A thesis entitled

Transcription and cell cycle control.

Presented by

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To

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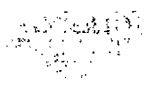
To my parents,



Abstract.

The antiproliferative effects of the tumour suppressor p53 stem from its ability to induce either cell cycle arrest or apoptosis and are regulated by the p300/CBP transcriptional co-activator proteins. In order to elucidate the mechanisms of p300 dependent transcriptional activation of the p53 response, the role of a newly identified protein co-factor, termed JMY, that physically associates with p300 was examined. JMY effectively assists p300 in p53-dependent *trans*activation of apoptotic promoting genes. Removal of the proline rich domain in the C-terminus of JMY produces a protein that switches the functional outcome of the p53 response from apoptosis to cell cycle arrest. Results presented here suggest that JMY collaborates with p300 to stimulate p53 apoptosis while the JMYΔP isoform collaborates with p300 to induce p53 *trans*activation of $p21^{Waf1/Cip1}$ and cell cycle arrest. Thus, the proline rich region of JMY modulates p53's role as a cell cycle arrest or apoptotic inducing protein.

Furthermore, JMY may functionally impact on the p53 pathway through its ability to associate with and influence the activities of human $p14^{ARF}$. JMY co-activates and assists p300 in E2F-1 mediated expression of $p14^{arf}$. In addition JMY is present in the $p14^{ARF}$ complex and a functional consequence of the interaction is the displacing of the nucleolar population of $p14^{ARF}$ into the nucleoplasm.

A tumour derived mutant of pRb, pRbΔ22, that has lost E2F regulatory activity collaborates with JMY in the co-activation of p53-dependent Bax expression, suggesting that cells can by-pass the loss of growth control through pRb by

stimulating apoptosis. JMY therefore acts as a potential regulator of the p53 response and may represent a novel target for the development of therapeutically useful modulators of p53 activity. Defining the mechanism through which JMY and pRb collaborate in apoptosis may prove useful in the understanding of the cells response to tumourigenesis.

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Abbreviations.

A	Adenine
Abl	Abelson virus
AML	Acute myeloblastic leukaemia
ARF	Alternative reading frame
Ap1	Activating enhancer-binding protein 1
Apaf-1	Apototic protease activating factor 1
Arg	Arginine
AS	Alternative splice
ATF2	Activating transcription factor 2
ATM	Ataxia-telangiectasia gene product.
ATP	Adenosine tri-phosphate
Bag	Bcl-2 associated athanogene-1
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
cAMP	Cyclic adenosine mono-phosphate
cDNA	Complementary deoxyribonucleic acid
CAK	Cyclin activating kinase
CBF	CCAAT binding factor.
CBP	CREB-binding protein
Cdc2	Cell division cycle 2
cdk	Cyclin dependent kinase
C/EBP	CCAAT-box/enhancer-binding protein

Ced-3	*C. elegans* cell death-3
CH	Cysteine/histidine-rich domain
Chk1	Checkpoint kinase protein 1
Cip1	Cdk-interacting protein 1
CK	Casein kinase
CMV	Cytomegalovirus
CpG	Cytosine-phosphate-Guandine
CR	Conserved region (in the adenovirus E1A protein)
CRE	cAMP-response element
CREB	CRE-binding protein
Cyc	Cyclin
DAPI	4,6-diamidino-2-phenylindole
DBD	DNA binding domain
DHFR	Dihydofolate reductase
DMEM	Dulbecco's modificatied eagle's medium
DMP1	Cyclin D interacting myb-like protein 1
DNA	Deoxyribosnucleic acid
DNA pol α	DNA polymerase α
DNA-PK	Double-stranded DNA-activated protein kinase
dNTP	Deoxy-nucleoside triphosphate
DP	DRTF interacting protein
DTT	Dithiothreitol
E	Exon
E7	Early protein 7
E1A	Adenovirus early protein 1A
E2F	E2 factor
EBV	Epstein Barr virus
EDTA	Ethylene diamine tetra-acetic acid
EKLF	Erythroid kruppel like factor

Elf-1	E74 like factor 1
ER	Estrogen receptor
FACS	Fluorescence activated cell scanning
FADD	Fas associated death domain
FasL	Fas ligand
FCS	Foetal calf serum
F9EC	F9 embryonal carcinoma
FITC	Fluorescein isothiocyanate
G	Guanine
G_1	Gap phase 1
G_0	Quiescence
GADD	Growth arrest and DNA damage gene
Gas1	Growth arrest specific protein 1
GATA-1	GATA binding protein 1
GCN5	General control of amino acid synthesis protein 5
Gly	Glycine
GR	Glucocorticoid receptor
GST	Glutathione-S-transferase
GTP	Guandine tri-phosphate
HA	Hemagglutinin protein (derived from influenza virus)
HAT	Histone acetyltransferase
HBS	HEPES-buffered saline
HBV	Hepatitis B virus
HDAC	Histone deacetylase
HEPES	N-[2-Hydroxethyl]piperazine-N'-[2-ethanesulfonic acid]
HMG1	High mobility group protein 1
HNF-4	Hepatocyte nuclear factor 4
HPV	Human papilloma virus
Hsp	Heat shock protein

IB	Immunoblotting
ICE	Interleukin 1β converting enzyme
IGF-1	Insulin like growth factor
IGF-BP3	Insulin-like growth factor-binding protein 3
IL-2	Interleukin-2
INF α	Interferon α
INF β	Interferon enhancer
IP	Immuno-precipitation
IPTG	Isopropyl-ß-D-thiogalactopyranoside
IVT	*In vitro* translated
JMY	Junction-mediating and regulatory protein
JNK	c-Jun NH_2-terminal kinase
KID	Kinase-inducible domain
KIX	Kinase inducible domain
LOH	Loss of heterozygosity
Luc	Luciferase
LXCXE	Leucine-X-Cysteine-X-Glutamic acid
M	Mitosis
MAP	Microtubule associated proteins
MAP4	Microtubule associated protein 4
MAPK	Mitogen activated protein kinases
MDM2	Murine double minute 2
MDMX	Mouse double minute X
MEF2	Myocyte enhancer factor
MEF's	Mouse embryonic fibroblasts
MLL	Mixed lineage leukaemia
MOZ	Monocytic-leukaemia zinc-finger
mRNA	messenger ribonucleic acid
MyoD	Myogenic HLH transcription factor

NADPH	Nicotinamide adenine dinucleotide phosphate
NES	Nuclear export signal
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor of immunoglobulin k locus in B cells
NLS	Nuclear localisation signal
N-CoR	Nuclear receptor co-repressor
Np-40	Nonidet P-40
NS	Normal splice
NuLS	Nucleolar localisation signal
P	Proline
PAGE	Polyacrylamide gel electrophoresis
PAR	Proteinase activated receptor
PBS	Phosphate buffered saline
P/CAF	p300/CBP-associated factor
PCIP	p300/CBP interacting protein
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PIG3	p53 induced gene 3
PKA	Protein kinase A
PKC	Protein kinase C
PMSF	Phenylmethysulfonyl fluoride
Pol	Polymerase
PP-1	Protein phosphatase 1
$pp90^{RSK}$	90kDa S6 kinase
PR	progesterone receptor
pRb	Retinoblastoma gene product
Pu	Purine
Py	Pyamanidne
Ran-GTP	Ran GTPase activating protein

RCE	Retinoblastoma control element
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
ROS	Reactive oxygen species
RT	Reverse transcription
RTS	Rubinstein-Taybi syndrome
RP-A	Replication associated protein-A
S	Serine
Sap1a	SRF accessory protein 1a
SAS2	Something about silencing 2
SCF	SKIP1/CUL1/F-Box protein complex
SDS	Sodium dodecyl sulphate
SH_3	Src homology domain 3
SnRNP's	Small nuclear ribonucleoproteins
SRC-1	Steroid receptor co-activator 1
SRE	Sterol regulatory element
SREBP	SRE binding protein
SRF	Serum response factor
STAT	Signal transducers and activators of transcription
S/T-P	Serine/Threonine-proline
SV40	Simian virus 40
T	Thymine
TAF	TBP-associated factor
TBP	TATA binding protein
TCF	T cell factor
TCR	T cell antigen receptor
TdT	Terminal deoxy-transferase
TF	Transcription factors
TGF-β	Transforming growth factor β

Tip60	Tat interacting protein
TK	Thymidine kinase
TNF	Tumour necrosis factors
TRAIL	TNF related apoptosis inducing ligand
TR	Thyroid receptor
TRE	Thyroid response element
TRID	Truncated intracellular death domain
TRITC	Tetramethylrhodamine isothiocyanate
Tris	Tris(hydroxymethyl)methylamine
TRUND	Truncated death domain
TRUNDD	Trail receptor with a truncated death domain
TS	Thymidylate synthetase
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 homolog 1
v/v	Volume per volume
Wip1	Wild-type p53 inducible phosphatase 1
wt	Wild-type
WT1	Wilm's tumour-1
w/v	Weight per volume
X	Any amino acid
YY1	Ying yang 1
Zn^{2+}	Zinc atom
(+/+)	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.

Stephen France

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1. Introduction.

Cells have acquired the ability to divide and replicate in order to allow the propagation of the species and to counter the loss of cells by damage. The replication of cellular material and DNA, which leads to cell division, takes place through a process known as the cell cycle. Understanding the cell cycle is essential in the comprehension of human diseases that originate from its breakdown and is vital in the search and design of anti-tumour therapies.

The cell cycle is regulated through the activity of transcription factors and their associated repressors and activators. The co-ordination of the signals from these components determines whether a cell proliferates, differentiates, enters quiescence or dies (La Thangue, 1994).

The integration of growth regulating signals occurs at the G_1 to S phase boundary after which point a cell is committed to divide. The transition from G_1 into S phase is controlled by a number of critical regulatory proteins that control gene expression, such as the transcription factors p53 and E2F (La Thangue, 1994; Levine, 1997). In addition these critical factors are themselves regulated by cellular proteins such as the tumour suppressor pRb and the transcriptional co-activator p300/CBP (Torchia *et al.*, 1998; Weinberg, 1995; Shikama *et al.*, 1997). The cellular importance of p53, pRb, and p300/CBP is emphasised by the observations that viral proteins that maintain proliferation directly interfere with their functions.

Under conditions where the integrity of the genome has been compromised the cells cycle is arrested. The failure of cells with unstable genomes to arrest is a defining point in the formation of cancer cells. Cellular mechanisms have evolved that repair damaged genomes but under conditions where the damage response is unable to initiate a recovery a second more potent mechanism that prevents the proliferation of cells has arisen, namely apoptosis. Apoptosis plays an indispensable role during development to eliminate unwanted potentially dangerous cells and consequently the mechanisms that control the apoptotic response are highly conserved (Burns and El-Deiry, 2000).

Additional, new, members involved in the regulation of the cell cycle and apoptotic pathways are continually being identified and with every discovery a more detailed understanding of the cell cycle emerges. Discussed here in more detail are the known functions of the cell cycle regulators, p53, p300/CBP, ARF and pRb.

1-1. p53, the cellular gatekeeper for growth and apoptosis.

1-1.1. Introduction.

A key regulator of cellular growth and neoplasia is the tumour suppressor p53. The gross over-expression 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, 1983).

p53 is a multi-functional protein that executes a variety of cellular outcomes in order to maintain a healthy cell. Numerous studies have elucidated a role for p53 in growth arrest, apoptosis and differentiation and defined p53 as a DNA damage-inducible protein that participates in genomic repair (Cross *et al.*, 1995; Wells, 1996).

The inactivation of wild-type p53 promotes genomic instabilities and is a key event in the formation of cancer cells. The high frequency of p53 mutations observed in human tumours clearly helps define p53 as the "guardian of the genome" (Levine, 1997).

1-1.2. Characteristics of p53.

The p53 gene is highly mutated in human cancer, with approximately 50% of tumours displaying a loss of p53 function as a result of germline mutations (Hollstein *et al.*, 1991; Greenblatt *et al.*, 1994). Many tumour types show deletion of one p53 gene allele and mis-sense mutation in the other (Hollstein *et al.*, 1994). The high frequency of mutations in p53 alleles lead to the discovery that p53 is a heritable

germ line mutated gene. Individuals suffering from Li-Fraumeni syndrome that are predisposed to cancer carry a germ line mutation in p53 (Donehower and Bradley, 1993). In addition individuals with independently arising neoplasms often display germ line mutations in the p53 locus.

Species and mutational comparisons identified that the vast majority of mis-sense mutations in p53 are clustered at hot spot regions in the highly conserved core domain. The hot spot regions are comprised primarily of amino acids that incapacitate p53 sequence specific DNA binding and underpin p53's ability to suppress tumourigenesis. The hot spot amino acids Arg248 and Arg273 directly contact DNA while Arg175, Arg245, Gly249, Arg273 and Arg282 are responsible for stabilising the structure of the DNA binding interface (Levine, 1997) (Figure 1.1a).

p53 is more susceptible to mis-sense mutations than nonsense mutations which suggests that p53 mutants that retain selective functions are advantageous to tumour cells. Consistent with the advantage of p53 mutations in tumour cells is the observation that mutant p53 introduced into p53 negative cells aids tumourigenesis (Dittmer *et al.*, 1993). In addition to the gain of function observed by mutant p53 the proteins is also able to disrupt cellular growth control by affecting the activity of the wild-type protein. In agreement, oligomerization domain mutations in p53 that dominate wild-type p53 function are negative in cell cycle control as a result of their inability to form tetramers (Unger *et al.*, 1993).

p53, in keeping with the observation that many oncogenes and tumour suppressor genes are arranged into families, is itself one member of a family. p53 has two known

family members, namely p63 and p73 (Kaghad *et al.*, 1997; Yang *et al.*, 1997). Interestingly p63 and p73 show more similarity to each other than to p53. Common to all three family members is the high number, large size and organisation of their non-coding introns (Soussi and May, 1996; Marin and Kaelin, 2000).

The p53 family of proteins is enlarged by the ability of all the family members to undergo splicing events that produce functionally distinct proteins. In mouse, two p53 splice forms are expressed, called normal splice (NS) and alternative splice (AS), while both p63 and p73 are multiply spliced (Wu *et al.*, 1995; Arai *et al.*, 1986). p63 can be spliced into α, β, and γ forms while p73 has five known forms namely α, β, γ, δ, and ε (De Laurenzi *et al.*, 1998; De Laurenzi *et al.*, 1999; Zaika *et al.*, 1999; Marin and Kaelin, 2000). Additionally a cryptic promoter located in exon three is utilised to produce three N-terminal deleted transcripts of *p63* (Yang *et al.*, 1997).

The cloning and sequencing of p53 from a variety of species in combination with functional interaction data has enabled a detailed structural analysis of p53. Human p53 is encoded by 393 amino acids and has four structurally conserved functional domains in addition to a number of other interesting features. The N-terminal 42 amino acids of p53 constitute its transcriptional activation domain, presumably as a result of its ability to contact directly members of the basal transcription machinery (Fields and Jang, 1990). The N-terminus is predominantly acidic in nature, however, two hydrophobic residues in this region mediate p53's ability to interact with the transcription machinery components $TAF_{II}70$ and $TAF_{II}31$ (Lu and Levine, 1995; Thut *et al.*, 1995). The *trans*activation domain of p53 is also the site targeted by the negative regulators E1B-55kDa and MDM2 (Lin *et al.*, 1994) (Figure 1.1b).

Active p53 exists as tetramers (dimer of dimers) and the domain that mediates oligomerization resides in the C-terminus (Shaulian *et al.*, 1992; Iwabuchi *et al.*, 1993; Wang *et al.*, 1994a) (Figure 1.1b). Interestingly, as a result of allosteric interactions between the core domain and the oligomermization domain through a flexible linker, p53 can also form multiples of tetramers (Stenger *et al.*, 1994).

p53 binds to specific DNA sequences through its central core domain (Zauberman *et al.*, 1993) (Figure 1.1b). A tetrahedrally co-ordinating Zn^{2+} atom in the core domain confers the self-folding and sequence specific binding properties of p53 (Pavletich *et al.*, 1993; Wang *et al.*, 1993). Analysis of multiple genomic p53 target sites has defined the consensus site 5'-PuPuPuC($^{A}/_{T}$)($^{T}/_{A}$)GPyPyPy-3' for tetrameric p53 binding (Strurzbecher and Deppert, 1994).

The highly basic, extreme, C-terminus of p53 negatively regulates the specific binding of p53 to its consensus site and can also non-specifically bind DNA and RNA (Hupp *et al.*, 1992; Pavletich *et al.*, 1993; Wang *et al.*, 1993) (Figure 1.1b). The ability of the C-terminal domain of p53 to recognise nucleotide mismatches, insertions and deletions to either sterically or allosterically alter the sequence specific binding capacity of p53 is undoubtedly an important feature of p53 (Levine, 1997). C-terminal domain associated catalysis of DNA and RNA re-association underlies the involvement of p53 in the mismatch repair process.

p53's functional diversity has been attributed to its ability to interact with a large number of different cellular proteins (Figure 1.1c). p53 interacts with the single

stranded binding protein RP-A and the interaction is believed to inhibit RP-A binding and stimulation of single stranded DNA condensation (Dutta *et al.*, 1993). The interaction of the RNA polymerase subunit $TF_{II}H$ with p53 results in the loss of its helicase activity and a modulation of its function in nucleotide excision repair (Wang *et al.*, 1995b). The Wilms' tumour suppressor gene product WT1 also interacts with p53. WT1 abrogates p53-mediated transcription to suppress apoptosis (Maheswaran *et al.*, 1995). In addition to the factors discussed above, p53 binds to and utilises a large number of transcriptional co-activator proteins, one such member being p300/CBP (Avantaggiati *et al.*, 1997; Gu *et al.*, 1997; Lill *et al.*, 1997b).

