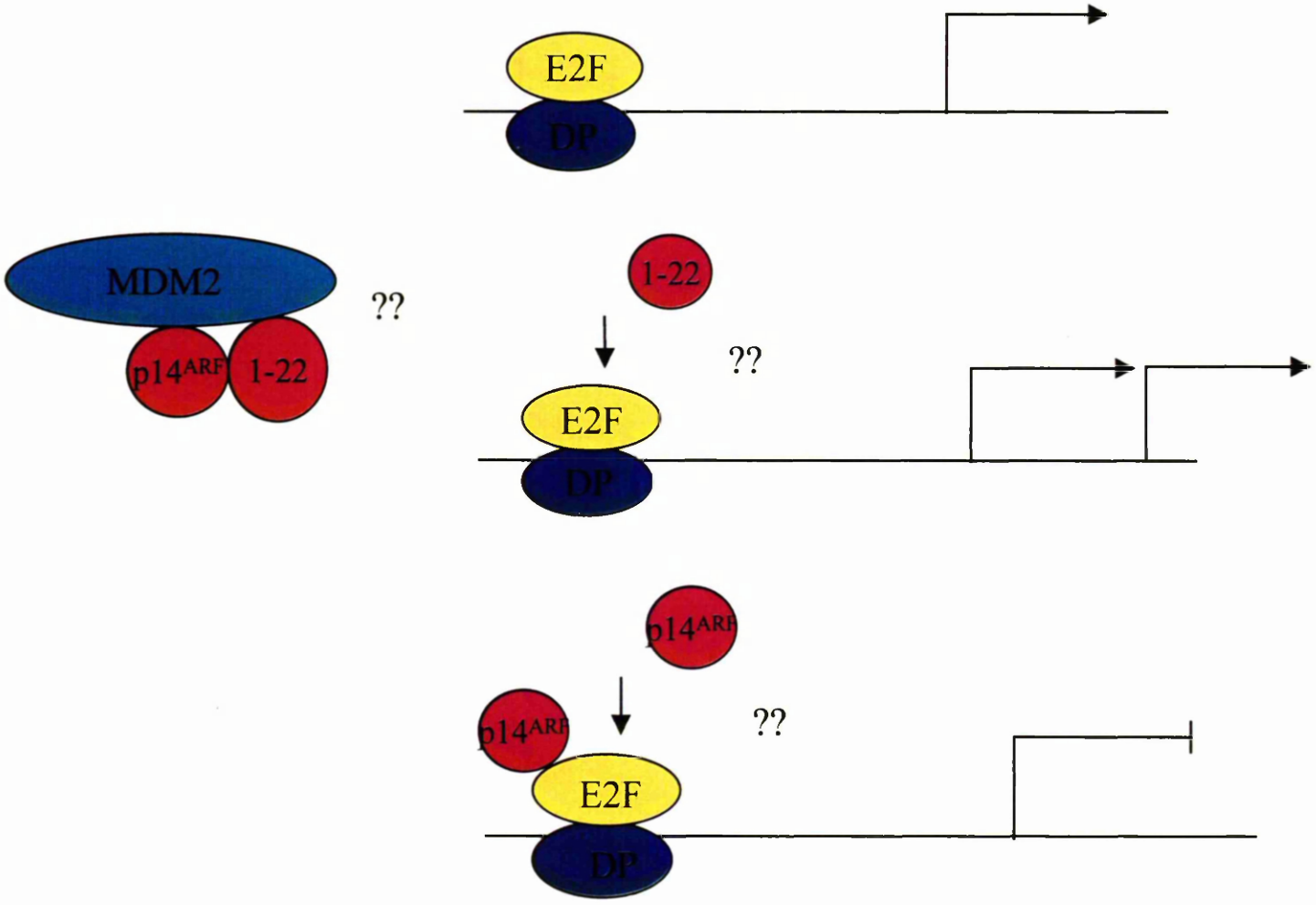

60
30
0
%BrDU
HA-E2F-1 : - + + + + +
DP-1 : - + + + + +
Tx-Myc-p14ARF1-132 : - - + - - -
Tx-Myc-p14ARF1-64 : - - - + - -
Tx-Myc-p14ARF65-132 : - - - - + -
Tx-Myc-p14ARF1-22 : - - - - - +

Figure 6.9. Model for $p14^{ARF}$ 1-22 Effect on E2F activity.

Wild-type $p14^{ARF}$ can bind to E2F-1 and DP-1 and down-regulate the activity of the E2F complex. $p14^{ARF}$1-22 is unable to bind to E2F-1 and cannot down-regulate E2F activity. $p14^{ARF}$1-22 does however, reproducibly enhance the activity of E2F.


E2F
DP
MDM2
??
p14ARF
1-22
1-22
??
E2F
DP
p14ARF
p14ARF
??
E2F
DP

Chapter 7: The $p14^{ARF}$ Regulation of E2F

7.1 Introduction

$p14^{ARF}$ has previously been shown to regulate the p53 pathway through MDM2. The mechanism of regulation described is thought to be partially due to the ability of $p14/p19^{ARF}$ to promote the degradation of MDM2 (Zhang *et al.*, 1998).

MDM2 normally shuttles between the nucleus and the cytoplasm in order to target p53 for degradation (Roth *et al.*, 1998). Honda *et al.*, demonstrated that association of $p19^{ARF}$ with MDM2 inhibits the ubiquitin ligase activity of MDM2 for p53, and that the activity of a $p19^{ARF}$-MDM2 complex in a ubiquitination assay is lower than the activity of free MDM2 (Honda *et al.*, 1999). These data highlight the importance of $p19^{ARF}$ mediated p53 stabilisation.

Co-expression of $p19^{ARF}$ and MDM2 prevents nuclear-cytoplasmic shuttling of MDM2, and as time increases, MDM2 can move to the nucleolus (Tao *et al.*, 1999). Much data supports this MDM2 nucleolar localisation and this relocalisation also occurs following Myc activation and replicative senescence (Weber *et al.*, 1999). MDM2, p53 and $p14/19^{ARF}$ are able to form nuclear bodies, blocking nuclear export of p53 and MDM2, or else retaining them in the nucleolus. Formation of these nuclear bodies requires both the N and C terminus of ARF (Zhang and Xiong. 1999). Studies by Weber et al. suggest that $p19^{ARF}$ contains two NrLS signals which both contribute to nucleolar localisation, however, MDM2 also contains a NrLS and so the nucleolar co-localisation of $p19^{ARF}$ and MDM2 can be viewed as a bi-directional interaction. Data implies that $p19^{ARF}$ binding to MDM2 may induce a conformational change that facilitates nucleolar import of the ARF-MDM2 complex (Weber *et al.*, 2000).

Results

7.2. Over-expression of $p14^{ARF}$ affects its cellular localisation.

Given that $p14/p19^{ARF}$ have been shown to re-localise p53 and MDM2 to the nucleolus (Zhang and Xiong 1998) it was of interest to investigate whether $p14^{ARF}$ could affect E2F cellular localisation. Normally, E2F-1 is nuclear, while DP-1 is cytoplasmic, but becomes nuclear in the presence of E2F-1 (de la Luna *et al.*, 1996; Allen *et al.*, 1997). $p14^{ARF}$ is localised mainly to the nucleolus (Stott *et al.*, 1998; Zhang *et al.*, 1998; Weber *et al.*, 1999). In order to investigate the localisation of $p14^{ARF}$ immuno-staining experiments were carried out in SAOS2 cells, using both endogenous $p14^{ARF}$and over-expressed $p14^{ARF}$.

Firstly, the localisation of $p14^{ARF}$ was determined by immuno-staining. SAOS2 cells were either mock transfected, or transfected with $p14^{ARF}$ and localisation of $p14^{ARF}$ was determined by staining with anti-$p14^{ARF}$ antibody (Figure 7.1A). As expected, endogenous $p14^{ARF}$ was detected almost exclusively in the nucleolus (Figure 7.1A). However, interestingly, different staining patterns were observed when using over-expression vectors. $p14^{ARF}$ when over-expressed was detected in the nucleolus and occasionally in the nucleus (Figure 7.1A). Stained cells were counted in each instance in order to determine precisely how much the localisation of $p14^{ARF}$ can change with expression (Figure 7.1B).

7.3 $p14^{ARF}$ does not affect E2F-1 or DP-1 cellular localisation.

In order to determine whether $p14^{ARF}$ could change the cellular localisation of E2F-1 or DP-1, immuno-staining experiments were again carried out in SAOS2 cells. Cells were transfected with E2F-1 and DP-1 or E2F-1, DP-1 and $p14^{ARF}$. Firstly, the localisation of E2F-1 and DP-1 was determined by staining cells transfected with E2F-1 and DP-1 with either anti-E2F-1 or anti-DP-1 antibody. As expected, when expressed together E2F-1 and DP-1 were nuclear.

The localisation of $p14^{ARF}$, E2F-1 and DP-1 were then examined in cells over-expressing combinations of all three proteins. When E2F-1 and $p14^{ARF}$ were expressed together, $p14^{ARF}$ was found to be nucleolar while E2F-1 was nuclear and excluded from the nucleolus (Figure 7.2A). Additionally, in cells expressing E2F-1, DP-1 and $p14^{ARF}$, E2F-1 was again nuclear, and excluded from the nucleolus while $p14^{ARF}$ was nucleolar (Figure 7.2B). Similarly, cells over-expressing all three proteins were co-stained with DP-1 and $p14^{ARF}$ antibodies. DP-1 was nuclear and $p14^{ARF}$ was nucleolar in this situation (Figure 7.2C).