In the N-terminus of p53 there are five copies of the SH_3 (Src homology domain 3) binding motif P-X-X-P. A potential role of the polyproline domains is in the regulation and binding of p53 to SH_3 signal transduction domain containing proteins. Consistent with the proposed role of p53 as a SH_3 domain binding protein and signal transduction cascade target is the ability of the SH_3 domain containing c-Abl protein to stimulate and activate a p53-dependent cell cycle arrest (Goga *et al.*, 1995). Interestingly, mutations in the P-X-X-P motifs have been detected in patients suffering Li-Fraumeni syndrome and correlates with the reduced ability of p53 in these suffers to induce apoptosis and cell cycle arrest (Sun *et al.*, 1996; Walker and Levine, 1996).

In addition to the sequence and structural features discussed above p53 also has a number of other interesting features, including potential sites that can be modified by phosphorylation and acetylation (Privies, 1998; Soutogloou *et al.*, 2000). The high

level of evolutionary conservation of p53 together with its structural characteristics underlies p53's vital cellular importance.

1-1.3. p53 mediated transcription.

In most cells p53 is a latent, short-lived protein with a rapid turnover rate. Consequently, in order for p53 to perform a specific function it must receive signals that alter its half-life. Several types of cellular stresses such as γ-irradiation, UV irradiation, nucleotide depletion and chemical damage all activate p53 function either by post-translationally stabilising p53 or increasing its profile of expression. Consistent with the activation of p53 in response to DNA damage is the observation that transgenic mice defective in nucleotide excision repair display an elevated level of p53 (McWhir *et al.*, 1993).

Given that divergent forms of DNA damage result in an identical cellular outcome it is possible that the cellular mechanisms that sense DNA damage cross-talk and converge on p53. Supportive of such a role for p53 is the observation that cells defective in the ATM gene product, that senses DNA damage, display a delayed p53 accumulation response following their treatment with ionising radiation (Kastan *et al.*, 1992).

The p53 response is also activated as a result of hypoxia. An interesting possibility is that tumour cells, as they reach a critical size, begin to undergo a p53 response that holds the tumour in a non-metastatic state (Graeber *et al.*, 1996). The anti-angiogenic factor thrombospondin-1 is induced by p53 suggesting the existence of a feedback mechanism of p53 activation in tumours. Thrombospondin reduces the blood supply

to tissues, which concurrently activates the p53 hypoxia response which in turn will feedback and activate thrombospondin expression (Sun *et al.*, 1999).

The ability of p53 to specifically bind DNA elements in the reporters of many cell cycle genes clearly demonstrates that p53 is a *bona fide* transcription factor. The *gadd45* gene, that encodes a protein which modulates PCNA function and is involved in nucleotide excision repair, is induced by p53 in response to DNA damage (Kastan *et al.*, 1991; Smith *et al.*, 1994). Induction of GADD45 by p53 stimulates a growth arrest phenotype to presumably allow damage repair.

Interestingly, p53 was also shown to induce the expression of the MDM2 proto-oncogene that, in turn, can repress p53 transcription by promoting its degradation (Momand *et al.*, 1992). The *mdm2* gene contains two distinct potential p53 responsive promoters, P1 is responsible for basal MDM2 expression and P2 is involved in the activated p53 response (Barak *et al.*, 1994). MDM2 gene expression is elevated following the radiation induced DNA damage stabilisation of p53 (Barak and Oren, 1992; Barak *et al.*, 1994) (Figure 1.2b).

The exposure of cells to a variety of DNA damaging agents led to the identification of $p21^{Waf1/Cip1}$ as a p53 responsive gene (El-Deiry *et al.*, 1993). The ability of $p21^{Waf1/Cip1}$ to inhibit cyclin-dependent kinases and thereby influence pRb phosphorylation is the mechanism by which p53 stimulates G_1 arrest (Gu *et al.*, 1993). The treatment of human fibroblasts with radiation confirmed that $p21^{Waf1/Cip1}$ expression and inhibition of the cell cycle was p53-dependent (Dulic *et al.*, 1994) (Figure 1.2c).

The regulation of cellular apoptosis by the proto-oncogene Bcl-2, that prevents apoptosis, and its homologous protein Bax, that accelerates apoptosis, were studied in relation to p53 transcriptional activation (Oltvia *et al.*, 1993). The Bcl-2 family of proteins can be divided into pro-survival members such as Bcl-2, Bcl-X_L, Bcl-w and CDE9 and pro-apoptotic members such as Bax, Baf and Bid (Burns and El-Deiry, 1999). The Bcl-2 family members form heterodimers, and it is the relative ratio of the survival versus apoptotic factors that determines whether the cell lives or dies. The anti-apoptotic members of the Bcl-2 family inhibit cyctochrome *c* release from the mitochondria which prevents Apaf-1 activation of initiator capase 9. The over-expression of p53 in a murine leukaemia cell line resulted in an increase in Bax expression and further analysis demonstrated that the actual gene promoter contains four potential consensus p53 sites. Physiologically p53 appears to specifically activate Bax in the context of p53-dependent apoptosis (Miyashita *et al.*, 1994a; Miyashita and Reed, 1995) (Figure 1.2a).

The insulin-like growth factor binding protein 3 (IGF-BP3) that inhibits mitogenic signalling by the insulin like growth factor (IGF-1) is effectively induced by p53 (Buckbinder *et al.*, 1995). The induction of IGF-BP3 occurs in response to DNA damage and lowers the receptiveness of cells to mitogenic signals (Levine, 1997). Given that IGF-BP3 protects cells from c-Myc induced p53-dependent apoptosis it has been proposed to act as a survival factor that sensitises cells to apoptotic signals.

The cyclin G gene is also transcriptionally activated by p53 although the functional significance of this over-expression is unknown (Zauberman *et al.*, 1995).

Interestingly in transient transfection assays the pRb promoter is dose-dependently p53 responsive, with high levels of p53 being repressive, and low levels activating (Shiio *et al.*, 1992; Osifchin *et al.*, 1994).

The N-terminal p53 polyproline domain is essential for efficient growth suppression and transcription of the *PIG3* (p53 induced gene 3) promoter but is dispensable for the transcriptional activation of $p21^{Waf1/Cip1}$, *mdm2* and *bax* (Walker and Levine, 1996). Conflicting evidence exists as to the ability of the polyproline region to affect p53's DNA binding affinity. The polyproline domain of p53 is believed to mediate p53's role in non-specific transcriptional repression, production of reactive oxygen species (ROS), and apoptosis but not its role in growth arrest (Sakamuro *et al.*, 1997; Venot *et al.*, 1998).

An interesting feature of p53 is its ability to repress both cellular and viral promoters that do not contain a consensus p53 site (Ginsberg *et al.*, 1991; Subler *et al.*, 1992; Jackson *et al.*, 1993). p53's non-specific repressive activity relies on both its N- and C- termini and is TATA box promoter dependent (Subler *et al.*, 1992; Mack *et al.*, 1993; Sang *et al.*, 1994). p53's ability to repress transcription may be an indirect consequence of activation domain binding factor competition. Consistent with such a model is p53's ability to repress the human *hsp70* promoter as a consequence of its ability to bind and sequester the transcription factor CBF (CCAAT binding factor) (Agoff *et al.*, 1993). p53's ability to act as a transcriptional repressor is also highlighted by its ability to repress a large number of genes following p53 induction (Polyak *et al.*, 1997). One such gene encodes the microtubule-associated protein MAP4 (Murphy *et al.*, 1996).

1-1.4. p53 induced cell cycle arrest.

The constitutive over-expression of wild-type p53 abrogates oncogene-mediated transformation and inhibits the growth of various tumour cell types (Eliyahu *et al.*, 1989; Finlay *et al.*, 1989; Baker *et al.*, 1990; Diller *et al.*, 1990). p53 inhibits cell cycle progression by holding cells in G_1 which allows time for the assessment of the DNA integrity of the cell before a commitment to divide, that helps maintain the genetic stability of the cell (Levine, 1997). Consequently the level of p53 is directly induced upon the treatment of cells with DNA damaging agents (Kastan *et al.*, 1991).

The ability of p53 to cause G_1 arrest is mediated by its ability to reduce the activity of the cyclin-dependent kinases that promote gene expression. p53 induction of the cyclin dependent kinase inhibitor $p21^{Waf1/Cip1}$ in response to DNA damage in human fibroblasts is the major event that mediates G_1 arrest. (Dulic *et al.*, 1994). $p21^{Waf1/Cip1}$ universal inhibition of the cyclin dependent kinases is mediated by its interaction with the cyclin fold and inhibition of CAK (CDK activating kinase) phosphorylation. Inactivation of cyclin/cdk's results in the hypophosphorylation of pRb which in turn represses the key cell cycle regulatory transcription factor E2F (Figure 1.2c). Interestingly however p53 transcriptionally inactive mutants are still able to induce a G_1 arrest and $p21^{Waf1/Cip1}$ deficient mice develop normally (Deng *et al.*, 1995). Additionally, mouse embryonic fibroblasts (MEF's) that are $rb^{-/-}$ can still undergo a radiation stimulated p53 mediated G_1 arrest supporting a role of p53 not only in pRb regulation but also in the regulation of the other pocket protein family members namely, p107 and p130 (Slebos *et al.*, 1994). The ability of p53 to mediate a G_1 arrest is, however, pivotal to the stability and regulation of the genome.

When mitotic spindle inhibitors, such as nocodazole, are added to cells in the presence of p53 cells arrest in G_2/M (Vikhanskaya *et al.*, 1994; Cross *et al.*, 1995). The ability of p53 to G_2/M arrest cells is believed to be a consequence of p53's role as a centrosome number and spindle checkpoint controlling protein (Fukasawa *et al.*, 1996). In support of p53's role as a spindle formation regulator is the observation that *p53*$^{-/-}$ cells and embryos from p53 knockout mice display a high degree of aneuploidy, tetraploidy and octaploidy (Harvey *et al.*, 1993; Cross *et al.*, 1995). Indeed, p53 directly associates with centrosomes (Brown *et al.*, 1994).

In addition to its role in cell cycle arrest p53 has been linked to a Gas1 associated G_O arrest. Gas1 is a membrane protein that is expressed during G_O and functions in maintenance of the G_O phenotype. p53's role in signalling, in a transcriptionally independent manner, may be the mechanism that p53 utilises to mediate a Gas1 associated G_O arrest (Del-Sal *et al.*, 1995; Ruaro *et al.*, 1997).

The flattened senescence like phenotype observed in some p53-induced cells indicates that the p53 response is cell type specific. The levels of p53 and p21$^{Waf1/Cip1}$ increase as cells age and senesce, and interestingly *p53*$^{-/-}$ cells escape a senescence check point and go on to form aneuploid immortalised cells (Bond *et al.*, 1995). The transient G_1 arrest or permanent senescence like arrest associated with p53 activation outlines two of the mechanisms that the cell has evolved to prevents its proliferation with an unstable genome (Gottlieb and Oren, 1996).

In addition to repression of the cell cycle machinery, another way that p53 may prevent cell cycle progression is through the inhibition of DNA replication. p53 interacts with the replication associated protein, RP-A, and stimulates *gadd45* transcription. GADD45 can bind and inhibit PCNA's (proliferating cell nuclear antigen) role in replication. The p53 transcriptional target gene $p21^{Waf1/Cip1}$, can also inhibit SV40 DNA replication potentially as a consequence of its ability to bind PCNA and block its role as a DNA processcivity factor in replication (Flores-Rozas *et al.*, 1994; Waga *et al.*, 1994). The importance of $p21^{Waf1/Cip1}$ in DNA damage-inducible G_1 arrest has been confirmed by the deficiency of $p21^{-/-}$ MEF's to undergo a G_1 arrest in response to DNA damage (Deng *et al.*, 1995). These observations clearly show that the molecular mechanism of p53 mediated cell cycle arrest may be either direct through the action of inhibitors such as $p21^{Waf1/Cip1}$ or indirect through proteins such as PCNA.

1-1.5. Induction of apoptosis by p53.

The introduction of p53 into cells can also induce a programmed cell death or apoptotic phenotype (Yonish-Rouach *et al.*, 1991). p53's role in apoptosis was confirmed by the finding that $p53^{-/-}$ mouse thymocytes and intestinal stem cells, unlike wild-type cells, are unable to undergo radiation induced apoptosis (Clarke *et al.*, 1993; Lowe *et al.*, 1993; Merritt *et al.*, 1994). p53's role as a cell death inducer is however stimuli specific as $p53^{-/-}$ thymocytes undergo a normal apoptotic response following their treatment with glucocorticiods or T cell receptor stimulants (Clarke *et al.*, 1993; Lowe *et al.*, 1993).

Consistent with the role of p53 in apoptosis, the introduction of p53 into quiescent cells produces an apoptotic response (Lowe and Ruley, 1993; Howes *et al.*, 1994). Adenoviral E1A and the Papilloma virus E7 proteins both induce p53-dependent apoptotic pathways (Vaux *et al.*, 1994). Both E1A and E7 stabilise p53 but conversely in the normal viral life cycle the anti-proliferative viral E1B and E6 proteins are expressed simultaneously with E1A and E7. The co-expression of E1B and E6 results in the prevention of apoptosis while simultaneously promoting proliferation (Reo *et al.*, 1992).

The cellular oncogenes E2F-1 and c-Myc are two other proteins that trigger p53-dependent apoptosis (Hermeking and Eick, 1994; Wagner *et al.*, 1994; Wu and Levine, 1994). The over-expression of c-Myc in $p53^{-/-}$ fibroblasts induces cell cycle progression while over-expression in a p53 positive background stimulates apoptosis (Hermeking and Eick, 1994). Similarly the over-expression of E2F-1 can induce a p53 apoptotic response (Qin *et al.*, 1994).

Given that p53-dependent apoptosis can occur in the presence of the RNA and protein synthesis inhibitors, actinomycin D and cycloheximide, it is possible that apoptosis is independent of p53 mediated transcription (Caelles *et al.*, 1994). In addition, p53 mutants, devoid of a transcriptional activation domain when introduced into cells, although more slowly than wild-type, still induce apoptosis (Haupt *et al.*, 1995).

Interestingly, pRb overcomes the anti-apoptotic function of MDM2 but does not prevent MDM2 from inhibiting p53-mediated transcription (Hsieh *et al.*, 1999).

Given that pRb forms a trimeric complex with p53 and MDM2 it is therefore plausible to assume that p53 transcriptional activation is dispensable for apoptosis (Hsieh *et al.*, 1999). The ability of p53 and pRb to cross-talk in the regulation of apoptosis points to a cellular mechanism by which apoptosis is tightly regulated by the two tumour suppressors, p53 and pRb.

However certain cell systems appear to require an intact p53 transcriptional activation capacity in order to induce apoptosis (Sabbatini *et al.*, 1995b). Consistent with the *trans*activation domain of p53 being necessary for the effective induction of apoptosis is the ability of p53 to transcriptionally activate *bax* expression (Miyashita and Reed, 1995). Bax protein expression accelerates apoptosis by overcoming the anti-apoptotic effects of Bcl-2. Interestingly and consistent with p53's role as a transcriptional repressor, p53 overexpressing cells display a lower level of the anti-apoptotic factor Bcl-2 (Miyashita *et al.*, 1994a; Miyashita *et al.*, 1994b).

Interestingly although *bax* is a p53 responsive gene that stimulates apoptosis a number of studies demonstrated that *bax* expression was dispensable for p53 mediated apoptosis (Knudson *et al.*, 1995). Another protein potentially involved in p53 mediated apoptosis is the tumour necrosis factor receptor superfamily member Fas/APO1. The binding of the Fas/APO1 receptor ligand FasL stimulates FADD binding (Fas-associated death domain) and the autocatalysis and activation of caspase 8, which results in apoptosis. Even through Fas/APO1 is transcriptionally and non-transcriptionally stimulated by p53 it is not essential for p53-dependent apoptosis (Fuchs *et al.*, 1997; Bennett *et al.*, 1998).

Consequently additional genes that participate in the DNA damage induced p53 apoptotic response have been identified. The gene encoding, the cathepsin-D aspartyl protease that contributes to cytokine mediated apoptosis, KILLER/DR5 a pro-apoptotic inducing member of the tumour necrosis related factor receptor family (TRAIL), PA26 a novel member of the GADD family, Wip1 a type 2C phosphatase, A28-RGS14 a GTPase activating protein, PAG608 a nuclear zinc finger protein and a human homologue of the *Drosophila sina* gene have all been implicated in the p53 apoptotic pathway (Nemani *et al.*, 1996; Buckbinder *et al.*, 1997; Fiscella *et al.*, 1997; Israeli *et al.*, 1997; Wu *et al.*, 1997; Wu *et al.*, 1998; Velasco-Miguel *et al.*, 1999).

The pro-apoptotic caspase cascade activating TRAIL receptors (TNF-related apoptosis inducing ligand) KILLER/DR4 and /DR5 function is counteracted by the anti-apoptotic or decoy receptors TRID (truncated intracellular domain) and TRUNDD (truncated death domain). Interestingly p53 also induces the expression of TRID and TRUND which provides a mechanism by which p53 modulates its own apoptotic response (Burns and El-Deiry, 1999).