These data together indicate that $p14^{ARF}$, E2F-1 and DP-1 do not affect the cellular localisation of one another, under the experimental conditions used.

7.4 $p14^{ARF}$ does not affect binding of the E2F complex to DNA.

$p14^{ARF}$ has been shown to overcome E2F mediated transcription, S-phase progression and apoptosis (Chapter 3), and also to bind to both E2F-1 and DP-1

(Chapter 4). One possible mechanism by which $p14^{ARF}$ could regulate these activities could be through binding to the E2F complex on DNA and somehow blocking transcription. Alternatively, $p14^{ARF}$ may be able to prevent the E2F-DP complex from forming and associating with DNA through direct binding to the individual partners. Therefore, it was of vital importance to investigate the mechanism by which $p14^{ARF}$ regulated the E2F complex on DNA.

In order to investigate the effect of $p14^{ARF}$on E2F complex DNA binding, *in vitro* bandshift assays were performed. It was decided to use *in vitro* translated proteins in the binding assays in order to define specifically E2F-1-DP-1 complexes. Firstly, E2F-1-DP-1 complexes were identified by binding to the E2A oligonucleotide. As expected, neither E2F-1 or DP-1 alone bound efficiently to the DNA probe while efficient binding was observed when both E2F-1 and DP-1 *in vitro* translates were incubated together with the same probe (Figure 7.3A).

Complexes were identified by antibody shifts with the relevant antibodies (Figure 7.3B). Complexes were incubated without (lanes 4&5) or with (lanes 6-14) antibody and then visualised on a non-denaturing polyacrylamide gel by auto-radiography. As expected, E2F heterodimers were effectively shifted with anti-HA, anti-E2F-1 and anti-DP-1 antibodies, demonstrating that the E2F-1 and DP-1 proteins were in the complex. In contrast, a Gal4 antibody did not shift the DNA bound complex, indicating that the observed bands were specific for E2F-1 and DP-1 (Figure 7.3B).

In order to investigate the effect of $p14^{ARF}$ on E2F complex binding to DNA, *in vitro* translated E2F-1 and DP-1 proteins were incubated with purified GST, GST-pRb or GST-$p14^{ARF}$ protein. As expected GST-pRb when incubated with E2F complexes caused a mobility shift, indicating that binding of pRb to E2F-1 had occurred (Figure 7.4 Lane 11). In contrast, addition of $p14^{ARF}$ to the E2F-1-DP-1 reaction had no effect on the size of the complex. A decrease in the binding activity of the E2F complex to DNA was observed in the presence of GST-$p14^{ARF}$, however, this was not significant when compared to the effect of GST protein or heat denatured GST-$p14^{ARF}$ on the complex (Figure 7.4 Compare lanes 5, 6, 7 to 9&10).

$p14^{ARF}$ has previously been shown to bind to E2F-1 and DP-1 directly *in vitro* (Chapter 4) and to E2F-1 in *in vitro* binding experiments (Figure 5.3). It was therefore expected that purified $p14^{ARF}$ would bind to the E2F heterodimer and have some effect on DNA binding. These data suggests that $p14^{ARF}$ may not affect E2F binding to DNA and may not form a complex with E2F on DNA. However, the experimental conditions used in these experiments did not reflect the physiological situation in the cell and $p14^{ARF}$ may still regulate E2F DNA binding activity under certain cellular conditions. Although $p14^{ARF}$ can bind to E2F it may alternatively regulate the activity of E2F at a level other than at the DNA binding level.

7.5 $p14^{ARF}$ degrades the E2F-1 protein.

E2F-1 is regulated in a number of different ways, including cellular localisation, acetylation, phosphorylation and degradation (de la Luna *et al.*, 1996; Marti *et al.*, 1999; Martinez-Balbas *et al.*, 2000; Morris *et al.*, 2000). Given that $p14^{ARF}$ has significant effects on the activity of E2F in cells, and that studies of cellular localisation and DNA binding activities showed no significant effect of $p14^{ARF}$ on E2F in these contexts, it became important to look at the post-translational effects of $p14^{ARF}$ on E2F-1.

It was decided to investigate whether over-expression of $p14^{ARF}$ affected E2F-1 protein levels in cells. Previously MDM2 has been shown to decrease levels of E2F-1 in cells (Loughran and La Thangue 2000). SAOS2 cells were transfected with E2F-1 and either $p14^{ARF}$ or MDM2. As expected MDM2 caused a decrease in E2F-1 protein levels compared to E2F-1 alone (Figure 7.5). Interestingly $p14^{ARF}$ also caused a dose dependent decrease in the level of E2F-1 protein compared to E2F-1 alone, and this decrease was greater than that observed with MDM2 (Figure 7.5).

7.6 Conclusions

These data show that under the experimental conditions used $p14^{ARF}$ does not regulate E2F cellular localisation or binding of the E2F complex to DNA. However $p14^{ARF}$ does decrease E2F-1 protein levels, indicative of a post-translational effect on E2F activity.

7.7 Discussion

$p14^{ARF}$ is thought to be involved in relocation of MDM2 to the nucleolus and this is the mechanism by which $p14^{ARF}$ is supposed to regulate p53 activity, through sequesterisation of MDM2 (Weber *et al.*, 1999). Recent evidence has suggested that this re-localisation of MDM2 to the nucleolus is not essential for its function and that further studies are required (Llanos *et al.*, 2001).

$p14^{ARF}$ has no effect on the cellular localisation of E2F-1, or its heterodimeric partner DP-1 (Figure 7.2). This result may be dependent on the particular cells, experimental conditions or time scales used and so cannot completely exclude the fact that $p14^{ARF}$ and E2F may co-localise in the cell under certain conditions. However the result does indicate that over-expression of $p14^{ARF}$ does not force re-location of E2F, and that under these particular experimental conditions the two proteins do not co-localise.

E2F-1 and its heterodimeric binding partner bind efficiently to E2F site in DNA (Zheng *et al.*, 1998) in order to activate or repress transcription of E2F target genes (Dyson 1998). $p14^{ARF}$ down-regulates E2F activity (Chapter 3) and so one possibility was that $p14^{ARF}$ could be binding to the E2F complex, either before DNA binding or while the complex is bound to DNA and preventing E2F from *trans*activating target genes. *In vitro* binding assays showed that $p14^{ARF}$ has no significant effect on E2F binding to DNA in that it does not cause a shift or a decrease in complex binding to DNA, compared to negative control proteins (Figure 7.4).

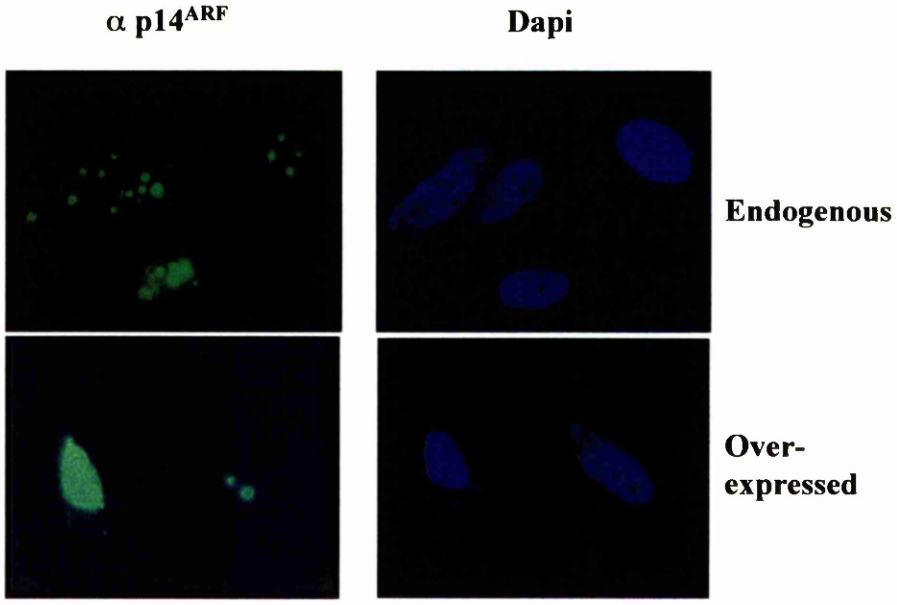
$p14^{ARF}$ does cause a reduction in the levels of E2F-1 protein levels in cells, suggesting that the observed effects of $p14^{ARF}$ on E2F activity may be due to a post-translational down-regulatory mechanism of E2F protein levels. It would be of interest to investigate how $p14^{ARF}$ can down-regulate E2F-1 protein levels, and whether it has intrinsic ubiquitin ligase activity or somehow targets E2F for degradation.

Figure 7.1 $p14^{ARF}$ localisation

(A) SAOS2 cells were either mock transfected or transfected with 6μg CMV-$p14^{ARF}$. Cells were then stained with an antibody to $p14^{ARF}$ and examined for immunoflourescence. Nuclear localisation was confirmed by DAPI staining.

(B) Cells were counted to determine the percentage of $p14^{ARF}$ present in the nucleus and nucleolus as a result of overexpression.

A.



B.

	Nucleolar	Nuclear
Endogenous $p14^{ARF}$	100%	0%
Overexpressed $p14^{ARF}$	84%	16%

Figure 7.2 $p14^{ARF}$ does not affect E2F cellular localisation.

SAOS2 cells were transfected with 6μg HA-E2F-1, CMV-DP-1, CMV-$p14^{ARF}$ together, or in combination. Cells were stained with either $p14^{ARF}$, HA, or DP-1 antibodies to detect the overexpressed proteins and examined for immunoflourescence. Nuclear localisation was confirmed by DAPI staining.