Recently the serial analysis of gene expression identified a number of p53 induced genes (PIG's) involved in the oxidative stress response of p53 (Polyak *et al.*, 1996). The *PIG3* gene identified by Polyak *et al.* (1996) encodes an apoptosis inducing protein based on its homology with a plant apoptotic promoting protein NADPH quinone oxidoreductase.

The ability of p53 to induce apoptosis is clearly cell type specific and depending on the stimuli received can be transcriptionally dependent or independent. Furthermore it appears that p53 not only acts as an activator but also potentially as a repressor in the induction of apoptosis.

1-1.6. Differentiation and development.

The ability of p53 to induce cell cycle arrest and apoptosis is well documented but additionally p53 may also be involved in differentiation and development. A number of differentiation associated markers are expressed following a rise in the level of p53 in hematopoietic cells (Feinstein *et al.*, 1992; Aloni-Grinstein *et al.*, 1995). Furthermore the level of p53 mRNA is increased alongside the level of differentiation markers (Aloni-Grinstein *et al.*, 1993). p53 has been linked with the differentiation of a number of cellular tissue, namely hematopoitic cells, skeletal muscle cells, epithelial cells, central nervous system cells and thyroid neoplasms. Interestingly the level of p53 in different cell types seems to bestow p53's role in differentiation, with a decrease in p53 responsible for differentiation in some linagaes while an increase is responsible in others.

p53's role in development is further emphasised by the observation that a fraction of female $p53^{-/-}$ mice embryos, that predominantly develop normally, display neural tube closure defects (Donehower *et al.*, 1992; Sah *et al.*, 1995). Consistent with a role for p53 in neural development is the finding that the central nervous system regulatory gene, PAX5, is a regulator of p53 gene expression (Stuart *et al.*, 1995). In particular the regulation of p53 and its family members as a result of alternative

splicing especially in the C-terminal non-specific DNA binding domain has been associated with differentiation control.

1-1.7. Cell cycle arrest or apoptosis.

The regulatory event that governs the decision to undergo apoptosis or cell cycle arrest in response to p53 expression is unknown. However the apoptotic and cell cycle arrest functions of p53 appear to be mutually exclusive functions as p53 mutations that retain apoptotic function but have lost growth arrest capabilities are found in tumours. Several factors have been proposed to play a role in shifting the balance between growth arrest and apoptosis including; cell type, the presence of survival factors and the presence of oncogenes (Gottlieb and Oren, 1996; Burns and El-Deiry, 1999).

Following the induction of a p53 mediated G_1 arrest the cell is held in a position that allows time for potentially damaging genetic events to be repaired. If components in the mechanistic pathway of p53 mediated G_1 arrest are deregulated or abrogated the cell will then undergo apoptosis. Thus p53-dependent apoptosis may be the favoured outcome when G_1 arrest is not possible or can not be maintained long enough. Consistent with the loss of G_1 arrest being a controlling mechanism in p53 mediated apoptosis is the observation that DA-1 irradiated cells undergo replicative DNA synthesis and cell cycle re-entry prior to apoptosis (Gottlieb and Oren, 1996).

The extent and severity of the cellular shock appears to be the major controlling event in growth arrest versus apoptosis. If the cell can not repair its genetic lesions then apoptosis ensues to prevent tumourigenesis.

1-1.8. Modulation of p53 function.

The high-risk Papilloma virus E6 protein inhibits p53 sequence specific transcription by promoting p53 degradation through the ubiquitin proteasome pathway (Werness *et al.*, 1990; Lechner *et al.*, 1992; Gu *et al.*, 1994; Mansur *et al.*, 1995; Thomas *et al.*, 1995). The adenovirus E1B proteins, 55kDa and 19kDa, both effectively promote transformation in co-operation with E1A by inhibiting p53 mediated apoptosis (Rao *et al.*, 1992; Lowe and Ruley, 1993). The 55kDa E1B protein acts as a non-specific transcriptional repressor that binds to and prevents p53 transcription (Yew *et al.*, 1994). Adenoviral E1B 19kDa component is believed to prevent apoptosis by mimicking Bcl-2 function (Sabbatini *et al.*, 1995a).

p53 was first identified through its ability to bind the Simian virus 40 large T antigen (Lane and Crawford, 1979; Linzer and Levine, 1979). SV40 large T binds to the central DNA binding core domain of p53 to prevent DNA binding and transcriptional activation (Farmer *et al.*, 1992). As a result of SV40 binding to p53 the stability of p53 is increased which may contribute to the abrogation of p53's apoptotic function.

In addition to the viral proteins discussed above a number of other viral proteins interact with p53. The hepatitis B virus X protein, human cytomegla virus IE84 protein and the Epstein-Barr virus (EBV) protein BZLF1 all bind p53 and inhibit its *trans*activation capacity (Speir *et al.*, 1994; Wang *et al.*, 1994b; Zhang *et al.*, 1994). Clearly the interaction and abrogation of p53 function imposed by viral proteins is an important event in the viral life cycle and the maintenance of viral infection.

The MDM2 protein that was originally identified by virtue of its amplification in a transformed mouse cell line also interacts with p53. (Fakharzadeh *et al.*, 1991; Momand *et al.*, 1992). MDM2 inhibits p53-mediated transcription by binding to the *trans*activation domain of p53 and promoting its ubiquitin dependent degradation (Oliner *et al.*, 1993; Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). Ubiquitin is a 76 amino acid protein that when attached to substrate proteins at free lysine residues marks them for 26S proteasome degradation (Momand *et al.*, 2000). p53 proteolysis mediated by MDM2 corresponds to a transfer of a ubiquitin molecule from the C-terminus of MDM2 to p53. Consequently the C-terminus of MDM2 is essential in the mediation of p53 degradation and mutants of p53 that are unable to bind MDM2 are constitutively more stable (Haupt *et al.*, 1997; Honda *et al.*, 1997; Kubbutat *et al.*, 1997).

MDM2's ability to stimulate p53 degradation stems not only from its inherent E3 ubiquitin ligase activity but also from its ability to shuttle p53 to the cytoplasm (Roth *et al.*, 1998; Tao and Levine, 1999a). MDM2 contains both a nuclear import (NLS) and export signal (NES) and so is constantly shuttled between the nucleus and cytoplasm (Roth *et al.*, 1998). It is this shuttling capability of MDM2 that allows the effective transport of p53 to the cytoplasm and its subsequent degradation. Interestingly p53 itself contains a nuclear export signal that is masked after tetramization and activation (Stommel *et al.*, 1999). In addition MDM2 interacts with p300 and p53 simultaneously to mediate p53 degradation (Grossman *et al.*, 1998). Presumably MDM2 utilises all its inherent activities in the regulation of p53.

Interestingly MDM2 and p53 function in an auto-regulatory feedback loop. p53 stimulates MDM2 expression which in turn degrades and down-regulates p53, which then subsequently leads to a reduction in the level of MDM2. The *in vivo* importance of the MDM2/p53 regulatory loop is emphasised by the rescue of the mice embryonic lethality phenotype of $mdm2^{-/-}$ when crossed with $p53^{-/-}$ mice (Jones *et al.*, 1995; Montes de Oca Luna *et al.*, 1995).

MDM2's ability to interact with p53 is undoubtedly an important regulatory step in p53 control. The p53/MDM2 interaction is directly regulated by the covalent phosphorylation and acetylation of p53 and indirectly through the actions of proteins such as $p14/19^{ARF}$ (Chin *et al.*, 1998; Prives, 1998). Interestingly, MDM2, in response to ionising radiation, is phosphorylated in an ATM dependent manner by DNA-PK (Momand *et al.*, 2000). This implies that ionising radiation which triggers an ATM/DNA-PK phosphorylation cascade results in the modification of both MDM2 and p53, that in turn regulates MDM2's association with p53 (Momand *et al.*, 2000).

The oncogenic properties of MDM2 stem not only from its ability to prevent a p53 mediated response but also from its ability to activate the E2F directed transcription of S-phase promoting genes (Martin *et al.*, 1995; Xiao *et al.*, 1995). Given that many tumours with MDM2 mutations retain wild-type p53 it is feasible to assume that inactivation of p53 or MDM2 is a mutually exclusive event in tumour formation.

The MDM2 family member, MDMX, although structurally almost identical to MDM2 can not substitute for MDM2 in early embryonic development (Shvarts *et al.*,

1996). MDMX can from stable heterdimers with MDM2 through its ring finger domain and is also able to associate with p53 (Tanimura *et al.*, 1999). The functional significance of MDMX function on p53 action are two fold, firstly p53 can be nuclear retained but transcriptionally inactivated when complexed with MDMX and secondly p53 can be stabilised and transcriptionally activated as a result of MDMX dimerisation with MDM2 (Jackson and Berberich, 2000). The regulation of p53's level and localisation by MDMX and MDM2 indicates a mechanism for the regulation of p53 function in cells.

1-1.9. Modification of p53.

Two distinct serine/threonine rich domains in the N- and C-termini of p53 are extensively post-translationally modified by phophorylation (Figure 1.1b). The kinases responsible for p53 phosphorylation are the DNA dependent protein kinase A (DNA-PK), ATM/ATR, casein kinase I and II (CKI, CKII), protein kinase C (PKC), mitogen activated protein (MAP), Chk1/Cds1 and the UV induced kinases JNK1 and raf-1. (Milne *et al.*, 1992; Milne *et al.*, 1994; Takenaka *et al.*, 1995; Woo *et al.*, 1998; Chebab *et al.*, 2000; Shiel *et al.*, 2000). Interestingly p53 is also a substrate for the cyclin dependent kinases, cyclin B/cdc2, and cyclin A/cdk2, both of which stimulate the sequence specific binding property of p53 (Ko and Prives, 1996). Casin kinase II phosphorylation of p53 also results in an increase in sequence specific binding of p53 to DNA (Hupp and Lane, 1994).

Intriguingly, ATM kinase is induced following DNA damage and this induction runs alongside p53 activation and N-terminal phosphorylation (Banin *et al.*, 1998; Canman *et al.*, 1998). Additionally, *atm*$^{-/-}$ cells show delayed phosphorylation and activation

of p53 following γ-irradiation but not UV irradiation suggesting that ATM is important for signalling to p53 in a DNA damage specific manner (Siliciano *et al.*, 1997). However, the prolonged exposure of *atm*$^{-/-}$ cells to γ-irradiation does eventually lead to the N-terminal phosphorylation of p53 suggesting that other stress activated kinases can substitute for ATM.

Potentially, a mechanistic function of p53's N-terminal phosphorylation is the loss of its negative regulation by MDM2. Shieh *et al.* (1997) demonstrated that DNA damage-induced N-terminal phosphorylation weakens the association of MDM2 with p53 and consequently stimulates transcriptional activation. Additionally DNA damage and stalled replication activates the Chk1 and Cds1 kinases which functionally phosphorylate the N-terminus of p53 to dissociate MDM2 (Chehab *et al.*, 2000; Shiel *et al.*, 2000). The actions of Chk1 and Cds1 on p53 result in p53 stabilisation and G_1arrest.

C-terminal phosphorylation of p53 actives the DNA binding capacity of p53 and is associated with a loss in C-terminal auto-repression. The C-terminus of p53 negatively regulates the sequence specific binding activity of p53 by interacting with the central core domain (Hupp *et al.*, 1995). In addition the C-terminus has a non-specific damaged induced DNA binding capacity that can allosterically stimulate the sequence specific binding of p53 to DNA (Bayle *et al.*, 1995; Jayaraman and Prives, 1995). The C-terminus of p53 is believed to maintain p53 in a latent form but upon either single stranded DNA binding or phosphorylation its ability to inhibit DNA binding is lost, which results in sequence specific p53 transcription. Interestingly the

C-terminus of p53 is phosphorylated in response to UV but not ionising irradiation (Kapoor and Lozano, 1998; Lu *et al.*, 1998).

Conversely, phosphatases that act on p53 have been proposed as regulators of its function. The C-terminal protein kinases C phosphorylation site on p53 is actually dephosphorylated following the treatment of cells with ionising irradiation (Waterman *et al.*, 1998). Dephosphorylation of this region induces a conformational change that correlates with an increase in the sequence specific binding of p53 (Prives, 1998). Indeed, the dephosphorylation of the C-terminus of p53, following an ATM response to ionising radiation, creates a consensus site for 14-3-3 proteins (Waterman *et al.*, 1998).

A second potent mechanism of p53 modification is acetylation. C-terminal acetylation of p53, by p300, induces a transcriptionally active DNA bound form of p53 (Gu and Roeder, 1997; Sakaguchi *et al.*, 1998). Presumably the post-translational acetylation of lysine residues in the C-terminus of p53 counteracts there highly positively charge and removes their negative affect on p53's sequence specific DNA binding activity.

The ability of p53 to function in the DNA repair mechanism of cells is consistent with the nuclear localisation of p53. It is conceivable that latent cytoplasmic p53 is activated and transported or translocated to the nucleus where it then regulates the cell cycle. The ability of viral and cellular oncogenes to influence p53 localisation and function also occurs through the regulation of $p14/19^{ARF}$ (deStanchina *et al.*, 1998; Palmero *et al.*, 1998). $p14/19^{ARF}$ is induced by viral oncoprotein and cellular

oncogenes, that deregulate the cell cycle, and functions to induce p53 by interfering with the MDM2/p53 pathway (Chin *et al.*, 1998; Sharpless and DePinho, 1998). p53 regulation by the $p14/19^{ARF}$ is a highly co-ordinated process that goes even further to underlie the cellular importance of p53.

1-1.10. Conclusions.

The role of p53 as the "guardian of the genome" is associated with its ability to enforce either cell cycle checkpoint arrest or in cases where the cell is unable to recover apoptosis (Levine, 1997) (Figure 1.3). Given the high level of germline mutations in cancer cells that either directly or indirectly target p53's role its importance in the maintenance and development of the healthy cell is seemingly unquestionable.

Even through p53 has been widely studied many of the environmental and cellular mechanisms that activate it remain unknown. Furthermore, the roles of phosphorylation, and acetylation are only now becoming understood. Recently, proteins which help mediate p53's function are becoming known and it is with the understanding of these proteins that p53's cellular activity will be unravelled.

Clearly the ability to fully understand p53's activation pathway will prove of insurmountable benefit in the design of drugs for the treatment of cancer.

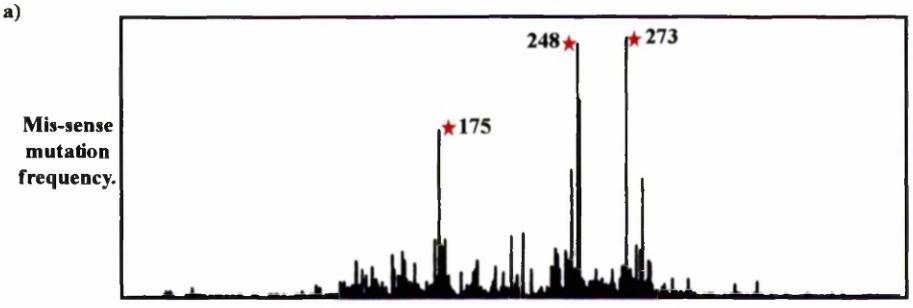
Figure 1.1. Structural and functional interactions of p53.

a). Diagram representing the amino acid mis-sense mutational frequency of human p53. Indicated are the core domain hot spot mutations Arg175, Arg248 and Arg273.

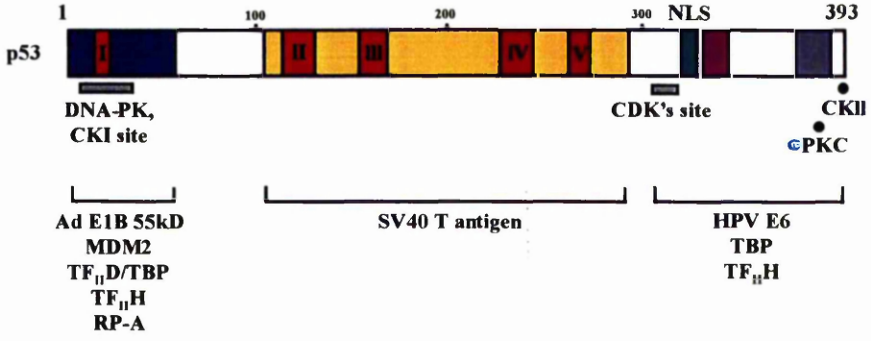
b). A diagrammatic representation of human p53. Indicated are the N-terminal *trans*activation domain (Blue), the central DNA binding core domain (yellow), the C-terminal non-specific DNA/RNA binding domain (grey), the nuclear localisation signal (Green) and the oligomerisation domain (Purple). The red boxes I, II, III, IV and V represent the evolutionarily conserved regions. The binding regions for the indicated cellular and viral proteins are shown together with the phosphorylation sites for DNA-PK, CKI, CKII, CDK's and PKC (Gottlieb and Oren, 1996; Levine, 1997; Prives *et al.*, 1998).

c). Proteins that have a functional relationship with p53. Some of the viral and cellular proteins that interact with p53 and their functional outcomes are listed.

a)



b)



c) **Functional Interactions of p53.**

Viral proteins;

SV40 large T antigen.	: Prevents p53 DNA binding.
Human papilloma virus (HPV) E6	: Blocks p53 transcriptional activation activity.
Adenovirus E1B 55kDa	: Promotes p53 degradation.

Cellular proteins;

c-Abl	: Induces p53 mediated cell cycle arrest.
$TF_{II}D$/TBP	: Activity modulated by p53
WT1	: Stabilises p53 but inhibits apoptosis.
$TF_{II}H$	: Helicase activity modulated by p53
MDM2	: Promotes p53 degradation and blocks *trans*activation.
RP-A	: Helicase activity modulated by p53

Figure 1.2. Model of p53 transcription activation.

The activation of p53 by cellular stimuli or external DNA damaging events promotes the activation and tetramerisation of p53. The active p53 tetramer can either activate the expression of apoptotic promoting proteins such as Bax (a), stimulate the expression of $p21^{Waf1/Cip1}$ that promotes cell cycle G_1 arrest (c), or self regulate its own level through the expression of MDM2 (b). The exact mechanism that controls the outcome of p53's activation whether it is apoptosis or cell cycle arrest is unknown.

The over-expression of Bax changes the composition of the Bcl-2 family heterodimers that in turn alters the cells pro- to anti-apoptotic signal ratio. The high level of Bax stimulates apoptosis (Burns and El-Deiry, 1999; Miyashita *et al.*, 1994a; Miyashita and Reed, 1995).

$p21^{Waf1/Cip1}$ binds and inhibits the activity of the cyclin/cdk enzyme complexes. The inactive cyclin/cdk complexes no longer promote the phosphorylation and release of pRb's repression of E2F. Consequently the expression directed through E2F, of the S-phase promoting genes is suppressed and cell cycle arrest occurs at the G_1 phase (El-Deiry *et al.*, 1993; Gu *et al.*, 1993).

The up regulation of MDM2, directed by p53 acting on the P2 responsive promoter, leads to a down regulation in the level of p53 which in turn leads to a reduction in the level of MDM2. MDM2 and p53 levels are therefore auto-regulatory (Barak and Oren, 1992; Barak *et al.*, 1994).

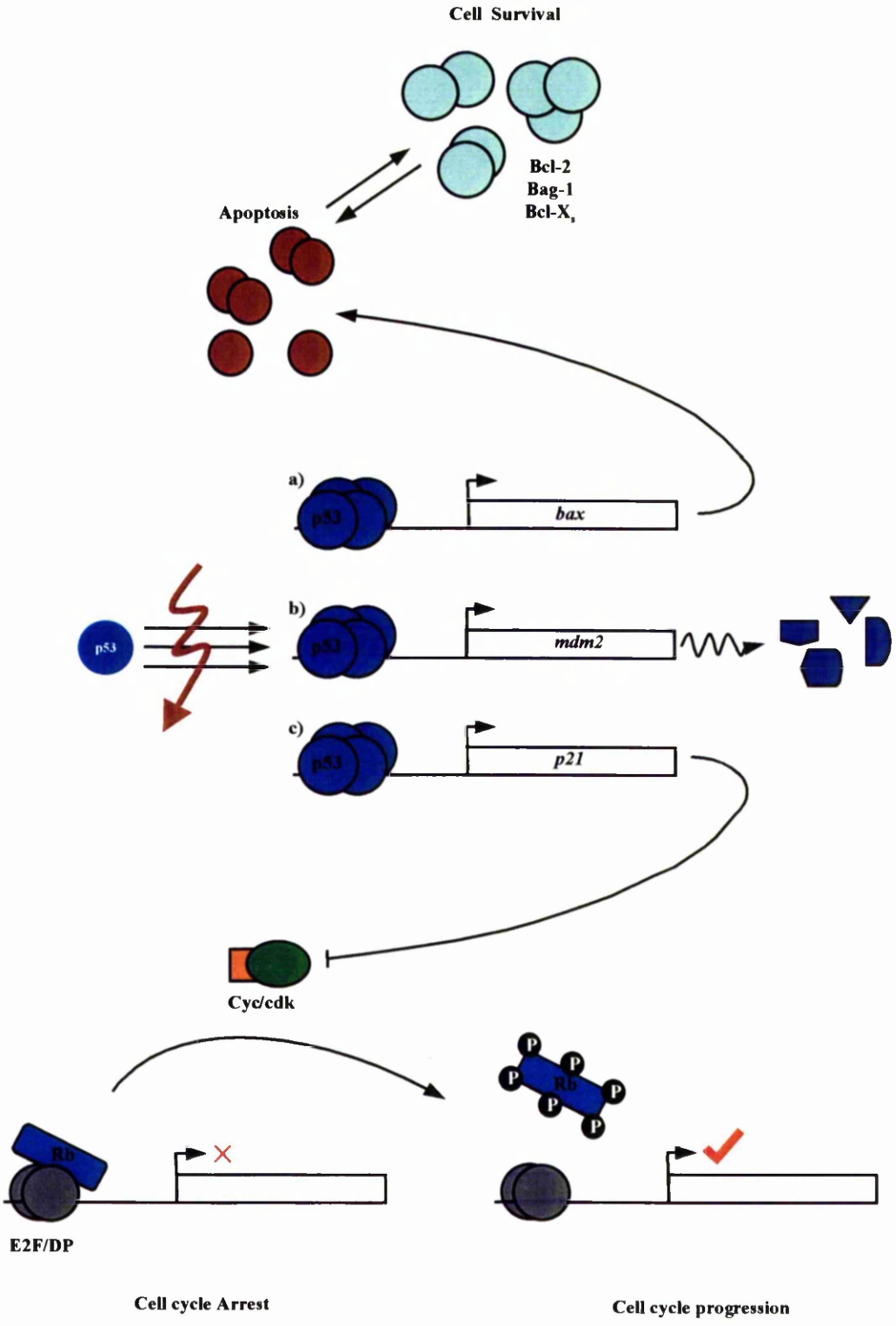
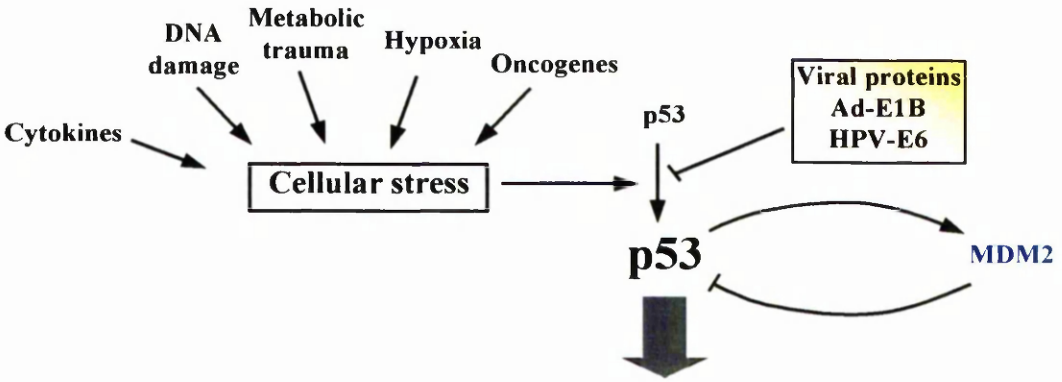

Cell Survival
Bcl-2
Bag-1
Bcl-X
Apoptosis
a)
p53
bax
b)
p53
mdm2
p53
c)
p53
p21
Cyc/cdk
P
P
P
Rb
P
P
Rb
E2F/DP
Cell cycle Arrest
Cell cycle progression

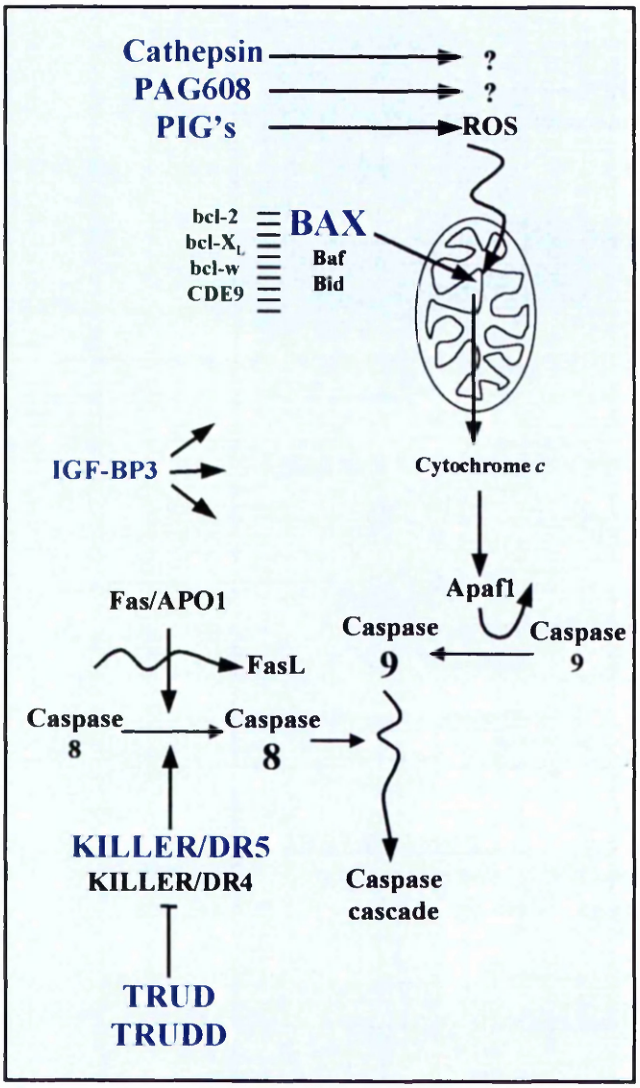
Figure 1.3. p53-dependent pathways of apoptosis and cell cycle arrest.

Summary of the molecular mechanisms that p53 uses to induce either apoptosis or cell cycle arrest. p53 is activated following the cellular response to a variety of stresses and it is this activation of p53 that viral proteins such as E1A target and prevent. Many of the cellular events and proteins that p53 influences are indicated (blue) and their downstream mechanisms of action outlined.

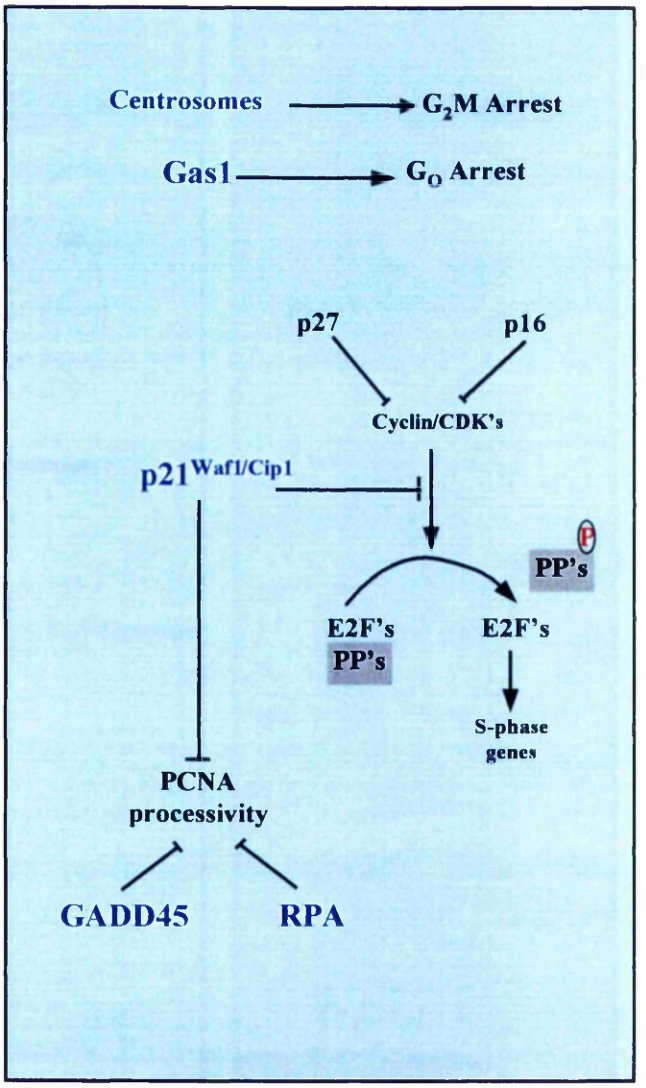
p53's induction of G_1 arrest is primarily associated with induction of $p21^{Waf1/Cip1}$ expression that in turn impacts on the pocket protein regulatory pathway as indicated. In addition p53 can induce cell cycle arrest in both G_2/M and G_o via its as yet poorly understood roles in centrosome formation and Gas1 expression respectively

The induction of apoptosis following the stimulation of p53 occurs in response to a variety of genetic stresses and involves the actions of a number of p53 responsive genes. The level and extent of gene activation required for p53 mediated induction of apoptosis is poorly understood, however it is clear that p53 induced apoptosis takes place through the sequential and cumulative effects of the genes which it activates.





APOPTOSIS



CELL CYCLE ARREST

1-2. The p300/CBP family of transcriptional co-activators.

1-2.1. Introduction.

p300 and CBP belong to a family of versatile transcriptional co-activators that function as regulators of a number of cellular processes; including proliferation, differentiation and apoptosis (Giordano and Avantaggiati, 1999). p300 and CBP were originally identified by their ability to interact with the adenovirus E1A and the transcription factor CREB proteins respectively (Whyte *et al.*, 1989; Chrivia *et al.*, 1993; Eckner *et al.*, 1994).

It is believed that co-activators, such as p300 and CBP, facilitate transcription by promoting the interactions between sequence specific activators and the RNA polymerase II transcription machinery (Roeder, 1996). The ability of p300/CBP to physically interact with an assorted number of activators and the basal transcription machinery components, together with topological factors such as RNA helicase A clearly supports its role as a transcriptional co-activator (Imhof *et al.*, 1997; Nakajima *et al.*, 1997; Sang *et al.*, 1997; Kim *et al.*, 1998; Felzien *et al.*, 1999).

The over-expression of p300 or CBP in cellular systems results in the transcriptional activation of both viral and cellular enhances and promoters which utilise a wide body of transcription factors (Lundbiad *et al.*, 1995; Janknecht and Hunter, 1996; Shikama *et al.*, 1997; Giordano and Avantaggiati, 1999). CBP, for instance, acts as a transcriptional co-activator of cAMP responsive elements as a consequence of its

ability to directly bind the transcription factor CREB. CREB is induced, by protein kinase A stimulated KID domain phosphorylation, to bind the cAMP response element (CRE) from where it recruits CBP to activate the transcription of target genes (Chrivia *et al.*, 1993).

In addition to the multi-faceted interaction capacity of p300/CBP the protein also possesses an intrinsic enzymatic histone acetyltransferase activity (HAT). The HAT activity of p300/CBP equally contributes to its ability to act as a transcriptional co-activator (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996a).

The cellular significance of p300/CBP is highlighted by the observation that p300/CBP may function as a tumour suppressor. Mutations in p300/CBP have been detected in a range of tumour types and genetic disorders, such as epithelial malignancies and Rubinstein-Taybi syndrome (Giles *et al.*, 1998; Gayther *et al.*, 2000).

1-2.2. p300/CBP.

The transcriptional co-activators p300 and CBP share a high degree of homology and genetic evidence suggests that the two proteins perform both overlapping and unique functions (Arany *et al.*, 1995) (Figure 1.4a). *p300* and *cbp* are both highly conserved genes in multi-cellular organisms with orthologs in organisms as diverse as human, *Drosophila* and *Caenorhabditis elegans* (Akimaru *et al.*, 1997). In particular the functional domains in p300 and CBP show a very high degree of sequence homology with each other (Figure 1.4a). The Bromodomain, the cysteine/histidine rich domains (CH1, CH2 and CH3) and the KIX domain are all regions of high homology.

Interestingly the p300/CBP family of co-activators is known to contain at least two other members, namely p270 and p400. The functions of p270 and p400 have not yet been reported but p270 is a known component of the mammalian Swi/Snf complex (Dallas *et al.*, 1998; Giles *et al.*, 1998).

The functional domains in p300/CBP thus far identified have a number of interesting features. The central Bromodomain, which is highly conserved in all known mammalian HAT proteins, in alliance with the CH2 and CH3 domains encompasses the acetyltransferase activity of p300/CBP (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996a) (Figure 1.4a). The KIX, CH1 and CH3 domains in p300/CBP mediate its protein interactions and are the regions targeted by viral proteins (Shikama *et al.*, 1997). It is the modular organization of p300/CBP that imparts its ability to act as a transcriptional co-activator as it allows the formation of muti-meric transcription mediating complexes. To this end both the N- and C-termini of p300/CBP are known to possess *trans*activation properties (Figure 1.4a).

The overlap in function seen between p300 and CBP is highlighted by the embryonic phenotype of $p300^{-/-}$, $cbp^{-/-}$ and $p300^{+/-}$;$cbp^{+/-}$ knock out mice. They all show similar neural tube closure, growth, and embryonic lethality defects (Yao *et al.*, 1998). The close overlap seen between the phenotypes of $cbp^{+/-}$ mice and RTS patients whom lack one *cbp* allele also suggests that p300 and CBP carry out dose-dependent functions. Consistent with these observations is the fact that a population of $p300^{+/-}$;$cbp^{+/-}$ mice suffer embryonic lethality as a consequence of the reduced level of p300/CBP (Tanaka *et al.*, 1997). Given that the level of p300/CBP in cells is limited

and that its absence is an inducer of lethality in embryonic mice, it is believed that the physical distribution and redistribution of p300/CBP by controlling signals is what permits its varied and wide ranging cellular influences (Torchia *et al.*, 1998; Yao *et al.*, 1998).