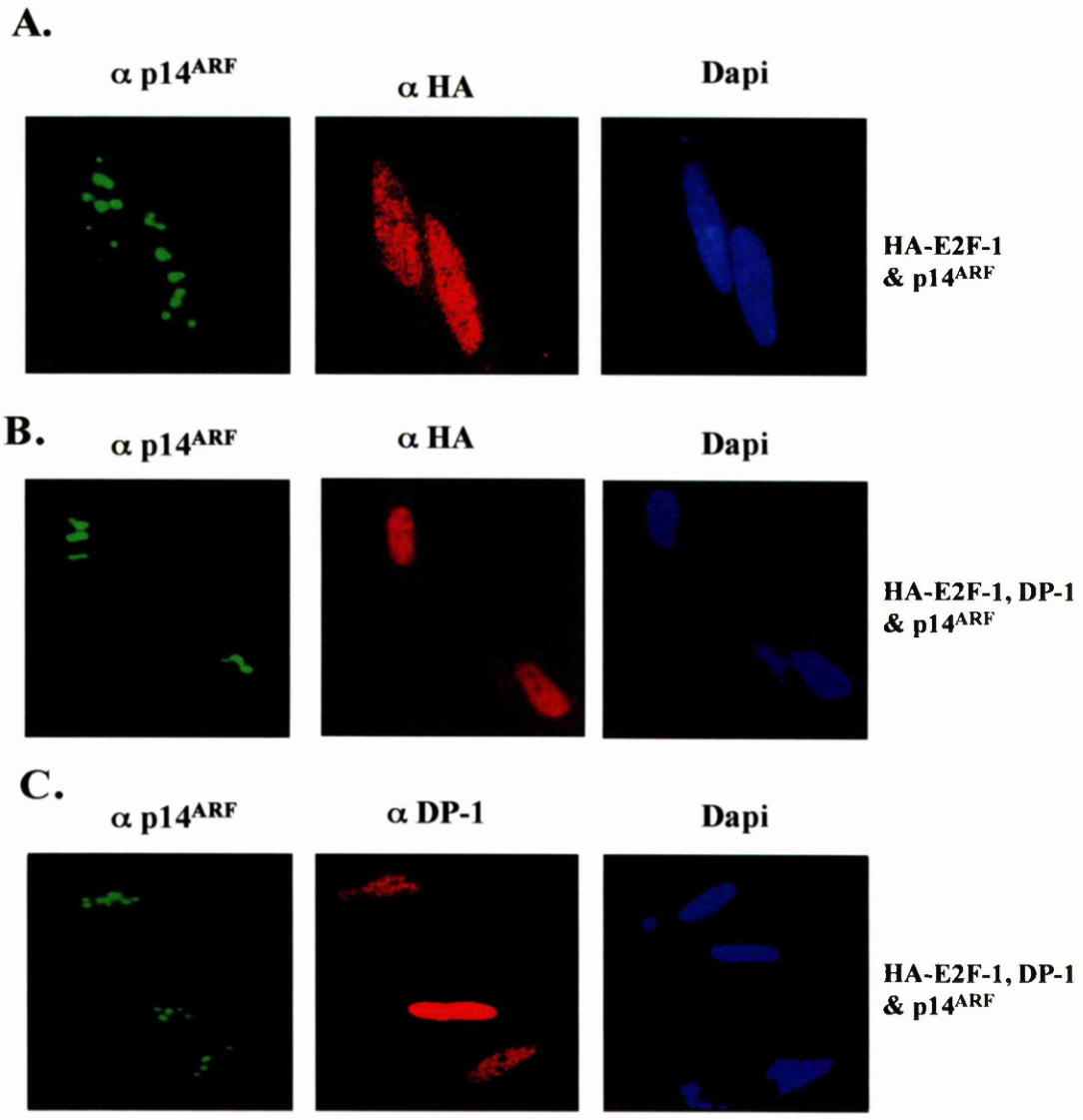

A.
α p14ARF
α HA
Dapi
HA-E2F-1
& p14ARF
B.
α p14ARF
α HA
Dapi
HA-E2F-1, DP-1
& p14ARF
C.
α p14ARF
α DP-1
Dapi
HA-E2F-1, DP-1
& p14ARF

Figure 7.3 Detection of E2F complexes.

(A) ^{32}P labelled probe containing the E2A E2F binding site was incubated with *in vitro* translated HA-E2F-1 and DP-1 or a mixture of the two proteins. Complexes bound to DNA were analysed by gel electrophoresis and autoradiography.

(B) ^{32}P labelled probe containing the E2A E2F binding site was incubated with *in vitro* translated HA-E2F-1 and DP-1 or a mixture of the two proteins. Additionally, antibodies specific for HA, E2F-1, DP-1 and Gal4 were added to the E2F complex in order to determine the nature of these complexes. Complexes bound to DNA were analysed by gel electrophoresis and autoradiography.

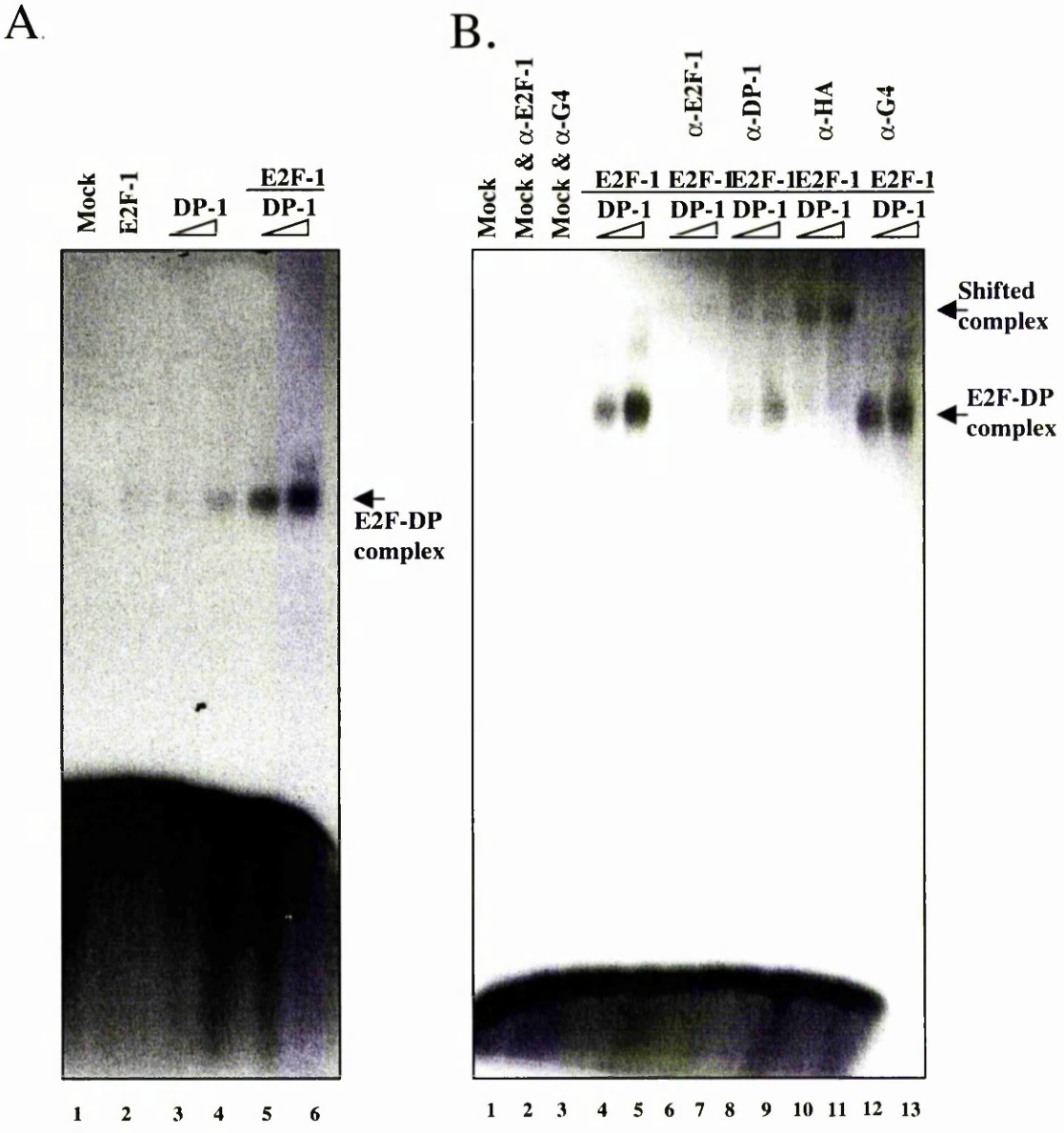

A.
Mock
E2F-1
DP-1
E2F-1
DP-1
E2F-DP complex
1 2 3 4 5 6
B.
Mock
Mock & α-E2F-1
Mock & α-G4
α-E2F-1
α-DP-1
α-HA
α-G4
E2F-1 DP-1
E2F-1 DP-1
E2F-1 DP-1
E2F-1 DP-1
E2F-1 DP-1
Shifted complex
E2F-DP complex
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 7.4. $p14^{ARF}$ does not affect E2F binding to DNA

^{32}P labelled probe containing the E2A E2F binding site was incubated with *in vitro* translated HA-E2F-1 and DP-1 or a mixture of the two proteins. Additionally, purified, GST-$p14^{ARF}$, denatured GST-$p14^{ARF}$, GST-pRb or GST was added to the E2F probe/mixture. Complexes bound to DNA were analysed by gel electrophoresis and autoradiography.

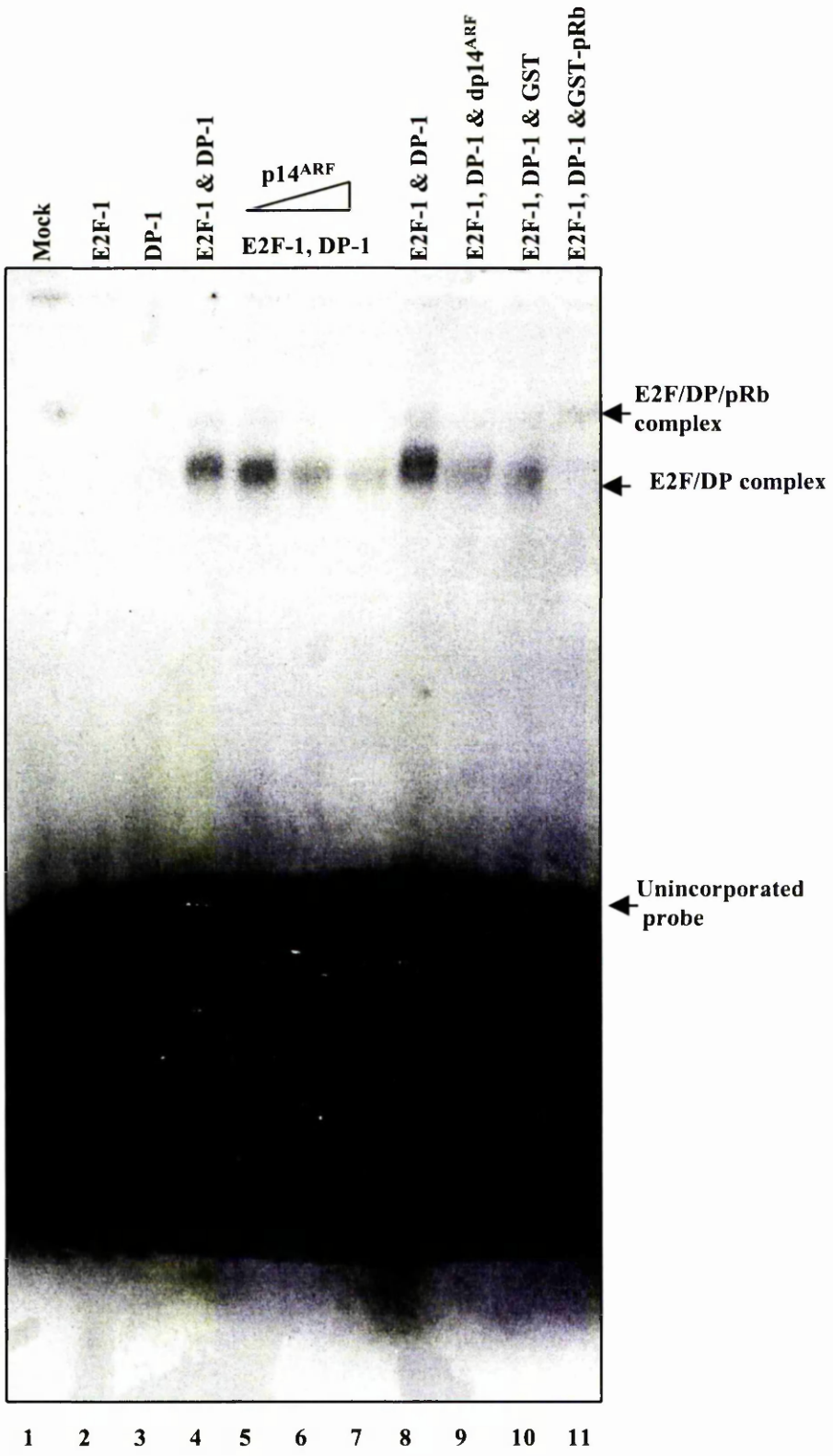
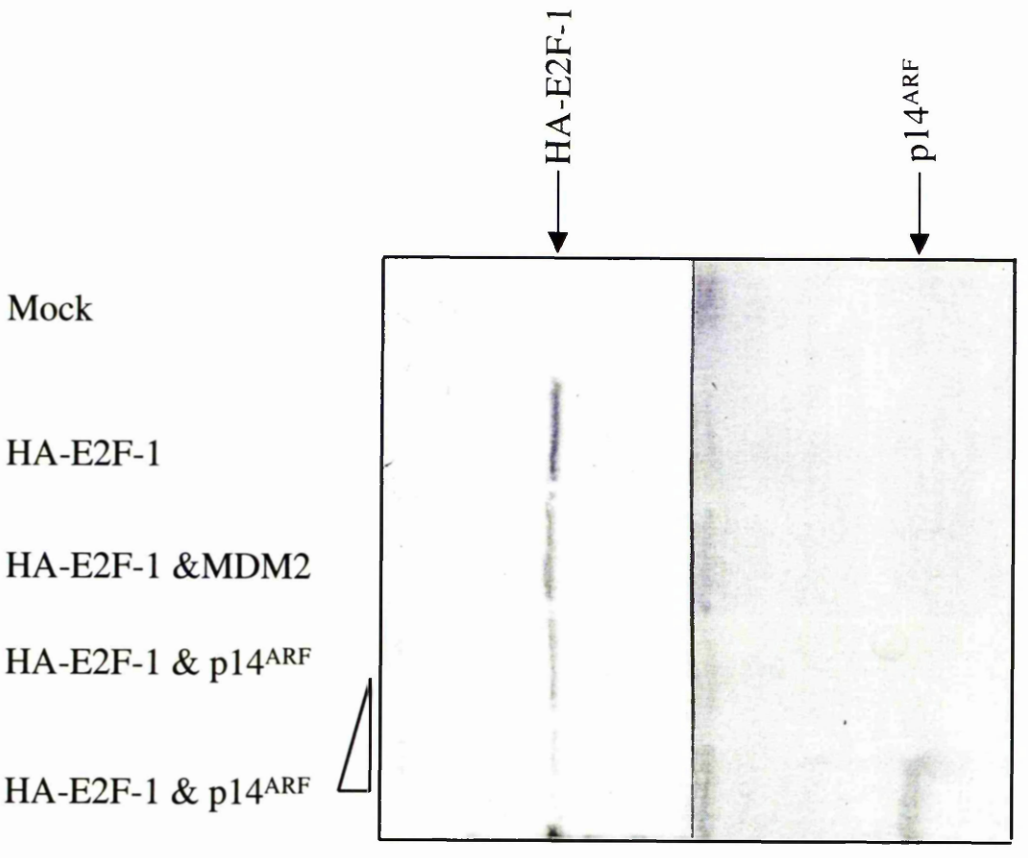

Mock
E2F-1
DP-1
E2F-1 & DP-1
p14ARF
E2F-1, DP-1
E2F-1 & DP-1
E2F-1, DP-1 & dp14ARF
E2F-1, DP-1 & GST
E2F-1, DP-1 &GST-pRb
E2F/DP/pRb complex
E2F/DP complex
Unincorporated probe
1 2 3 4 5 6 7 8 9 10 11

Figure 7.5 $p14^{ARF}$ causes a decrease in E2F-1 protein levels.

SAOS2 cells were mock transfected or transfected with 15μg of HA-E2F-1, 1μg CMV-βgal and CMV-$p14^{ARF}$, (15, 30μg) or pCMDM2 (30μg). Extracts were prepared and normalised for βgal activity to ensure equal loading. Proteins were analysed by SDS-page and western blotting.


HA-E2F-1
p14ARF
Mock
HA-E2F-1
HA-E2F-1 &MDM2
HA-E2F-1 & p14ARF
HA-E2F-1 & p14ARF

Chapter 8. Discussion

8.1 Activation of $p14^{ARF}$.

$p14^{ARF}$ is a protein of central importance in cell cycle control. Expression of $p14^{ARF}$ is induced by the action of a diverse array of oncogenic signals (Bates *et al.*, 1998; De Stanchina *et al.*, 1998; Palmero *et al.*, 1998; Radfar *et al.*, 1998; Zindy *et al.*, 1998; Lin and Lowe, 2001), which results in either cell cycle arrest, apoptosis or senescence (Sherr and Weber, 2000). An established mechanism by which $p14^{ARF}$ can prevent aberrant cellular proliferation is through its physical interaction with MDM2 to prevent the destabilisation of p53 and therefore facilitate the p53 response (Pomerantz *et al.*, 1998).

MDM2 is clearly an important target in mediating the effects of $p14^{ARF}$, however, some studies have suggested that mechanisms other than the direct regulation of MDM2 may exist (Carnero *et al.*, 2000; Weber *et al.*, 2000). Probably the most interesting study regards the effects of $p19^{ARF}$ in triple knock-out mice for MDM2/p53/$p19^{ARF}$, indicating a role for $p19^{ARF}$ independent of MDM2 (Weber *et al.*, 2000). These mice develop tumours at a greater frequency than $p53^{-/-}/mdm2^{-/-}$ mice, demonstrating that $p19^{ARF}$ can interact independently of the p53-mdm2 axis in tumour surveillance (Weber *et al.*, 2000).

Previous studies have documented a role for MDM2 in the regulation of the E2F/pRb pathway (Martin *et al.*, 1995; Loughran and La Thangue., 2000), and MDM2 can increase E2F transcriptional activity and S-phase progression while simultaneously overcoming E2F mediated apoptosis (Martin *et al.*, 1995;

Loughran and La Thangue., 2000). Given that MDM2 can regulate E2F activity, and that $p14^{ARF}$ is a modulator of MDM2 activity, it was of interest to investigate the role of $p14^{ARF}$, if any, in the control of E2F activity. The results presented here identify E2F-1 as a new target for $p14^{ARF}$ and provide evidence that $p14^{ARF}$ can arrest cell growth in a p53 independent manner, probably through modulation of E2F activity.