The ability of p300 and CBP to perform unique non-overlapping cellular functions has been demonstrated using ribozyme technology that specifically inactivates one member of the family. The ribozyme study demonstrated that p300 but not CBP was responsible for retinoic acid induced F9 embryonic carcinoma cell differentiation (Kawasaki *et al.*, 1998). In addition the ability of ionising radiation to induce apoptosis is impaired in $p300^{+/-}$ cells but remains unaffected in CBP deficient cells (Yuan *et al.*, 1999). Consistent with these observations is the ability of $p300^{+/-}$ mice but not $cbp^{+/-}$ mice to retain a normal hematopoeitic differentiation phenotype and the fact that CREB function is unaffected in $p300^{-/-}$ mice that show impaired retinoic acid induced transcription (Kung *et al.*, 1999).

Together these observations suggest that transcriptional co-activators, such as p300 and CBP, perform synergistic functions in the regulation of cellular gene transcription. Interestingly and consistent with their family status it is also recognized that both p300 and CBP do however perform unique tasks that can not be compensated by the other family members.

1-2.3. Transcription and p300/CBP.

The formation of a molecular bridge by p300/CBP is highlighted by its ability to interact with a wide variety of transcription factors and basal transcription machinery

components, namely TBP, $TF_{II}B$, $TF_{II}E$, and $TF_{II}F$ (Imhof *et al.*, 1997; Sang *et al.*, 1997; Felzien *et al.*, 1999) (Figure 1.4b). p300/CBP's ability to allow the cross-talk of transcription factors with the RNA polymerase II holoenzyme is undoubtedly a cellular mechanism by which it regulates transcription (Roeder, 1996).

p300/CBP's role in transcription is in part connected with its ability to act as a rate-limiting factor. The rate-limiting level of p300/CBP is highlighted by hormone dependent transcriptional activation that utilises p300/CBP to indirectly inhibit mitogen activated transcription factors such as AP-1. p300/CBP is also utilised by p53 in the repression of TRE regulated promoters (Kamei *et al.*, 1996; Avantaggiati *et al.*, 1997; Shikama *et al.*, 1997). Interestingly p300/CBP association with the mitogen regulated S6 kinase $pp90^{Rsk}$ has been linked with the repression of CREB dependent transcription during Ras signaling (Nakajima *et al.*, 1996). The physiological induction of differentiation and block of proliferation by hormone treatment intriguingly points to competition for p300/CBP as the determining factor in differentiation versus proliferation. Furthermore the transcription factor E2F-1 blocks p53 *trans*activation in a p300/CBP dependent manner (Lee *et al.*, 1998).

p300/CBP's role as co-activator that responds to a wide range of signal transduction pathways to specifically promote a cellular outcome is well documented. It would appear that a major regulatory event involved in p300/CBP mode of action is promoter specific targeting and it is this promoter specific targeting that viral oncoproteins, such as E1A and SV40 large T, employ in order to induce proliferation and not differentiation.

The ability of p300/CBP to form a large nuclear co-activator protein complex is also associated with its ability to act as a transcriptional co-activator (Korzus *et al.*, 1998; Westin *et al.*, 1998; Xu *et al.*, 1999). Signal transduction pathways that induce a number of different cellular outcomes potentially regulate p300/CBP's ability to recruit and be recruited to specific promoters (Carey 1998; Kim *et al.*, 1998; Korzus *et al.*, 1998; Xu *et al.*, 1999). The ability of p300/CBP to recruit factors such as p50/p65, NFκB, P/CAF and HMGI (high mobility group proteins) to the human interferon enhancer (INFβ) provides credence to the role of p300/CBP as a complex protein recruiter (Kim *et al.*, 1998; Munshi *et al.*, 1998).

The recruitment of p300/CBP into a transcriptional enhancer element complex is further complicated by its intrinsic HAT activity. p300/CBP is also found complexed with the cellular HAT's, P/CAF (p300/CBP associated factor), SRC-1 (steroid receptor co-activator 1) and PCIP (p300 cellular interacting protein) (Yang *et al.*, 1996; Chen *et al.*, 1997; Spencer *et al.*, 1997). It is attractive to speculate that the different HAT's present in the DNA bound co-activator complex confer target specificity and mediate the cellular outcome. In support of co-activator HAT specific roles the INFβ enhancer is known to require p300/CBP mediated acetylation of HMG-1 for transcriptional termination (Munshi *et al.*, 1998). It is therefore plausible to associate HAT activities with functional specificity and to assume that co-ordination of the antagonistic or synergistic effects of acetylation are a mechanism by which transcription is regulated.

DNA is highly wound and compressed into nucleosomes that consist of octomers of histone H2A, H2B, H3 and H4. The acetylation of histones, on lysine tails,

neutralizes the attractive charges in the nucleosome and is associated with a hyper-relaxed chromosome structure and active transcription (Brownell and Allis, 1996; Grunstein, 1997; Hassig and Schrieber, 1997; Wade *et al.*, 1997) (Figure 1.5). The co-activation of transcription has been linked to p300/CBP's HAT activity, that preferentially acetylates histone H3 and H4, although the acetylation of H2A and H2B still occurs *in vitro* (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996a). Interestingly the HAT activity of p300/CBP shows promoter specificity, with the adenovirus major late (AdML) and E4; but not E1B or SV40 promoters, requiring the HAT activity of p300/CBP for co-activation (Martinez-Balbas *et al.*, 1998). The ability of p300/CBP to acetylate nuclosomes and histones *in vivo* still remain unclear.

Both p300/CBP and its associated factor P/CAF utilise their HAT activity in the acetylation of non-histone targets such as p53, E2F, c-Myb, MyoD, GATA-1, EKLF and HNF-4 (Gu and Roeder, 1997; Boyes *et al.*, 1998; Sakaguchi *et al.*, 1998; Zhang and Bieker, 1998; Liu *et al.*, 1999; Sartorelli *et al.*, 1999; Martinez-Balbas *et al.*, 2000; Marzio *et al.*, 2000; Soutogloou *et al.*, 2000; Tomita *et al.*, 2000). The acetylation of p53 and E2F-1 neutralizes positive charges on the ε-amino group of lysine residues. This promotes a charge induced conformational change that leads to an increase in sequence specific DNA binding and potentially transcriptional activation (Gu and Roeder, 1997; Gu *et al.*, 1997; Liu *et al.*, 1999; Martinez-Balbas *et al.*, 2000). Further to these observations, Lambert *et al.* (1998) demonstrated that ionising radiation induces the phosphorylation of p53, that in turn increases its affinity and consequently acetylation by p300/CBP.

Another functional significance of p300/CBP HAT activity is in the acetylation of the HIV-Tat protein. HIV-Tat protein acetylation induces transcription of the HIV-1 LTR by enhancing its binding to the Tat associated kinase CDK9/P-TEFb (Keirnan *et al.*, 1999). Interestingly one report of p300/CBP protein acetylation suggests a role for HAT activity in transcriptional down-regulation. The acetylation of the dTCF (T-cell factor) transcription factor by p300/CBP inhibits its association with the beta catenin/Armadillo co-activator and therefore transcription activation of the Wnt/Wingless genes (Waltzer and Bienz, 1998).

Interestingly p300/CBP undergoes auto-acetylation and is able to acetylate the basal transcription machinery components $TF_{II}E\beta$ and $TF_{II}F$ (Imhof *et al.*, 1997). The functional significance of p300/CBP HAT activity in these situations however is unclear.

Clearly the ability of p300/CBP to acetylate core histones and members of the transcriptional apparatus, as well as transcription factors is closely associated with its role as a transcriptional co-activator. Further studies that elucidate the pattern of protein acetylation in gene regulation will aid in the true identification of the importance of post-translation acetylation in gene transcription.

1-2.4. Differentiation, proliferation and apoptosis by p300/CBP.

The adenovirus E1A protein is a multi-functional pleiotropic protein that mediates its biological effects through its ability to modulate the activity, either directly or indirectly, of target genes. The ability of E1A to block differentiation, in a wide variety of lineage's such as myogenesis, neurogenesis and karatinocyte differentiation

correlates with its ability to inactivate enhancer activity in a p300/CBP dependent manner (Stein *et al.*, 1990; Puri *et al.*, 1997a; Puri *et al.*, 1997b). Consistently, E1A's increase of YY1 transcriptional activity involves the loss of p300/CBP mediated transcriptional repression (Lee *et al.*, 1995). E1A's ability, through its N-terminus and CH1 region, to bind p300/CBP and promote S-phase entry and cell cycle progression clearly supports p300/CBP's role as a mediator of the cell cycle (Whyte *et al.*, 1989; Stein *et al.*, 1990; Yaciuk and Moran, 1991; Arany *et al.*, 1995).

The requirement of p300 and CBP for both cellular proliferation and differentiation is highlighted by the inability of mutant E1A, that is unable to bind p300, to induce transformation and the embryonic lethality of $p300^{-/-}$ knock out mice due to defective cardiac myocyte differentiation (Wang *et al.*, 1995a; Yao *et al.*, 1998). In addition knock out mice studies have also defined a role for p300/CBP in the differentiation of haematopoietic tissues (Eckner *et al.*, 1996b).

Both p300/CBP and the associated protein, P/CAF, were shown by their ability to induce MyoD dependent transcription, to promote cell cycle withdrawal in muscle and B cells. The regulation of the myogenic factors such as myogenin and MEF2 (Mycoyte enhancer factor 2) in differentiation is also controlled by p300/CBP (Yuan *et al.*, 1996; Puri *et al.*, 1997a; Puri *et al.*, 1997b; Sartorelli *et al.*, 1999). Interestingly the HAT domain of p300 is not essential for MyoD dependent transcription although P/CAF's HAT activity is (Sartorelli *et al.*, 1999).

Consistent with the role of p300/CBP in differentiation is the observation that inactivation of *Caenorhabditis elegans cbp-1* gene, that is homologous to p300/CBP,

leads to severe neuronal cell differentiation defects (Shi and Mello, 1998). The differentiation defects seen in *cbp-1* knockouts can be overcome by the use of deacetylase inhibitors, which directly links HAT activity and suppression of deacetylases with p300/CBP differentiation regulation (Shi and Mello, 1998). The ability of p300/CBP to participate in cellular differentiation strongly supports its ability to act as a tumour suppressor.

A role for p300/CBP in cellular proliferation was initially suspected given that $p300^{-/-}$ embryos and $p300^{-/-}$ MEF's proliferate slower than wild-type and are significantly smaller with a phenotype reminiscent of senescent cells (Yao *et al.*, 1998). More detailed studies demonstrated that p300/CBP directly mediates E2F-1 transcriptional activation (Trouche *et al.*, 1996). In addition the ability of E1A to bind p300 under conditions that promote DNA synthesis illustrates an interesting model whereby E1A prevents p300 induced cellular differentiation while simultaneously using p300 to stimulate cellular proliferation (Stein *et al.*, 1990). Given that p300/CBP also associates with a number of cellular HAT proteins, namely P/CAF, SRC-1 and PCIP it is plausible to assume that transcription factors and viral oncoproteins utilise a multiple HAT containing complex in order to stimulate transcription (Yang *et al.*, 1996; Chen *et al.*, 1997; Brown *et al.*, 2000).

The analysis of p300 and CBP deficient cells indicated that the ionising radiation induced cellular shock response is impaired in p300 knock out cells but not CBP deficient cells (Yuan *et al.*, 1999). The inability of retinoic acid induced apoptosis to occur in p300/CBP deficient cells together with the ability of E1A to sequester p300/CBP and block p53-dependent apoptosis provides an interesting link of

p300/CBP with apoptosis (Kawasaki *et al.*, 1998). T-cell antigen receptor (TCR) induced thymocyte apoptosis triggered by calcium dependent signaling and MEF2/NFAT (nuclear factor of activated T cells) mediated transcription also utilises p300 (Youn *et al.*, 2000). Interestingly studies on the cell cycle effects of p300 and p53 showed that p300/CBP induced a p53 mediated G_1 arrest together with E2F-1 dependent apoptosis (Lee *et al.*, 1998). Together these results suggest a role for p300 in p53 and E2F-1 dependent mechanisms of cell cycle arrest and apoptosis, possibly as a cellular shock responder.

p300/CBP is also involved in the cellular mechanisms that control DNA damage repair as the expression of the human proliferating cell nuclear antigen (PCNA) requires p300/CBP. PCNA in response to serum and mitogenic growth factors is required for δ polymerase activity and DNA replication and repair (Lee and Mathews, 1997). Furthermore p300/CBP as a consequence of its interaction with p53 can induces the expression of the p53 responsive genes, *mdm2*, *Waf1/Cip1* and *bax* (Avantaggiati *et al.*, 1997; Gu *et al.*, 1997; Lill *et al.*, 1997a). Thomas and White (1998) demonstrated that p53 mediated transcription of *mdm2* is dependent on p300/CBP and proposed a model in which p300 regulation of MDM2 levels, through p53, determines whether the physiological response of p53 is growth arrest or apoptosis.

As MDM2 is known to regulate p53's stability it was interesting to recognize that p300 can mediate the formation of a p53/MDM2/p300 ternary complex in which p53 is targeted for degradation (Grossman *et al.*, 1998). The inability of MDM2 mutants, that retain p53 binding but have lost p300 binding capabilities, to degrade p53 clearly

points to p300 as a mediator of p53/MDM2 dependent degradation (Grossman *et al.*, 1998). Taken together it appears that p300/CBP plays a dual role, in one hand as a p53 transcriptional co-activator and in the other as a regulator of p53 stability.

Clearly p300/CBP and their family members appear to be key regulators of cell development, differentiation and proliferation. It would seem plausible to assume the exact cellular consequence of a p300/CBP response, whether it is proliferation, apoptosis or differentiation, will be a tightly controlled process depending on both the level of p300/CBP and also the stimuli that p300/CBP is exposed to.

1-2.5. Functional regulation of p300/CBP.

p300/CBP are phosphorylated in a cell cycle regulated fashion, with hyperphosphoryled forms being observed during mitosis (Yaciuk and Moran, 1991). The treatment with retinoic acid or the introduction of E1A into F9 cells, both of which induce differentiation, also induces p300 phosphorylation (Kitabayashi *et al.*, 1995). Interestingly although E1A stimulates p300 hyperphosphorylation, probably through cyclin/CDK recruitment, the SV40 large T antigen that binds to the same region of p300 as E1A is associated with and induces p300 hypophosphorylation (Banerjee *et al.*, 1994; Eckner *et al.*, 1996a). The cyclin dependent kinases cdk2 and cdc2 are known to phosphorylate p300 *in vivo* (Banerjee *et al.*, 1994). The bromodomain and associated HAT activity of p300/CBP is potentially a target of phosphorylation, as the similar bromodomain in GCN5 is known to undergo repression as a result of DNA-PK phosphorylation (Barlev *et al.*, 1998).

Perkins *et al.* (1997) determined that p300 was negatively regulated by cyclin E/cdk2 and that $p21^{Waf1/Cip1}$ expression, induced by p300, inhibited this regulation pathway. The inhibition of cyclinE/cdk2 by $p21^{Waf1/Cip1}$ results in p300 increasing NFκB mediated transcriptional activation. The ability of $p21^{Waf1/Cip1}$ to act in a positive feedback loop on p300/CBP cyclin E/cdk2 action proposes the existence of a potential G_1/S phase checkpoint involving p300/CBP (Missero *et al.*, 1995; Perkins *et al.*, 1997).

A role for p300/CBP in signal transduction was demonstrated when CBP was defined as a target for the MAPK (mitogen activated protein kinases) and PKA (protein kinase A) phosphorylation cascades (Janknecht and Hunter, 1996). Both MAPK and PKA were shown to upregulate CBP transcriptional co-activation potential. Undoubtedly p300 and CBP are both targets for a large and varied number of signal transduction pathways.

The ability of p300/CBP to acetylate histone tails in a cell cycle regulated fashion, with a peak in HAT activity at the G_1/S transition, points to a functionally important mechanism of p300/CBP (Ait-Si-Ali *et al.*, 1998). Indeed p300/CBP's HAT activity is potentially regulated and stimulated upon phosphorylation by the cyclinE/cdk2 complex. Interestingly E1A is believed to affect p300/CBP acetylation function in a dose-dependent manner, with low levels enhancing and high level suppressing the acetylation activity of p300/CBP (Ait-Si-Ali *et al.*, 1998; Chakravarti *et al.*, 1999; Hamamori *et al.*, 1999).

1-2.6. p300/CBP in human diseases.

A number of clinical observations have defined a role for p300/CBP as a tumour suppressor given that mutations in p300/CBP are closely linked to tumour formation and progression. The congenital autosomal dominant Rubinstein-Taybi syndrome that is characterised by mental retardation, skeletal abnormalities and an increased incidence of neoplasia is associated with a inactivating germline mutation of one *cbp* allele (Petrij *et al.*, 1995). The predisposition of RTS patients to cancer, given that only one *cbp* allele is deleted, point to a gene-dosage role for CBP in normal development and suggests that p300 can not rescue CBP insufficiency. Furthermore, $cbp^{+/-}$ mice are phenotypically similar to RTS patients as they show an abnormal skeletal development pattern with the developmental consequences being dependent on the genetic background (Tanaka *et al.*, 1997). The role of p300/CBP is further underscored by the link with RTS of two other congenital malformation syndromes, namely Greig cephalosyndactyly syndrome and Saethre-Chotzen syndrome. Both these congenital malformations, although not directly as a result of genetic p300/CBP alterations, are associated with p300/CBP development pathway disturbances (Giles *et al.*, 1998).

Interestingly the *p300* gene is subjected to bi-allelic inactivating somatic mutations in a number of gastric and colon cancers (Muraoka *et al.*, 1996). In 80% of examined glioblastomas a loss of heterozygosity (LOH) markers at the *p300* gene locus on chromosome 22q13 have been observed. Similarly a loss of heterozygosity around the *cbp* gene locus is associated with hepatocellular carcinomas (Sakai *et al.*, 1992). The observation that $cbp^{+/-}$ mice are prone to haematologic malignancies as a result of defects in haematopoietic differentiation suggests that CBP is a tumour suppressor.

In a study of human cancer cell lines by Gayther *et al.* (2000) a number of p300 truncation mutations were observed in epithelial cancers, together with p300 somatic in-frame insertions in primary breast cancer and mis-sence alterations in colorectal cancers. Taken together these observations clearly demonstrate that p300/CBP behaves as classic tumour suppressor protein and is consistent with the ability of viral proteins such as E1A and SV40 large T to target and antagonize p300/CBP function.

The p300/CBP gene locus, in addition to germline mutations is subject to somatic translocations that are associated with various types of malignancies. The acute myeloid leukaemia associated translocation t(8;16)(p11;p13) results in a disruption of the *cbp* and *moz* genes and in at least one case a MOZ-CBP fusion protein (Borrow *et al.*, 1996; Giles *et al.*, 1998). MOZ is a protein of unknown function although based on its homology with the mammalian Tip60 protein (human immunodeficiency virus tat-interacting protein) and the yeast silencing protein SAS2 (something about silencing) is has been assigned as a putative acetyltransferase with potentially a gene silencing role (Reifsnyder *et al.*, 1996). The 5'-MOZ-CBP-3' fusion protein retains the HAT domain from both proteins but is no longer able to act as a nuclear receptor co-activator, this alters CBP mediated transcriptional control and is at least in part responsible for transformation.

Another translocation event associated with CBP that arises, as a consequence of anti-cancer chemotherapeutic treatments, such as the topoisomerase II inhibitors etoposides, is known to occur in chronic myeloid leukaemia and myelodysplastic syndrome. The translocation results in the fusion of CBP to the mixed lineage

leukaemia (*mll*) gene product. MLL is believed to function as a chromatin modulator and Swi/Snf complex component based on its SET domain homology and likeness to the *Drosophila* trithorax group genes (Sobulo *et al.*, 1997; Taki *et al.*, 1997). A p300-MLL in frame fusion protein has also been identified in therapy related acute myeloid leukaemia (Ida *et al.*, 1997).

The ability of CBP-MOZ, p300-MLL and CBP-MLL to contribute to hematological malignancy through their gain of function and presumably inactivation of un-rearrange CBP or p300 provides a mechanism whereby an uncontrolled and deregulated cell cycle results. Given that MLL and MOZ as well as p300/CBP are associated with chromatin remodeling it is plausible to assume that the translocation events produce an altered acetylation pattern that presumably contributes to their oncogenic activity (Giles *et al.*, 1998).

Additional to chromosomal aberrations p300/CBP are also involved with the products of some leukaemogenic chromosomal translocation events. p300/CBP is known to function as a transcriptional co-activator in the induction of differentiation by the leukaemia-associated transcription factors AML-1 and TAL-1 (Kitabayashi *et al.*, 1998; Huang *et al.*, 1999).

The underlying genetic instability of sequences within the *p300/cbp* alleles together with the susceptibility of the genes to translocations, inversions and deletion as well as the ability of the proteins to interact with the p53 tumour suppressor and E2F oncogene clearly supports the importance of p300/CBP in human malignancies.

1-2.7. Conclusions.

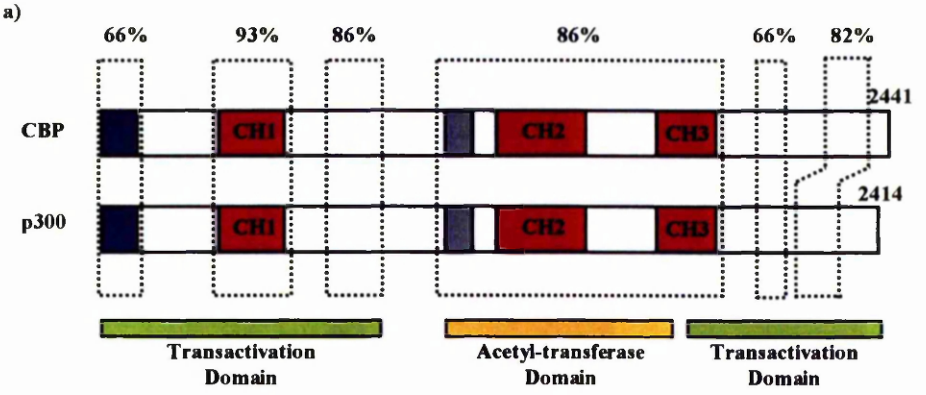
The cellular, transcriptional co-activator, role of p300/CBP is clearly not as simple as first believed given that both proteins display contradictory transcriptional properties. The paradoxical functions of p300/CBP on proliferation and differentiation highlight's the potential for cross-talk between the separate cellular systems.

However, the absolute requirement for p300/CBP in the actions of many transcription factors underlies their importance in the control of cell growth and differentiation. Given the function of p300 and CBP as interconnecting proteins that regulate transcription by integrating signaling pathways their importance in cellular control is indisputable. Elucidating p300/CBP's role in the regulation of a cells fate will not only benefit our understanding of the cell cycle but will help in the design of new cell cycle related therapies.

Figure 1.4. Schematic representation of p300 and CBP.

a). The alignment of human CBP and p300. The CH1, CH2 and CH3 regions are indicated (Red) together with other areas of high homology. Percentages refer to amino acid identity between the two proteins. The region encompassing the acetyltransferase activity of p300 and CBP is indicated (Yellow) together with the N- and C-terminal *trans*activation domains (Green) (Giles *et al.*, 1998). The N-terminal nuclear hormone receptor binding domain is indicated (Blue) together with the central Bromodomain (Grey).

b). Functional interaction domains in p300/CBP. Indicated are the binding domains for the previously identified factors that interact with p300/CBP. The N-terminal nuclear hormone receptor-binding region is indicated (Blue) together with the cysteine/histidine rich regions, CH1, CH2, and CH3 (Red). Also indicated are the central Bromodomain (Grey) and the N-terminal CREB binding KIX domain (Janknecht and Hunter, 1996; Shikama *et al.*, 1997).


a)
66%
93%
86%
86%
66%
82%
CBP
p300
CH1
CH2
CH3
2441
2414
Transactivation Domain
Acetyl-transferase Domain
Transactivation Domain

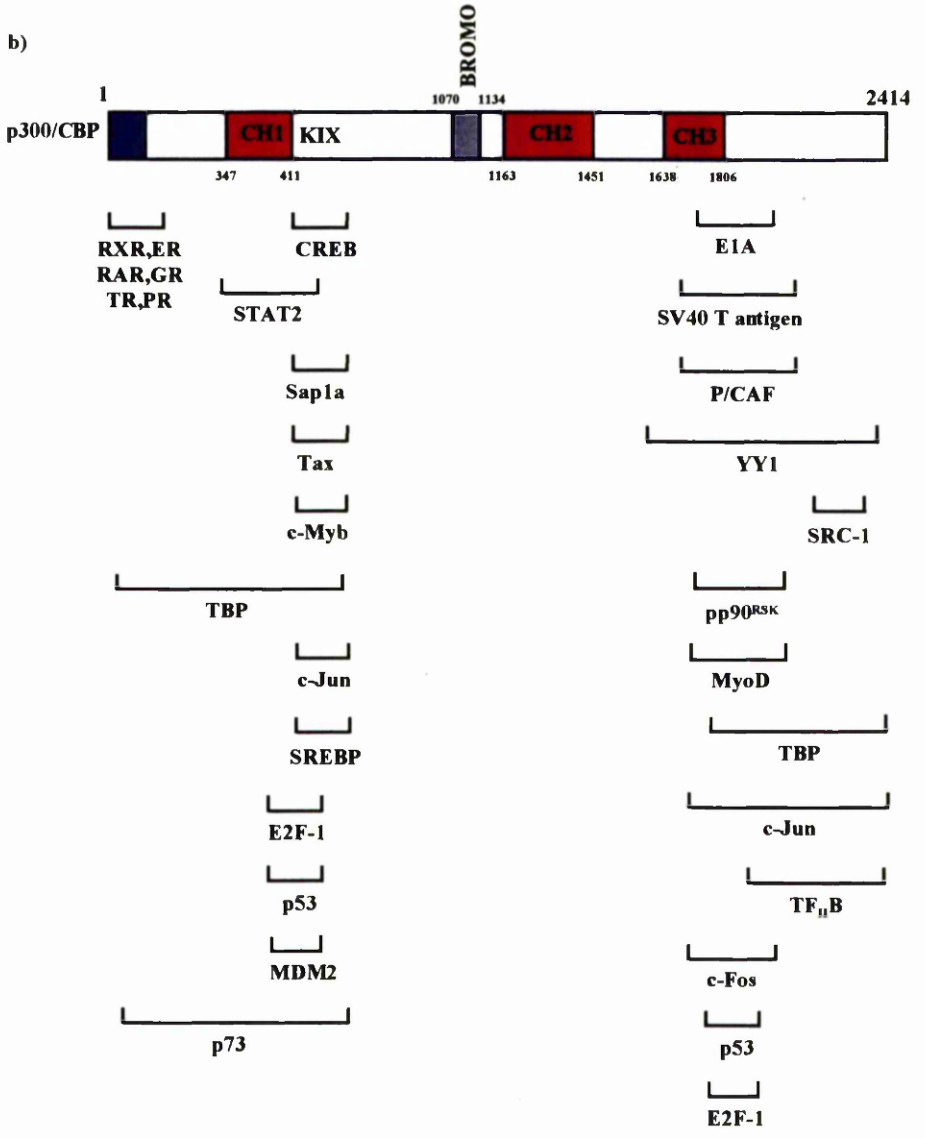
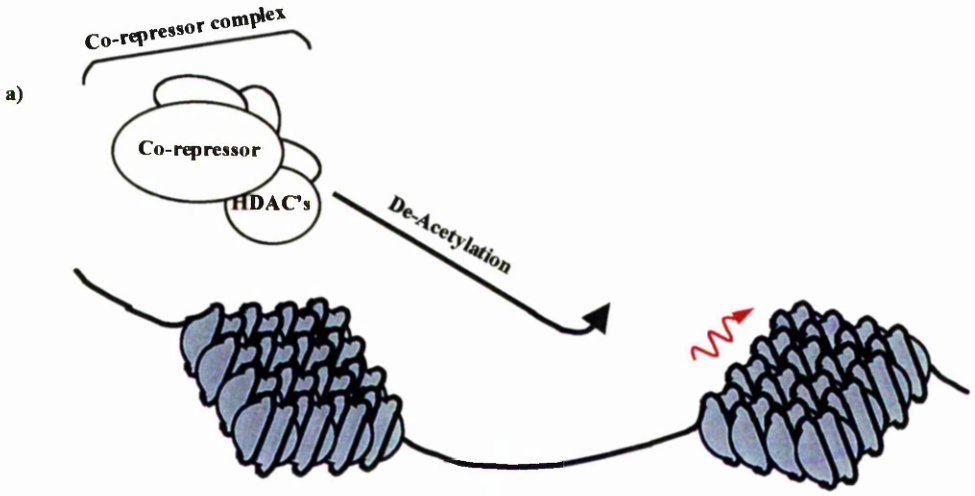
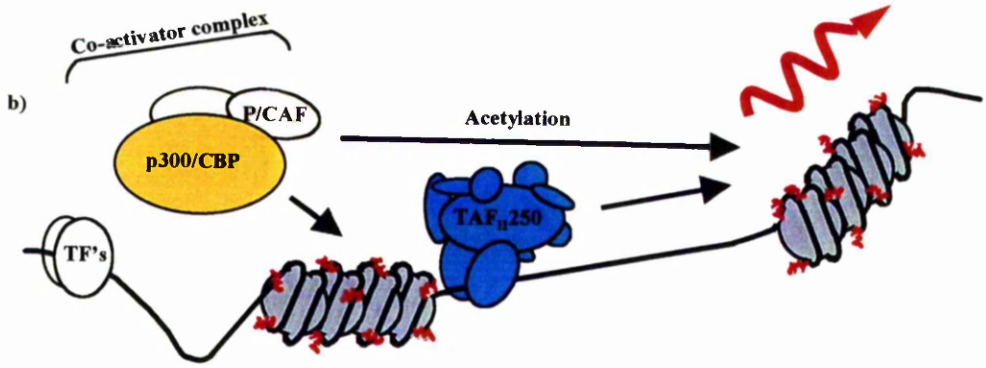

b)
BROMO
1
1070
1134
2414
p300/CBP
CH1
KIX
CH2
CH3
347
411
1163
1451
1638
1806
RXR,ER
RAR,GR
TR,PR
CREB
STAT2
Sap1a
Tax
c-Myb
TBP
c-Jun
SREBP
E2F-1
p53
MDM2
p73
E1A
SV40 T antigen
P/CAF
YY1
SRC-1
pp90RSK
MyoD
TBP
c-Jun
TFIIB
c-Fos
p53
E2F-1

Figure 1.5. Co-activator function.

a). A diagrammatic representation of transcriptional repression. As a result of particular cellular stimuli or a lack of stimuli a co-repressor complex is targeted to genetic promoter elements where it actively represses transcription. Co-repressor complexes, which contain histone deacetylases (HDAC's), promote the condensation of DNA and histones into nucleosomes. The nucleosome contains a highly condensed inaccessible DNA topology and as a result the level of transcription in such a situation is basal.

b). Transcription factors (TF's) actively recruit the co-activator complex that contains p300/CBP, to the gene locus. The co-activator complex acetylates the histone tails (Red) that results in a loss of charge and a breakdown of the nucleosome topology. The altered chromatin integrity now allows the entry of RNA polymerase II transcription machinery components such as TBP and $TF_{II}B$, which results in the active induced transcription of target genes. The ability of the co-activator complex to acetylate the basal transcription component may in addition help activated transcription. Interestingly the $TAF_{II}250$ component of the transcription machinery has intrinsic HAT activity so the activation of gene transcription may proceed exponentially once begun (Mizzen *et al.*, 1996).


a)
Co-repressor complex
Co-repressor
HDAC's
De-Acetylation


b)
Co-activator complex
P/CAF
p300/CBP
Acetylation
TF's
TAF$_{II}$250

1-3. The *INK4a/ARF* locus and its two gene products.

1-3.1. Introduction.

Growth control in mammalian cells is facilitated by the retinoblastoma (pRb) protein regulating exit from the G_1 phase and the p53 protein that triggers growth arrest and apoptotic events in response to cellular stress. Consequently the loss of cell cycle control by the inactivation of the pRb and p53 pathways appears to be a vital step in the rite of passage for all cancer cells. The *INK4a/ARF* locus and its gene products stand at the nexus of both these cell cycle growth controlling pathways (Chin *et al.*, 1998).

The *INK4a/ARF* locus encodes $p16^{INK4a}$, an inhibitor of cyclin D-dependent kinases and $p14/19^{ARF}$ which blocks MDM2 inhibition of p53 activity. It is therefore not surprising that the *INK4a/ARF* locus is one of the most frequently mutated genes in cancer irrespective of the tumour type (Sharpless and DePinho, 1998).

1-3.2. The *INK4a/ARF* locus.

The *INK4a/ARF* locus, which stretches over ~20Kb, is located at position p21 on the short arm of chromosome 9 in humans, and the cognate loci on chromosome 4 in mouse and 5 in rat (Sharpless and DePinho, 1998; Stott *et al.*, 1998). The 9p21 chromosomal hot spot region is frequently subject to deletions and point mutations in a broad spectrum of cancer types ranging from familial melanomas to non-small lung carcinomas (Caldas *et al.*, 1994; Kamb, 1995). *ink4a/arf*$^{-/-}$ locus knock out mice

exhibit a cancer prone phenotype and fibroblasts from these mice have an enhanced potential for spontaneous immortalization and are efficiently transformed by activated Ras (Serrano *et al.*, 1996). Together these observations point clearly to the *INK4a/ARF* locus as a *bona fide* tumour suppressor (Chin *et al.*, 1998; Kamijo *et al.*, 1999b).

Two transcripts, which are driven by distinct promoters that encode two functionally separate potential tumour suppressor proteins, are expressed from the *INK4a/ARF* locus, the $p16^{INK4a}$ CDK inhibitor and the $p14/19^{ARF}$ protein (Quelle *et al.*, 1995). Within the *INK4a/ARF* locus there are four exons, E1β, E2 and E3 encode $p14/p19^{ARF}$ and E1α, E2 and E3 encode $p16^{INK4a}$. Splicing of exon 1β to exon 2 allows translation to continue in the –1 reading frame relative to $p16^{INK4a}$, giving rise to a 132 amino acid protein termed $p14^{ARF}$ in humans or a 169 amino acid $p19^{ARF}$ protein in mouse (Figure 1.6). The mouse $p19^{ARF}$ and human $p14^{ARF}$ proteins although functionally almost identical show only a 50% identity over the region of overlap (Stott *et al.*, 1998).

The ability of the $p14/19^{ARF}$ and $p16^{INK4a}$ proteins to be encoded by distinct reading frames within a common coding sequence, such that the two products share no amino acid identity, although common in viruses and bacteria is an exceedingly rare event in eukaryotes where an evolutionarily advantage as yet remains unclear (Quelle *et al.*, 1995). The observation that the *INK4a/ARF* locus is the genetic target for specific mutational events which affect only one member of the gene locus gives credence to the finding that each protein monitors a separate essential cellular function (Quelle *et al.*, 1995; Kamijo *et al.*, 1997; Gardie *et al.*, 1998).