8.2 $p14^{ARF}$ overcomes E2F mediated transcription

E2F-1 activates $p14/19^{ARF}$ in a transcriptionally dependent fashion as previously described (Bates *et al.,* 1998). I also found that E2F-1 can activate the $p14^{ARF}$ promoter and using the $p14^{ARF}$ promoter in a reporter gene assay I further showed that $p14^{ARF}$ can negatively regulate E2F-1 mediated *trans*activation (Figure 3.2). These data suggest that $p14^{ARF}$ is able to act to down-regulate E2F transcription in a dose-dependent, p53-independent manner and, as a result of this down-regulation may consequently act to regulate its own expression and steady state levels via an as yet, undescribed negative feedback loop.

E2F transcriptionally activates a number of cellular genes, which control many cellular processes including apoptosis, cell cycle progression, and differentiation (chapter 1) so it was of interest to investigate the effects of $p14^{ARF}$ mediated suppression of E2F transcription on other E2F target genes. Experiments were performed to investigate these effects and here I showed that $p14^{ARF}$ can overcome E2F mediated transcription on a number of different E2F responsive reporter gene constructs, including the cellular target genes *cyclinE* (Figure3.3C)

and *Apaf-1*(Figure 3.4B). $p14^{ARF}$ was unable to repress basal transcription on the reporter gene constructs tested, and also fails to negatively regulate p53 activity on the Apaf-1 –871/+208, reporter gene construct which contains a p53 binding site and is responsive to p53 *trans*activation (Figure 3.5). Taken together these observations imply a specific effect for $p14^{ARF}$ on E2F transcription.

During the course of this thesis Eymin *et al.*, (2001a) published similar data showing that $p14^{ARF}$ inhibited E2F transcriptional activity. These authors showed that $p14^{ARF}$ can overcome E2F activity on exogenous reporter gene constructs, including the *cyclinE* gene. These data further support a role for $p14^{ARF}$ in targeting E2F-1 and down-regulating its transcriptional activity.

Intriguingly, $p14^{ARF}$ can also act to up-regulate E2F-mediated transcription, although on the majority of E2F responsive promoters tested, $p14^{ARF}$ down-regulated E2F mediated transcription. Surprisingly, $p14^{ARF}$ itself acted as a transcriptional activator on both the Apaf-1 reporter gene constructs –871/+208 and +35/+208, and additionally co-activated both E2F-1 and p53 transcription (Figure 3.5). Although only one of these reporter genes contains a p53 site, p53 could activate both, and this observation could be due to some secondary p53 independent cell cycle affect of $p14^{ARF}$. The co-activation observed with p53 and $p14^{ARF}$ is probably due to $p14^{ARF}$ binding to MDM2 and preventing MDM2 from degrading p53. $p14^{ARF}$ could be stimulating E2F transcriptional activity through binding directly to the transcription factor, or by some other cellular mechanism. As yet there have been no reports on the ability of $p14^{ARF}$ to bind to DNA,

however, it may be possible that $p14^{ARF}$ activates transcription by binding to other transcription factors or other cellular proteins.

The transcription factor p53 has also been described as being able to act as both a transcriptional activator and a transcriptional repressor (Ryan *et al.*, 2001). p53 can repress promoters that lack its response element and p53 transcriptional repression is in part mediated by an interaction with the mSin3a and histone deacetylase complex (Zilfou *et al.*, 2001). Additionally, binding of MDM2 to the *trans*activation domain of p53 directly inhibits both transcriptional activation and repression by p53 (Haines *et al.*, 1994). p53 is also a general down-regulator of RNA polymerase III transcription (Cairns and White, 1998). p53 can specifically inactivate TFIIIB, however, only before its assembly into the pre-initation complex (Cairns and White, 1998). It is possible that $p14^{ARF}$ could act as both a transcriptional activator and repressor, through similar interactions with cellular, transcriptional regulators.

8.3 $p14^{ARF}$/E2F, an example of a negative feedback loop.

E2F-1 activates $p14/19^{ARF}$ in a transcriptionally dependent manner as previously described (Bates *et al.*, 1998). I showed that $p14^{ARF}$ can overcome E2F-1 mediated *trans*activation. These data suggest that $p14^{ARF}$ is able to down-regulate E2F transcription and, as a result of this down-regulation may consequently act to regulate its own expression and steady state levels via a negative feedback loop. A number of cellular activities are regulated in this way, and one example of a negative feed back loop is the p53/MDM2 pathway. p53 transcriptionally

activates expression of its negative regulator MDM2, which in turn, mediates the degradation of high levels of p53 protein (Haupt, *et al.*, 1997; Honda *et al.*, 1997). When the MDM2-p53 interaction is prevented, through $p14^{ARF}$ binding to MDM2 (Honda *et al.*, 1999) for example, then p53 activity is released. It may be that $p14^{ARF}$ acts in a similar way on E2F and removal of $p14^{ARF}$-E2F binding would allow release of E2F activity. Another example of E2F regulation of its own activity is the E2F-p107 interaction. E2F activates transcription of the p107 gene and p107 binds to members of the E2F family to repress E2F mediated transcription.

Recently more evidence is emerging which suggests that the HIF-1 transcription factor and its negative regulator the VHL tumour suppressor may exist as a negative feedback loop (Blagosklonny, 2001). An intriguing model is that an oncoprotein and a tumour suppressor may form functional pairs and couple *trans*activation with feedback protein degradation (Blagoskonny., 2001). In this context, E2F-1 could be the oncoprotein and $p14^{ARF}$ the tumour suppressor of a functional pair. These functional pairs must also overlap however, for example, $p14^{ARF}$ and MDM2 target both p53 and E2F-1.

8.4 $p14^{ARF}$ regulates E2F mediated apoptosis.

Here I showed that $p14^{ARF}$ can decrease the levels of E2F mediated apoptosis in cells (Figure 3.1). The initial objective of this experiment was to examine whether $p14^{ARF}$ could regulate the effect of MDM2 on E2F in a manner analogous to its effect on p53. The fact that $p14^{ARF}$ is able to overcome MDM2 mediated

reduction of the E2F apoptotic response implies that this may indeed be the case. However, $p14^{ARF}$ is also able to overcome E2F mediated apoptosis and these data suggest that $p14^{ARF}$ may be able to directly down-regulate E2F mediated apoptosis in a mechanistic pathway separate to the MDM2 mediated regulation of E2F apoptosis. In this situation, either $p14^{ARF}$ is able to overcome MDM2 mediated reduction of E2F apoptosis or MDM2 is able to overcome $p14^{ARF}$ mediated reduction of E2F apoptosis. This can be viewed as a bi-directional phenomenon, where either protein could be affecting the other, or merely sequestering each other away from the E2F heterodimer.

It is of interest to question whether the effects of $p14^{ARF}$ on E2F mediated apoptosis are specific or whether this may be a result of cell cycle arrest imposed by $p14^{ARF}$. Indeed, when expressed in combination with the E2F and DP components of the E2F heterodimer, $p14^{ARF}$ caused a decrease in cell cycle progression (Figure 3.6) and this cell cycle arrest may be connected to the observed reduction in apoptosis.

Additionally, the data showing that E2F-1 can activate the Apaf-1 promoter, and that $p14^{ARF}$ can overcome this transcriptional activation on the Apaf-1 –396/+208 reporter gene construct also implies a situation where $p14^{ARF}$ could directly regulate E2F-1 mediated apoptosis through its transcriptional down-regulatory effect. p53 transcriptionally activates the Bax pro-apoptotic factor and this partially mediates the ability of p53 to induce apoptosis (Miyashita *et al.*, 1994). Although it has been shown that the *trans*activation domain of E2F-1 is not required for apoptosis (Hsieh *et al.*, 1997), the fact that $p14^{ARF}$ could decrease the

level of Apaf-1 *trans*activation by E2F-1 may explain the partial effect of $p14^{ARF}$ in reducing apoptosis. It should also be noted, however that $p14^{ARF}$ can also activate transcription of some Apaf-1 reporter genes, this may suggest a role for $p14^{ARF}$ where under certain conditions it acts to induce apoptosis, while under others it can suppress apoptosis. Perhaps under normal cellular conditions $p14^{ARF}$ can suppress apoptosis by inhibiting E2F activity, but when oncogenic signalling occurs it then acts to induce apoptosis to prevent tumour development.

8.5 $p14^{ARF}$ regulates E2F cell cycle progression

Experiments were carried out to examine the effect of $p14^{ARF}$ and E2F in cell cycle control. Considering that $p14^{ARF}$ induces a G1 arrest, in part through p53 but also independently of p53 (Quelle *et al.*, 1995, Pomerantz *et al.*, 1998, Zhang *et al.*, 1998). While E2F over-expression forces S phase progression (Johnson *et al.*, 1994) it was interesting to note that, together with $p14^{ARF}$ the two proteins caused a decrease in S-phase progression, compared to E2F alone when examined in a cell cycle assay (Figure 3.6). Interestingly, in these experiments an increase in the number of cells undergoing S-phase was observed with the overexpression of $p14^{ARF}$ alone. This result is surprising as $p14^{ARF}$ is documented as causing a G1 arrest, however, as I had observed that $p14^{ARF}$ can sometimes act as a transcriptional activator then this may explain this observation. Although, the fact that this may be an artefact of $p14^{ARF}$ overexpression cannot be excluded in this type of experiment.