1-3.3. INK4a/ARF Expression.

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 $p14/19^{ARF}$ observed during both mouse and human development and ageing suggests that the transcriptional regulation of the two products differ (Quelle *et al.*, 1995; Zindy *et al.*, 1997). In fact differential controlled expression of $p16^{INK4a}$ and $p14/19^{ARF}$ is born out by the ability of cells to control promoter specific transcription of E1α and E1β independently of each other.

The high levels of CpG islands present within both the $p16^{INK4a}$ and $p14/19^{ARF}$ promoters are a characteristic of many cellular house keeping genes (Robertson and Jones, 1998). It is these CpG islands, as a consequence of hyper-methylation, that are often associated with tumour-derived promoter silencing. However the presence of Sp1 sites, within the *INK4a* and *ARF* promoters, may maintain the expression of both proteins under physiological cellular conditions by a mechanism that retains the promoters in an unmethylated form (Robertson and Jones, 1998).

In $p53^{-/-}$ cell lines or in cells in which p53 has been functionally compromised by the over expression of MDM2 the level of $p14^{ARF}$ is significantly elevated (Quelle *et al.*, 1995). Studies performed on p53 over expression in a number of cell lines showed that p53 itself is able to down regulate transcription from both the $p14^{ARF}$ and $p16^{INK4a}$ promoters (Robertson and Jones, 1998; Stott *et al.*, 1998). Consistent with these observations is the up regulation of $p14^{ARF}$ expression by the viral proteins HPV

E6 and SV40 large T, both of which deregulate p53 (Hara *et al.*, 1996; Stott *et al.*, 1998). These observations propose the existence of an auto regulatory feedback loop, reminiscent for that of p53 and MDM2, in which p53 levels are controlled by $p14^{ARF}$, whose expression is in turn controlled by p53. As yet the modulation of mouse $p19^{ARF}$ by p53 levels remains to be documented.

Interestingly the TATA less $p14/19^{ARF}$, but not the $p16^{INK4a}$ promoter, in a region upstream of exon 1β, contains several potential consensus E2F sites. The $p14^{ARF}$ promoter contains at least four potential negative strand E2F binding sites, two of which are high affinity while two are poor matches (Roberston and Jones, 1998). $p19^{ARF}$ contains only two high affinity E2F sites, one negative and one positive strand coded (Inoue *et al.*, 1999).

Initial studies demonstrated the potential of E2F to induce $p14/19^{ARF}$ expression (Bates *et al.*, 1998; Roberston and Jones, 1998; Inoue *et al.*, 1999). Over expression of E2F-1 activates the transcription of $p14^{ARF}$ mRNA in a transcriptionally-dependent but non cell cycle regulated manner (Bates *et al.*, 1998; Inoue *et al.*, 1999). Specificity studies carried out on the E2F family members indicated that E2F-1 and E2F-2, but not E2F-3 to E2F-5, were able to increase $p19^{ARF}$ mRNA levels (DeGregori *et al.*, 1997). The induction of $p14/19^{ARF}$ mRNA expression by E2F is paralleled by a marked increase in the level of $p14^{ARF}$ protein, although to date there is no indication whether this corresponds to a change in stability of $p14/19^{ARF}$ or an increase in translation (Bates *et al.*, 1998). It is interesting to note that pRb has been shown to repress $p16^{INK4a}$ expression under certain cellular circumstances (Moran, 1993).

The influence of E2F on the $p14/19^{ARF}$ pathway provides a mechanism whereby proliferative oncogenic stimuli are detected in such a way that cell cycle arrest or apoptosis is induced through p53. E2F deregulation via the activation of oncogenes, such as Ras or c-Myc, results in the activation of p53 and the enhanced expression of $p14/19^{ARF}$. Such a model, linking E2F to p53 activation, is strongly supported by the ability of $p14^{ARF}$ mutant tumours to tolerate the retention of wild-type p53 (Bates *et al.*, 1998). Given that the regulation of $p14/19^{ARF}$ and $p16^{INK4a}$ expression occurs through both p53 and pRb, via E2F, it is feasible to assume that both pathways cross talk, with p53 being the dominant component for $p14/19^{ARF}$ and pRb the dominant partner for $p16^{INK4a}$ (Robertson and Jones, 1998) (Figure 1.6).

The polycomb group of proteins, comprises a set of proteins that maintain the stable expression of specific target genes, such as homeo-box cluster genes, during development (Gould, 1997). The Bmi-1 oncognene is a member of the polycomb group which acts as a specific transcriptional repressor (Van Lohuizen *et al.*, 1991; Van der Lugt *et al.*, 1994). In addition to the severe neurological defects seen in *bmi-1*$^{-/-}$ mice, MEF's from these animals exhibit an impaired S phase entry and premature senescence phenotype (Jacobs *et al.*, 1999a). The impaired S phase and early senescence observed in MEF's from these animals corresponds to a highly elevated level of both the *INK4a/ARF* locus products. Over expression of Bmi-1 down regulates the expression of both $p16^{INK4a}$ and $p19^{ARF}$ in *bmi-1*$^{-/-}$ mice which results in fibroblast immortalisation. The full rescue of the proliferation defects and early senescence onset together with the loss of the neurological defects in *bmi-1*$^{-/-}$;*ink4a*$^{-/-}$ mice clearly point to Bmi-1 as an important regulator of *INK4a/ARF* expression in development and cell cycle regulation (Jacobs *et al.*, 1999a). Bmi-1 clearly functions

by suppressing both the $p16^{INK4a}$/pRb and $p19^{ARF}$/MDM2/p53 pathways, thereby allowing progression through the cell cycle.

Insertional mutagenesis in transgenic mice with c-Myc identified Bmi-1 as a collaborator in the onset of B-cell lymphomas (Haupt *et al.*, 1991; Alkema *et al.*, 1997). Given that *myc*$^{-/-}$ mice are embryonic lethal, and that c-Myc over expression induces apoptosis and tumourigenesis, the importance of c-Myc in proliferation and differentiation during embryogenesis is unquestionable (Davis *et al.*, 1993; Prendergast, 1999). Myc induced apoptosis is both p53 and $p19^{ARF}$ transcription dependent (Zindy *et al.*, 1998) and is prevented by Bmi-1 over expression (Jacobs *et al.*, 1999b). The synergistic and dose-dependent increase in proliferation and decrease in apoptosis observed between Bmi-1 and c-Myc indicates that their co-operation in oncogenic transformation is mediated by the ability of Bmi-1 to prevent Myc activation of $p19^{ARF}$ (Jacobs *et al.*, 1999b).

The $p19^{ARF}$ promoter has been shown to contain a single responsive consensus site for the transcription factor and potential tumour suppressor DMP1. Enforced expression of DMP1 in mouse fibroblasts induces cell cycle arrest (Inoue and Sherr, 1998). It is via the induced expression of $p19^{ARF}$ that DMP1 exerts its p53-dependent anti-proliferative effects (Inoue *et al.*, 1999). Studies performed on *dmp1*$^{-/-}$ animals suggests that $p19^{ARF}$ function is in fact compromised but not eliminated, in the absence of DMP1, raising the possibility that DMP1 contributes but is not essential for $p19^{ARF}$ regulation (Inoue *et al.*, 2000).

DMP1's ability to bind DNA and drive transcription is lost in the presence of over expressed D-type cyclins, in the absence of CDK's. It is therefore possible to assume that D-type cyclins can act negatively on the cell cycle through repression of $p19^{ARF}$ expression, as well as positively through the release of E2F-pRb repression.

The tumour suppressor BRCA1 is able to trigger transcription by both p53-dependent and independent pathways. Interestingly neither $p14^{arf-/-}$ cells nor $p19^{arf-/-}$ MEF's are able to induce p53 stabilisation in response to BRCA-1 over-expression (Somasundaram *et al.*, 1999). BRCA1's ability therefore to stabilise p53 is $p14/19^{ARF}$ dependent. It has also been noted that the level of $p14^{ARF}$ mRNA is substantially increased following BRCA1 introduction into cells, although the mechanism of such induction remains unclear (Somasundaram *et al.*, 1999). Given that BRCA1 transcription is itself activated by E2F it is attractive to imagine other potential synergistic pathways that act on $p14/19^{ARF}$ expression (Wang *et al.*, 2000).

Other potential regulators of $p14/19^{ARF}$ expression and their potential consequences include co-activators such as p300, that regulates MDM2 and co-activates E2F. In addition to the regulators of ARF expression previously discussed undoubtedly the Ap-1 and YY1 sites present in the $p14^{ARF}$ promoter have a significant but as yet unknown role (Roberston and Jones, 1998).

1-3.4. $p16^{INK4a}$.

Mutational analysis revealed that $p16^{INK4a}$ is commonly mutated or deleted in human cancer, in particular in pancreatic adenocarcinoma and melanomas, with some mutations mapping specifically to exon 1α (Hussussian *et al.*, 1994; Sharpless and

DePinho, 1998). Interestingly mutations in *cdk4*, which abrogate $p16^{INK4a}$ function, are also found in melanomas further strengthening the observation that the $p16^{INK4a}$-cdk interaction plays a role in tumour susceptibility (Zuo *et al.*, 1996).

$p16^{INK4a}$ was first identified by its ability to block cell passage from G_1 into S phase, as a consequence of the inhibition of cyclin D-dependent cdk4/6 kinase activity (Serrano *et al.*, 1993). The inhibition of cdk4/6 leads to the hypophosphorylation of pRb that in turn represses E2F and blocks G_1 progression and exit (Figure 1.6). Crystallographic studies of $p16^{INK4a}$ bound to cdk6 demonstrated that binding distorts the cyclin binding site and prevents ATP binding (Russo *et al.*, 1998). The lack of pRb and $p16^{INK4a}$ mutations in the same tumour together with the observation that $Rb^{-/-}$ cells generally display very high levels of $p16^{INK4a}$ indicates the lack of a selective advantage in deregulation of two genes in the same pathway.

1-3.5. $p14/19^{ARF}$.

Structurally $p14/19^{ARF}$ is a highly basic protein that shares no homology with known proteins in the databases and lacks any decisive functional protein motifs (Quelle *et al.*, 1995). $p14/19^{ARF}$ can induce cell cycle arrest in a p53-dependent cdk-independent manner as cells-lacking p53 are refractory to $p14/19^{ARF}$ arrest (Pomerantz *et al.*,1998; Zhang *et al.*, 1998; Kurokawa *et al.*, 1999).

Given the ability of $p14^{ARF}$ to suppress MDM2/Ras induced transformation it was suspected that $p14^{ARF}$ induced cell cycle arrest by preventing MDM2 mediated p53 ubiquitin degradation. Studies have demonstrated that $p14^{ARF}$ activates p53 by binding and sequestering MDM2 into nuclear bodies where it is unable to target p53

for nuclear export and degradation (Zhang and Xiong, 1999) (Figure 1.6). Expression of p14/19^{ARF} exon 1β alone (amino acids 1-64) is sufficient to stabilise p53, stimulate p53-dependent transcription of p21$^{Waf1/Cip1}$ and *mdm2*, and induce cell cycle arrest (Kamijo *et al.*, 1998; Stott *et al.*, 1998; Zhang *et al.*, 1998). Indeed p14/19^{ARF}, via at least two independent exon 1β encoded sequences, interacts directly with the central acidic domain of MDM2 at a site overlapped by the p300 binding domain but distant from the p53 site (Momand *et al.*, 1992; Zhang *et al.*, 1998; Zhang and Yiong, 1999; Weber *et al.*, 1999; Lohrum *et al.*, 2000a; Midgley *et al.*, 2000;). Given that p300 complexed with MDM2 may mediate ubiquitination of p53 and its subsequent degradation (Grossman *et al.*, 1998). It is plausible to assume that p14/19^{ARF} may compete with p300 for MDM2 in a manner that reduces degradation dependent down regulation of p53, simultaneously releasing p300 to act as a p53 transcriptional co-activator.

Although p14/19^{ARF} is functionally devoid of any previously characterised domain, regions within the protein have been shown to confer its characteristic nucleolar localisation. Nucleolar localisation of p14/19^{ARF} under basal conditions is poorly understood, although nucleolar structures are known sites of rDNA localisation, rRNA synthesis and ribosomal assembly (Scheer and Weisenburger, 1994; Fomproix *et al.*, 1998).

Nucleolar localisation of p14/19^{ARF} is imposed by specific sequence motifs present within both exon 1β and exon 2 of the protein (Weber *et al.*, 1999; Zhang and Xiong, 1999). Within the nucleolar localisation coding region from exon 2 of p14/19^{ARF} a number of mutational events have been noted, ranging from microdeletions to single

nucleotide insertions, which would presumably affect both localisation and consequently function of p14/19^{ARF} (Soufir *et al.*, 1998; Holland *et al.*, 1999). Recent observations have identified basic amino acid stretches present in both the N- and C-terminal of p14/19^{ARF} as being important for nucleolar localisation. Such basic amino acid stretches are also found in RNA binding proteins as well as the human immunodeficiency viral proteins Rev and Tat, where they mediate an importin β interaction (Henderson and Percipalle, 1997; Rizos *et al.*, 2000). The identification of these regulatory domains suggests that p14/19^{ARF} may be a multi-functional protein participating in several aspects of the controlled growth of cells.

Mutational analysis revealed that the formation of p53-MDM2-p14^{ARF} nuclear bodies, within which p53 is stabilised, requires to some degree the presence of the nucleolar localisation signal encoded by exon 2 (Zhang and Xiong, 1999). The presence of nuclear bodies and their function as yet remains unclear, although it is interesting to speculate that they are sites of DNA synthesis or are regions involved in structure-related events. The importance of nucleolar localisation on p14/19^{ARF} function is further highlighted by the presence of a second potential nucleolar localisation signal (NuLS) within the exon 1β encoded N-terminus which although less efficiently is still able to drive p53 stabilisation (Lohrum *et al.*, 2000a; Midgley *et al.*, 2000). Murine p19^{ARF} differs from human p14^{ARF} in that p19^{ARF} appears not to form nuclear bodies with p53 and MDM2 but to actually sequester and retain MDM2 but not p53 into the nucleolus of cells (Weber *et al.*, 1999).

In addition to the nuclear structures formed by the interaction of p14/19^{ARF} with MDM2 it appears that MDM2 itself can be sequestered, or located into the nucleolus.

As a result of its interaction with $p14^{ARF}$ a cryptic nucleolar localisation signal within the C-terminus of MDM2 is revealed that is effective in promoting MDM2 nucleolar localisation (Weber *et al.*, 1999; Lohrum *et al.*, 2000a; Lohrum *et al.*, 2000b).

The ability of MDM2 to regulate the cellular level of p53 is a direct consequence of its ability to undergo nucleo-cytoplasmic shuttling (Roth *et al.*, 1998). It is the targeting of p53 to the cytoplasmic proteasome by MDM2 that is blocked by $p14/19^{ARF}$, which tethers MDM2 into nuclear structures (Tao and Levine, 1999b). As MDM2 nuclear export is dependent upon its interaction with exportin-1 (CRM-1) and Ran-GTP it is feasible to assume that $p14/19^{ARF}$ effects these interactions either directly by holding MDM2 or indirectly through an as yet undefined secondary modification mechanism (Freedman and Levine, 1998).

$p14^{ARF}$ was originally believed not to participate in the p53 DNA damage response (Zhang *et al.*, 1998). However studies performed by Khan *et al.* (2000) demonstrated that $p19^{ARF}$ responds, in a p53-dependent manner, to microtubule disruption and ionising radiation, whereas ribonucleotide depletion and actinomycin induced RNA synthesis inhibition were completely independent of $p19^{ARF}$. Clearly these specific roles for $p14/19^{ARF}$ in the DNA damage response require clarification.

The direct interaction of the $p19^{ARF}$ exon 1β encoded N-terminal domain (amino acids 1-62) with p53 was demonstrated, and although the interaction can occur in a DNA context it does not increase the affinity of p53 for its consensus site (Kamijo *et al.*, 1998). Mutant forms of p53, that exhibit a prolonged half life and have a reduced

affinity for MDM2, and the stress activated kinase JNK show an increased affinity for p14/19^{ARF} (Buschmann *et al.*, 2000).