Other cell cycle arresting proteins such as the CDKIs, $p21^{Waf1/Cip1}$ and $p27^{Kip}$ may also cause cell cycle arrest in combination with E2F. Indeed previous studies have outlined a role for p21 in down-regulating E2F-1 transcription, independently of pRb (Delavaine and La Thangue, 1999). It would be of interest to investigate the effects of these proteins on E2F activity and compare them to the effects of $p14^{ARF}$. This would further elucidate the contribution of $p14^{ARF}$ to the modulation of E2F activity and to rule out that the observed effects are not a general consequence of cell cycle arrest.

Additionally, in a growth assay, $p14^{ARF}$ and E2F both act as growth suppressors, and $p14^{ARF}$ had a highly potent effect (Figure 3.7). When examined together, the observed effect is less than the effect of E2F but more than that of $p14^{ARF}$ (Figure 3.7), indicating that while $p14^{ARF}$ is overcoming E2F activity, it could also be described as E2F overcoming the negative growth activity of $p14^{ARF}$. These data are further supported by the paper from Eymin *et al.*, (2001), where the authors also observed a decrease in growth with $p14^{ARF}$ and E2F-1 in comparison to E2F alone, whilst an interesting study from Martelli *et al.*, (2001) also highlights a similar role for E2F-1 and $p19^{ARF}$ in the regulation of cell growth.

These results suggest a general role for $p14^{ARF}$ modulating E2F activity, however the possibility that $p14^{ARF}$ may be targeting E2F at the transcriptional level cannot be excluded. As E2F is responsible for the activation of so many genes required for cell cycle progression and apoptosis, then the results implied by these assays could be a consequence of the transcriptional down-regulation of cellular genes such as *cyclin E*.

8.6 Physical interaction between E2F-1 and $p14^{ARF}$

$p14^{ARF}$ has previously been described as interacting with the cellular proteins MDM2 (Pomerantz *et al.*, 1998) and topoisomerase I (Karayan *et al.*, 2001). The results presented in this thesis show that $p14^{ARF}$ and E2F-1 can interact both directly *in vitro* and under physiological cellular conditions (Chapter 4). Additionally, $p14^{ARF}$ also interacts with the E2F-1 heterodimeric binding partner, DP-1. These data provide evidence for E2F being a physiological target of $p14^{ARF}$ as the two proteins are found complexed together in cells. Eymin *et al.*, also described an interaction between $p14^{ARF}$ and E2F-1, mapping the N-terminal domain of ARF as binding to E2F-1. It has also recently been shown that $p19^{ARF}$ can bind to E2F-1, 2 and 3 (Martelli *et al.*, 2001), but not E2F-6. It would be of interest to investigate whether $p14^{ARF}$ can bind to other members of the E2F family, and to elucidate a role for more specific regulation of the E2F family members by $p14^{ARF}$.

Mapping data identified a number of sites in E2F for binding to $p14^{ARF}$ and excluded the *trans*activation domain from being required for binding. $p14^{ARF}$ binds to mutants in E2F-1 which are deficient in pRb and cyclin A binding, suggesting that the binding site is not involved in phosphorylation or negative regulation by pRb. $p14^{ARF}$ also bound to domains encompassing the central DNA binding region of E2F-1 (Figure 4.3). These data indicate that $p14^{ARF}$ may not compete with pRb for E2F-1 mediated binding and transcriptional repression, and that the E2F-1 *trans*activation domain is not required for $p14^{ARF}$ binding.

Although not conclusive, these data suggest that $p14^{ARF}$ mediated E2F-1 transcriptional repression is probably not regulated directly by binding to the *trans*activation domain of E2F but may be due to a secondary non-transcriptional activation domain masking effect. Additionally, $p14^{ARF}$ binds to a region of DP-1, which encompasses the heterodimerisation domain further suggesting that the binding of $p14^{ARF}$ to E2F may prevent formation of the heterodimeric complex. Further work is required to map the exact binding domain of $p14^{ARF}$ on E2F-1 and DP-1, in order to correlate binding with function.

8.7 Mutational analysis of $p14^{ARF}$

MDM2 has been shown previously to bind to both the N and C terminal regions of $p14^{ARF}$, however only the N-terminal region is able to regulate MDM2 activity on p53 (Zhang *et al.*, 1998). Furthermore, detailed mapping of the MDM2/$p14^{ARF}$ interaction identified an N-terminal region of $p14^{ARF}$ within the first 22 amino acid residues which was capable of interacting with MDM2, sequestering it to the nucleolus and inhibiting its ability to degrade p53 (Lohrum *et al.*, 2000). Although these data suggest that nucleolar relocalisation of MDM2 by $p14^{ARF}$ is required for the activation of p53, contradictory evidence has also been published indicating that this is actually not required and that $p14^{ARF}$ can still inactivate MDM2 when not relocalised to the nucleolus (Llanos *et al.*, 2001).

My data show that while the 1-34 region of $p14^{ARF}$ can interact with E2F-1 and regulate its transcriptional activity, the 1-22 region in contrast can neither bind E2F-1 nor have a negative effect on transcriptional activity or S-phase progression. Experiments failed to identify whether this was a dominant negative

effect and it is possible that $p14^{ARF}$s effect on E2F is directly related to binding. It is possible that the 1-22 region of $p14^{ARF}$ may be having a dominant negative effect on E2F transcription, or may be removing other, as yet undefined, negative regulatory complexes which are affecting E2F activity.

Given that E2F-1 is unable to bind to the TxMyc-$p14^{ARF}$ 1-22 mutant, while MDM2 does bind to this region (Figure 4.4B and Figure 4.5), this result is indicative of the possibility that E2F-1 and MDM2 may be able to bind to $p14^{ARF}$ and form a ternary complex. Alternatively, separate binding of E2F-1 and MDM2 may be required for the distinct control and function of $p14^{ARF}$.

An interesting feature of $p14^{ARF}$ is the ability of the C-terminal region of the protein to bind to both MDM2 and E2F-1 in cells, while at the same time having little effect on the activity of either protein. A nucleolar localisation signal has been identified in the C-terminus of $p14^{ARF}$, and tumour associated mutations have been mapped to the C-terminus which affect $p14^{ARF}$ nucleolar localisation (Zhang *et al.*, 1998). This has interesting implications for the regulation of E2F activity. It could be that the C terminal region of $p14^{ARF}$ has other, as yet undefined effects on E2F activity. Possibly, the C terminal half of $p14^{ARF}$ is more stable, or active, than the full-length protein, indicating that the C-terminal region of $p14^{ARF}$ may be required for protein stability and the increased activity observed with the N-terminus of $p14^{ARF}$ may be due to an as yet undefined release of activity from the $p14^{ARF}$ C-terminus.

8.8 The role of MDM2

The MDM2 oncogene has been shown to be an important cellular target of p14/p19^{ARF} and its effects on p53 activity are mediated through regulation of MDM2 (Pomerantz *et al.*, 1998). Previous studies however, have documented MDM2 independent pathways for p14^{ARF} cell cycle regulation (Carnero *et al.*, 2000; Weber *et al.*, 2000). The data presented show that the interaction between E2F-1 and p14^{ARF} is at least in part independent of MDM2, and that the effect of p14^{ARF} on E2F activity occurs in the absence of MDM2. This suggests the presence of a novel regulatory mechanism where p14^{ARF} is able to directly regulate E2F activity.

We cannot exclude the possibility, however that in a *p53*$^{-/-}$*/mdm2*$^{-/-}$ background, the MDM2 homologue MDMX may be able to compensate for the activity of MDM2 and act in a similar manner. Deletion of MDMX has recently been shown to cause embryonic lethality due to loss of cellular proliferation and this phenotype can be rescued by deletion of p53 (Parant, *et al.*, 2001), in a manner similar to that of MDM2. These data indicate that MDM2 and MDMX are non-overlapping regulators of p53 activity and so define a new pathway of p53 regulation. It would be of interest to further investigate the role of MDM2 and its homologue MDMX in the regulation of E2F activity by p14^{ARF}.

The binding sites for E2F-1 and p14^{ARF} on MDM2 are distinct, suggesting that the three proteins may exist in a trimeric complex, which indeed appears to exist in immunoprecipitation experiments (Figure 4.2). This could also mean that both

E2F-1 and $p14^{ARF}$ can bind to MDM2 independently of one another and that the components of this pathway could depend on specific cellular states. This situation is particularly indicative in that Eymin *et al.* (2001a) suggest that MDM2 is required for the modulation of E2F activity by $p14^{ARF}$.

This observation by Eymin *et al.* (2001a) contrasts with the data presented here which outlines an MDM2 independent role for $p14^{ARF}$ in the E2F pathway. Interestingly however, this study also identifies the E2F-1/$p14^{ARF}$ interaction as being independent of MDM2 despite their inability to see an effect of $p14^{ARF}$ on E2F activity in reporter assay experiments in *p53*$^{-/-}$*/mdm2*$^{-/-}$ cells. In my experiments I saw a clear effect of $p14^{ARF}$ on E2F activity in two different functional assays, and previous studies have indicated an MDM2 independent role for $p14^{ARF}$ in cell cycle regulation (Carnero *et al.*, 2000; Weber *et al.*, 2000). Together these data imply that $p14^{ARF}$ can act on E2F activity independently of MDM2 under certain cellular conditions. There also exists the potential for MDM2 to regulate $p14^{ARF}$s effect on E2F-1. MDM2 has been previously shown to interact with E2F-1 and DP-1 and modulate E2F activity (Martin *et al.*, 1995, Loughran and La Thangue 2000). Given that $p14^{ARF}$ regulates p53 through MDM2 it seems probable that MDM2 could be involved in some aspects of E2F regulation by $p14^{ARF}$.

8.9 Mechanism of $p14^{ARF}$ regulation of E2F.

E2F activity is tightly regulated during the cell cycle and is subject to a number of modifications including; binding to pRb (Helin *et al.*, 1993), cellular localisation

(de la Luna *et al.*, 1996) and ubiquitin mediated degradation (Marti et al., 1999; Loughran and La Thangue., 2000). It was of interest to investigate whether $p14^{ARF}$ could regulate the activity of E2F in a manner analogous to any of the previously described E2F regulatory cellular proteins, such as NPCD-1 (Sansal *et al.*, 2000) and Pur α (Darbinian *et al.*, 1999). These proteins bind to the E2F heterodimerisation domain and decrease E2F DNA binding, which in turn reduce the *trans*activation of E2F responsive genes.

Initially, experiments were carried out using both endogenous and over-expressed $p14^{ARF}$ in SAOS2 cells and it was interesting to discover that while endogenous $p14^{ARF}$ is nucleolar, over-expressed $p14^{ARF}$ is localised to both the nucleolus and the nucleus. These data correlate with another recent study which also questions the use of over-expressed $p14^{ARF}$ in immunostaining studies, and questions the true cellular localisation of over-expressed proteins (Llanos *et al.*, 2001).

The ability of $p14^{ARF}$ to regulate E2F cellular localisation was investigated and my experiments indicated that in a p53 negative background $p14^{ARF}$ and E2F stayed in their separate cellular compartments when over-expressed together (Figure 7.2). At no point could I observe co-localisation of $p14^{ARF}$ and E2F-1 or DP-1, indicating that the regulation of E2F activity by $p14^{ARF}$ may not be through relocalisation of the proteins as implied for p53/MDM2 (Zhang and Xiong, 1999). However, the possibility that $p14^{ARF}$ may move E2F to the nucleolus or itself move into the nucleus is not excluded as this may only occur at specific cell cycle stages or for brief time windows that are beyond the scope of immunostaining experiments.

It has been published however that $p19^{ARF}$ and E2F-1 can co-localise to the nucleolus and this result opens a number of questions (Martelli *et al.*, 2001). It may be that $p14^{ARF}$ can relocalise E2F to the nucleolus under certain cellular conditions, or indeed that $p14^{ARF}$ may move out into the nucleus in order to interact with E2F. The importance of $p14^{ARF}$ localisation is yet to be elucidated and recent studies have questioned previous data suggesting that $p14^{ARF}$/MDM2 co-localisation is required for $p14^{ARF}$ regulation of p53 (Llanos *et al.*, 2001). Much more work is required to investigate the effects of nuclear and nucleolar localisation of $p14^{ARF}$ and its ability to relocalise proteins such as MDM2 (Zhang and Xiong, 1998), and then to further question how E2F and $p14^{ARF}$ may co-localise in the cell.

The *in vitro* binding data presented in this thesis suggested that $p14^{ARF}$ may bind to E2F-1 and DP-1 through the DNA binding and heterodimerisation domains of these proteins. As heterodimerisation of the partners occurs before DNA binding (Zheng *et al.*, 1999), if $p14^{ARF}$ could interfere with this binding, then down-regulation of transcription would occur. Bandshift assays were performed to investigate whether $p14^{ARF}$ could bind to E2F on DNA or disrupt the E2F complex. Unexpectedly, $p14^{ARF}$ had no effect on E2F binding to DNA *in vitro* (Figure 7.4). As the proteins clearly bind to one another under various different assay conditions this result was not expected (Chapter 4). It may be possible that the conditions used in these assays were not conducive to allow $p14^{ARF}$ to bind E2F and that this interaction may indeed occur under physiological conditions, and may be a tightly regulated process which only occurs at specific cell cycle

stages. Alternatively, $p14^{ARF}$ may not act directly on E2F activity at the transcriptional level but may have a post-translational affect.

The fact that $p14^{ARF}$ showed no immediate effects upon E2F DNA binding activity points to a post-translational affect for $p14^{ARF}$ on E2F activity. A number of cellular proteins such as SCF and MDM2 have been shown to bind and degrade E2F at various stages of the cell cycle (Marti et al., 1999; Loughran and La Thangue, 2000), and it was possible that $p14^{ARF}$ could have been having a similar effect. My data showed that overexpression of $p14^{ARF}$ and E2F together results in a decrease in the levels of E2F-1 protein (Figure 7.5), suggesting a role for $p14^{ARF}$ in E2F degradation. $p14^{ARF}$ may have E3 ubiquitin ligase activity and could target E2F for degradation in a manner analogous to MDM2.

The degradation of E2F-1 by $p14^{ARF}$ could provide an explanation for the observed $p14^{ARF}$ down-regulation of E2F transcription, cell cycle progression and apoptosis. Over-expression of $p14^{ARF}$, may degrade E2F-1 therefore preventing it from performing its usual functions, namely transcriptional activation and cell cycle progression (Figure 8.1). It would be of great interest to investigate the mechanism by which $p14^{ARF}$ causes degradation of E2F, and whether it can degrade other cellular proteins.

Recently, $p19^{ARF}$ has been shown to bind and downregulate the E2F family members E2F-1, 2 and 3 at the post-translational level, however does not effect E2F-6 levels (Martelli *et al.,* 2001). This E2F downregulation is proteasome dependent, suggesting that $p19^{ARF}$ may act as an adapter for ubiquitination. These

data may help elucidate the mechanism by which $p14^{ARF}$ degrades E2F. In combination with these data, the authors also showed that E2F-1 mediated proliferation is suppressed by $p19^{ARF}$, an effect that can be partially reversed by the re-introduction of E2F-1, and that $p19^{ARF}$ could relocalise E2F-1 to the nucleolus (Martelli *et al.,* 2001). It seems that $p19^{ARF}$ targeting of certain E2F species may co-operate with a stimulation of the p53 pathway to counteract oncogenic growth signals.

8.10 General Discussion

The majority of these experiments were carried out in SAOS2 cells, which do not contain functional p53 or pRb in order to study p53 independent $p14^{ARF}$ effects on E2F. These data define a p53 and MDM2 independent effect for $p14^{ARF}$, namely in the regulation of E2F activity. It would be of great interest to create a *p19*$^{-/-}$ */E2F-1*$^{-/-}$ mouse and investigate the phenotype of these animals. However, the complexities of the many E2F family members would make this study difficult. It should also be remembered that although $p14^{ARF}$ and $p19^{ARF}$ are homologues of each other, the two proteins only share 50% amino acid similarity and many differences do exist between them.

It is becoming apparent that many human tumours that retain wild-type p53 suffer loss of $p14^{ARF}$, and are therefore unable to activate p53 in response to abnormal proliferative signals. It would be useful to examine the levels of E2F-1 and $p14^{ARF}$ in tumours and see whether a similar situation exists and indeed, whether loss of both p53 and E2F within the same tumour occurs frequently. A recent paper

published by Eymin *et al.* (2001b) shows that E2F-1 levels are frequently increased or lost in some lung carcinomas, and it would be interesting to investigate the levels of $p14^{ARF}$ in these tumours, which contain high levels of E2F.

The ability to self-replicate is a fundamental basis of life, reflected at the cellular level by the highly regulated process of committing to a round of DNA replication and cell division at the G1/S phase of the cell cycle. The ability of $p14^{ARF}$ to regulate E2F activity and prevent S-phase progression identifies another cellular pathway, which infringes upon the G1/S-phase transition. A number of pathways have been identified which converge at this stage of the cell cycle including the p53 pathway and the parallel pathways of E2F-pRb and Myc; both of which control the activity of cdk2 at the S-phase boundary (Figure 8.2) (Bartek and Lukas, 2001).

It would be of interest to investigate the effect of $p14^{ARF}$ on the Myc pathway, given that Myc also activates $p14^{ARF}$ and is involved in S-phase progression. The cyclin E gene is *trans*activated by both E2F and Myc, and is emerging as a key regulator of S-phase progression (Duronio *et al.*, 1998; Bartek and Lukas, 2001). $p14^{ARF}$ can repress E2F mediated transcription of the cyclin E reporter gene (chapter 3 and Eymin *et al.*, 2001a) and it would be of interest to see whether this was also the case for Myc mediated transcription. Cross talk exists between the pRb/E2F and Myc pathways, for example through Myc-mediated transcriptional activation of E2F-2 and E2F-3, which are negatively controlled by pRb and again, $p14^{ARF}$ may be involved in these cellular pathways.

$p14^{ARF}$ causes cell cycle arrest at both the G1 and G2 phases of the cell cycle (Kamijo *et al.*, 1998; Stott *et al.*, 1998). However, cells lacking p53 alone are refractory to ARF-induced arrest, and in this setting ARF protein expression is greatly increased. This suggests that p53 suppresses ARF through negative feedback, and consistent with this observation reintroduction of p53 into these cells returns ARF protein expression to lower levels (Sherr, 2001). Perhaps a similar effect would be observed in *e2f* $^{-/-}$ cells and it would be interesting to investigate the levels of $p19^{ARF}$ protein in these cells, and vice versa, the levels of E2F-1 protein in ARF knock out MEFs.

ARF acts as a fuse to filter inappropriate mitogenic signals flowing through the cyclin D-cdk-pRb-E2F circuit (Sherr, 2001), inducing p53 under conditions in which abnormal proliferative signals are generated. ARF normally acts to protect cells from Myc over-expression by facilitating Myc-induced, p53-dependent apoptosis. Cells corrupted in the ARF-MDM2-p53 pathway are resistant to Myc-induced killing, enabling Myc to act as a pure growth promoter in this setting. It is likely that $p14/19^{ARF}$ activity will also be tightly interlinked with the E2F and Myc regulatory pathways and future studies on human tumours and transgenic mice should help elucidate these pathways.

8.11 Overall conclusions and future work.

These data and the studies published during the course of this thesis have clearly identified E2F as a cellular target of $p14^{ARF}$. It is becoming increasingly apparent that $p14^{ARF}$ is a highly important tumour suppressor protein, and these data

highlight a role for an E2F-$p14^{ARF}$ pathway, which may regulate the cell cycle. Furthermore, this pathway is independent of MDM2. These data highlight $p14^{ARF}$ as a particularly important cell cycle regulator, in that it targets both the p53 and E2F pathways, albeit in opposite ways. While the previously described role of $p14^{ARF}$ is to upregulate p53 activity to cause cell cycle arrest and apoptosis, it down-regulates E2F activity, leading to a decrease in apoptosis but also a cell cycle arrest.

Much more work is required, both to elucidate the role for $p14^{ARF}$ in cell cycle regulation and to identify those activities which impinge upon both the p53 and E2F pathways.

Figure 8.1 Summary diagram

Summary diagram showing $p14^{ARF}$-E2F cellular effects. E2F *trans*activation leads to increased levels of $p14^{ARF}$. In turn, the high levels of $p14^{ARF}$ down-regulate E2F activity, possible through binding and/or degradation of E2F-1.

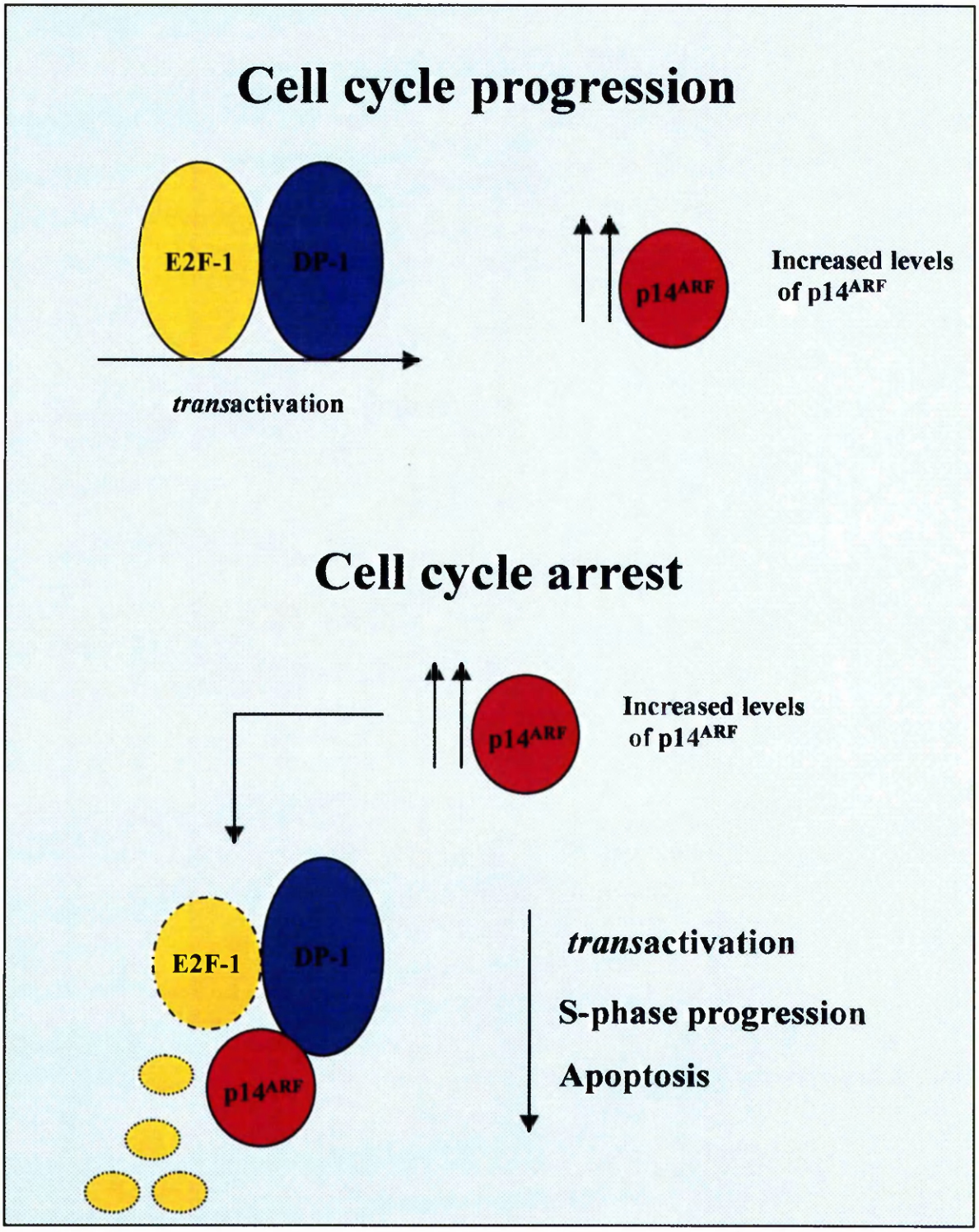
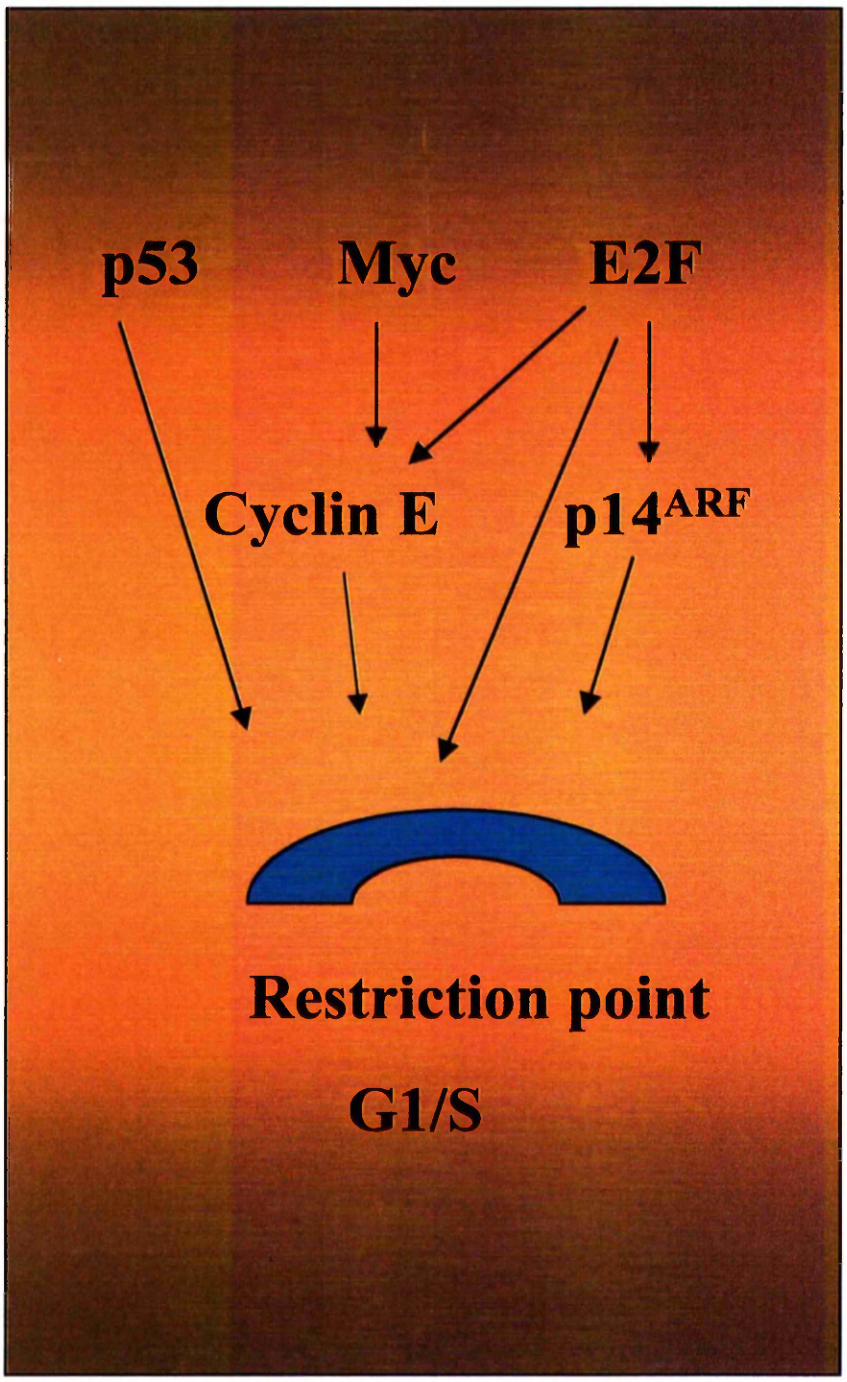

Cell cycle progression
E2F-1
DP-1
transactivation
p14ARF
Increased levels of p14ARF
Cell cycle arrest
p14ARF
Increased levels of p14ARF
E2F-1
DP-1
p14ARF
transactivation
S-phase progression
Apoptosis

Figure 8.2 G1-S-phase regulatory pathways.

Summary diagram outlining the pathways regulating the G1-S-phase transition. The p53, Myc and E2F transcription factors all regulate the cell cycle at the G1 stage. Both E2F and Myc target Cyclin E which itself is important for regulation of the restriction point.


p53
Myc
E2F
Cyclin E
p14ARF
Restriction point
G1/S

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