The forced induction of p19^{ARF} in mouse fibroblasts causes a p53-dependent G_1 and G_2/M arrest in the cell cycle (Kurokawa *et al.*, 1999). Fibroblasts that express high levels of p19^{ARF} prior to G_1 arrest show high mobility forms of p19^{ARF} that may correspond to either p19^{ARF} degradation intermediates or post-translational intermediates (Kurokawa *et al.*, 1999). Cells expressing high levels of p19^{ARF} retain high molecular weight forms of p53 (Pomerantz *et al.*, 1998) that possibly correspond to polyubiquitinated forms. Given this observation it is foreseeable that p19^{ARF} might not inhibit MDM2 mediated ubiquitination but instead might prevent the degradation of ubiquitinated p53.

p19^{ARF} reduces the level of pRb phosphorylation and forces pRb into a hypophosphorylated form. The induction of pRb hypophosphorylation corresponds with the ability of p19^{ARF} to down-regulate cdk activity. p19^{ARF} down-regulates the activity of cdk 2 and 4 by reducing the level of cyclin A, B1 and E. Interestingly the level of the mid G_1 cyclin, D1, is increased by p19^{ARF} as well as the p53 induced gene p21$^{Waf1/Cip1}$ which functionally inhibits cyclin D/cdk complex kinase activity. The increased expression of cyclin D and p21$^{Waf1/Cip1}$ as a consequence of p19^{ARF} alters the cellular composition of the cyclin/cdk complexes in such a way that usually undetected cyclin cdk complexes become prominent (Labaer *et al.*, 1997; Kurokawa *et al.*, 1999). The p27^{KIP1} cyclin dependent kinase inhibitor is down regulated by p19^{ARF} together with the cdc2 kinase, which is absolutely required for G_2/M

progression. The repression of cdc2 expression may provide a mechanism by with which $p19^{ARF}$ induces G_2/M cell cycle arrest (Kurokawa *et al.*, 1999).

mdm2 gene expression, as a consequence of p53 stabilisation, is upregulated by $p14/19^{ARF}$, but interestingly the stability of MDM2 in complex with $p19^{ARF}$ is reduced (Zhang *et al.*, 1998; Kurokawa *et al.*, 1999; Zhang and Xiong, 1999). The exact mechanism by which MDM2 stability in complex with $p19^{ARF}$ is reduced is unknown, although it is potentially by the cytoplasmic proteasome pathway in an analogous way to p53 stability regulation by MDM2.

$p19^{ARF}$ plays an important function in the p53 mediated pathways involved in G_1 arrest, but several lines of evidence point to mechanisms by which $p19^{ARF}$ can induce cell cycle arrest via a p53 independent mechanism (Kurokawa *et al.*, 1999; Carnero *et al.*, 2000).

1-3.6. Oncogenes and $p14/19^{ARF}$.

Given that $p14/19^{ARF}$ shows little or no response to DNA damaging conditions it is interesting to note the response to oncogenic stimuli. The oncogenes Ras, Myc and E1A are all able to induce a $p14/19^{ARF}$ response (de Stanchina *et al.*, 1998; Palmero *et al.*, 1998). Interestingly Ras can also elicit and make use of the $p16^{INK4a}$ tumour suppressor in order to arrest cells (Serrano *et al.*, 1997). The enforced expression of $p14^{ARF}$ induces cell cycle arrest however in the presence of E1A or Myc cells undergo apoptosis (deStanchina *et al.*, 1998; Zindy *et al.*, 1998). E1A requires both the capacity to bind pRb and p300 in order to induce cellular apoptosis, so it is possible that $p14/19^{ARF}$ activation is a consequence of these activities (de Stanchina

et al., 1998). The action of oncogenes in the targeting of $p14/19^{ARF}$ is further emphasised by the observation that $p14/19^{arf-/-}$ cells are resistant to E2F-1 induced apoptosis (Zindy *et al.*, 1998).

The ability of the Abelson virus (Ab-MLV) oncoprotein to induce pre-B cell transformations is a direct consequence of loss of p53 function. The normal cellular defence mechanism against v-Abl appears to be governed by $p19^{ARF}$, given that Ab-MLV induces c-Myc and Ras expression that in turn induce $p19^{ARF}$ (Zou *et al.*, 1997; Radfar *et al.*, 1998).

The actions of oncogenes on $p16^{INK4a}$ and $p19^{ARF}$ together with the lack of a clear involvement in the DNA damage response of the two proteins implies that the *INK4a/ARF* locus may be a specific cellular controlling mechanism against the actions of oncogenic stimuli.

1-3.7. Roles of $p16^{INK4a}$ and $p14/19^{ARF}$ in senescence.

Senescence is the loss of the ability to proliferate after the completion of a finite number of cellular divisions and is characterised by a growth arrest, apoptotic resistance and an altered spectrum of differentiation phenotypes (Campisi, 1996; Campisi, 1997). A loss of, or shortening of, telomeres from eukaryotic chromosome ends results in an inability to induce DNA replication and a senescence phenotype (Campisi, 1996; Campisi, 1997; Bodnar *et al.*, 1998). The senescence phenotype has also been shown to be induced under conditions of DNA damage, the introduction of deacetylase inhibitors and by oncogenic forms of Ras and Raf (Ogryzko *et al.*, 1996b; Serrano *et al.*, 1997; Chen *et al.*, 1998). As senescence entails an irreversible growth

arrest it has been suggested as a fail-safe program that curtails tumourigenesis and age related pathologies. The growth arrest associated with senescence is closely associated with the down regulation of many cell cycle associated genes, such as c-fos, Cdc2, cyclin A and E2F1, and the up regulation of growth inhibitors like $p16^{INK4a}$ and $p21^{Waf1/Cip1}$ (Alcorta *et al.*, 1996; Hara *et al.*, 1996; Stein *et al.*, 1999).

Viral oncoproteins immortalise cells, by inactivating the telomere length checkpoint control system which prevents the initiation of senescence, as a consequence of their ability to target the cellular proteins, p53 and pRb (Chen *et al.*, 1998). Consistent with the role of pRb and p53 as mediators of senescence is the observation that both $p21^{Waf1/Cip1}$ and $p16^{INK4a}$ are able to induce premature senescence phenotypes when expressed in human fibroblasts (McConnell *et al.*, 1998). A role for $p16^{INK4a}$ in senescence is further enforced in that primary cultured cells express increasing amounts of $p16^{INK4a}$ as they approach senescence and inactivation of $p16^{INK4a}$ prevents Ras induced senescence (Hara *et al.*, 1996; Haber, 1997).

Introduction of transcriptionally active E2F-1 into human fibroblasts is able to induce a senescence like phenotype in a p53 and $p14^{ARF}$ dependent manner. Given that E2F induces $p14^{ARF}$ expression and the introduction of $p14^{ARF}$ into fibroblasts produces a senescence phenotype it is likely that E2F's ability to induce a senescence phenotype is at least in part due to its ability to upregulate $p14^{ARF}$ (Kamijo *et al.*, 1999a; Dimri *et al.*, 2000). The senescence phenotype is therefore to a degree controlled by $p14^{ARF}$, although not maintained by induced expression from E2F, as E2F levels are low in senescence cells, possibly as a consequence of the low level of Bmi-1 expression seen in senescence cells (Jacobs *et al.*, 1999a).

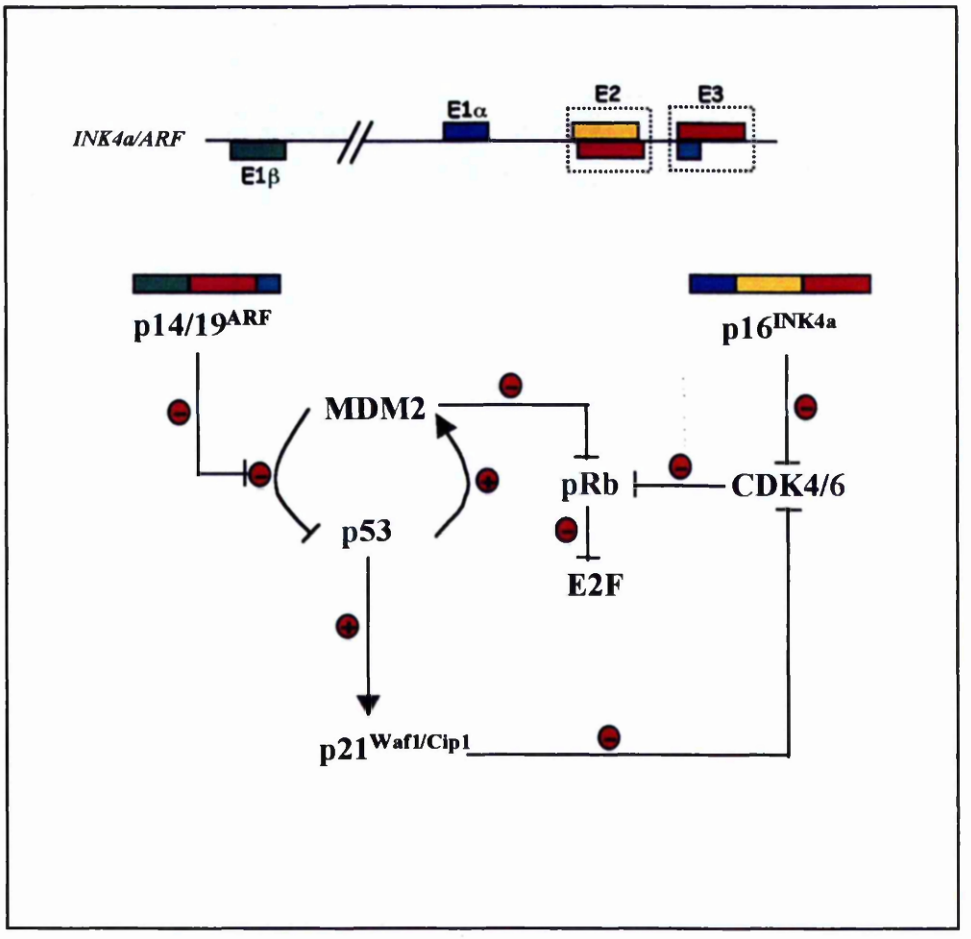
Carnero *et al.* (2000) performed an elegant study using anti-sense knock out vectors in which they examined the role of both $p19^{ARF}$ and $p16^{INK4a}$ in senescence and cell cycle arrest. Interestingly $p19^{ARF}$ could, in a p53 independent fashion, negatively regulate the cell cycle. The p53 independent mechanism of cell cycle arrest by $p19^{ARF}$ was lost upon pRb inactivation, and required MDM2. It was demonstrated that $p19^{ARF}$ was more effective at inducing senescence than $p16^{INK4a}$, as $p19^{ARF}$ deficient cells can proliferate with high levels of $p16^{INK4a}$, but cells without $p16^{INK4a}$ can not proliferate with high levels of $p19^{ARF}$. It is interesting to speculate that $p19^{ARF}$ and $p16^{INK4a}$ have developed as a mechanism by which senescence is activated by targeting both p53 and pRb not only potentially directly but also through MDM2 (Yap *et al.*, 1999; Carnero *et al.*, 2000; Lloyd, 2000).

1-3.8. Conclusions.

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. $p16^{INK4a}$ targets the pRb pathway to induce a cell cycle arrest and $p14/19^{ARF}$ the MDM2/p53 pathway (Figure 1.6). The transcription of two unrelated genes from the same genetic locus such that both function to mediate the same cellular consequences has undoubtedly come about through a genetic selection event. The reason for such conservation in genetic information in mammalian cells is unknown but will prove an exciting avenue for future research.

Figure 1.6. The *INK4a/ARF* locus and its two gene products.

Diagram of the exon organisation of the *INK4a/ARF* locus. Alternative splicing of E1β to E2 gives rise to $p14/19^{ARF}$ and transcription driven from E1α forms the CDI $p16^{INK4a}$ protein. $p14/19^{ARF}$ neutralises MDM2 to stabilise p53 that in turn results in p53 mediated cell cycle arrest as a result of induction of genes such as $p21^{Waf1/Cip1}$. $p16^{INK4a}$ inhibits the phosphorylation of pRb by directly inhibiting cyclin/CDK activity that in turn results in the repression of E2F and cell cycle arrest (Chin *et al.*, 1998).


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

1-4. The Retinoblastoma tumour suppressor protein (pRb).

1-4.1. Introduction.

The retinoblastoma tumour suppressor gene (*rb)* is frequently mutated in a broad spectrum of human tumours, including retinoblastoma, osteosarcoma, and prostate, breast and lung carcinomas (Weinberg, 1995; Sherr, 1996). In addition, a number of genes, such as $p16^{INK4a}$ and cyclin D1 that regulate pRb function are also frequently associated with tumour progression (Caldas *et al.*, 1994; Kamb *et al.*, 1995; Hall and Peters, 1996). The high frequency of mutations in the pathway that controls pRb activity underscores the importance of this tumour suppressor pathway in tumourigenesis. More precisely loss of pocket protein function, either through mutagenesis, viral infection or phosphorylation results in the de-regulation of the E2F transcription factor family and is a major step in the loss of cell cycle control and tumour formation (Dyson, 1998).

pRb's cellular importance is highlighted by the plethora of interactions, with transcription factors, kinases, phosphatases, structural proteins and deacetylases which it uses to suppress cell cycle progression. Clearly the role of pRb in suppressing growth, facilitating differentiation and inhibiting apoptosis defines pRb as a master regulator of the cell (Sellers and Kaelin, 1996; Dyson, 1998).

1-4.2. Rb family.

The retinoblastoma gene (*Rb*) located on chromosome 13 encodes a 110kDa nuclear phosphoprotein that behaves as a classical tumour suppressor protein (Lee *et al.*, 1987; Riley *et al.*, 1994). pRb is frequently inactivated in a variety of tumour types and sporadic or familial inherited inactivating mutations in both copies of the *rb* gene are associated with retinoblastoma (Friend *et al.*, 1986). Single allelic mutations in the *rb* gene predisposes suffers not only to retinoblastoma but also tumours such as osteosarcomas and fibrosarcomas (Horowitz *et al.*, 1990; Riley *et al.*, 1994).

pRb is a member of a family of proteins termed "the pocket proteins" that includes p107 and p130 (Ewan *et al.*, 1991; Hannon *et al.*, 1993; Weinberg, 1995; Whyte, 1995). The pocket protein family members are highly homologous proteins and their genes are highly conserved in multi-cellular organisms. The family share a region of very high homology termed the pocket region and it is this region that is targeted by the negative cell cycle viral regulators, SV40 large T, E1A and E7 (La Thangue, 1994; Chow and Dean, 1996).

The pocket region of pRb, p107 and p130 mediates many of the cellular binding properties of the proteins and is essential for their growth suppression properties. An LXCXE binding region imparts the ability of many cellular proteins that contain LXCXE motifs such as, histone deacetylases (HDAC's) and cyclin D to interact with the pocket proteins (Kaelin, 1999). Viral oncoproteins such as E1A, E7 and SV40 large T also contain LXCXE motifs in their protein sequence and similarly it is this region that mediates their interactions with pocket proteins (Dyson and Harlow, 1992; Moran, 1993). The pocket region that encompasses the LXCXE motif is also

responsible for the interaction of pRb with the transcription factor E2F. In addition pRb utilises its C-terminal pocket region to mediate interactions with the cellular proteins MDM2 and c-Abl. The binding of c-Abl to pRb prevents ATP binding and abrogates pRb's growth arrest function as a consequence of a loss in c-Abl's kinase activity (Welch and Wang, 1993; Welch and Wang, 1995). Conversely pRb binding to MDM2 inhibits its anti-apoptotic function (Martin *et al.*, 1995; Xiao *et al.*, 1995; Hsieh *et al.*, 1999) (Figure 1.7a).

To date only the pRb member of the pocket protein family has been observed to be mutated in tumour cells. The generation of knockout mice has indicated that pRb, p107 and p130 perform both distinct and overlapping functions in the regulation of cellular proliferation (Hurford *et al.*, 1997). *rb* knockout mice are embryonic lethal while $p107^{-/-}$ or $p130^{-/-}$ mice are normal and survive to term (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). $rb^{-/-}$ mice suffer from the defective differentiation of many tissues and an increased level of apoptosis and embryonic fibroblasts from these animals display inappropriate S-phase entry (Herrera *et al.*, 1996). Specifically, it appears that p107 and p130 in a $rb^{-/-}$ background are unable to compensate for the loss of pRb with respect to the differentiation and proliferation of certain tissue types. The unique tumour suppressor status of pRb is also highlighted by $p107^{-/-};p130^{+/-}$ and $p107^{+/-};p130^{-/-}$ mice that develop normally and show no increase in the level of tumour formation.

The non-lethal phenotype of p107 and p130 knockouts is potentially due to functional redundancy between the two family members and in agreement $p107^{-/-};p130^{-/-}$ mice die *in utero* due to cartilage and bone malformation (Cobrinik *et al.*, 1996).

Interestingly the overlap in function of the pocket protein family is highlighted by the observation that $rb^{+/-}$ mice, that do not develop retinoblastoma, when crossed with $p107^{-/-}$ mice, to generate $rb^{+/-};p107^{-/-}$ mice, begin to suffer from retinal dysplasia (Lee *et al.*, 1996). In addition $rb^{-/-};p107^{-/-}$ mice die at an earlier stage *in utero* than $rb^{-/-}$ mice (Lee *et al.*, 1996).

Given these observations it is interesting to speculate that p107 and p130 are essential cellular genes and that mutations in their regulatory mechanisms are intolerable even in tumour cells. Potentially the physiological role of p107 and p130 in tumourigenesis is confined to mutational events that inactive their functions as regulators of gene expression while maintaining other necessary functions they perform. In agreement such mutations are found in the pocket protein regulatory protein, $p16^{INK4a}$ (Caldes *et al.*, 1994; Kamb *et al.*, 1995; Koh *et al.*, 1995; Hall and Peters, 1996).

1-4.3. Cell cycle regulation by pRb.

pRb acts as a potent negative regulator of cellular proliferation through its ability to regulate the activity of a variety of nuclear proteins and transcription factors such as E2F (Weinberg, 1995). E2F is a heterodimeric transcription factor that consists of an E2F and DP component which regulates the transcription of a number of target genes involved in DNA replication, chromosomal replication and cell cycle control. Distinct cellular mechanisms control the timing of E2F target gene activation potentially by ordering the family composition of the E2F/DP DNA binding complexes (Bandara *et al.*, 1993; La Thangue, 1994; de La Luna *et al.*, 1996; Allen *et al.*, 1997). E2F target genes involved in DNA replication include DNA polymerase α, thymidine kinase (TK), dihydrofolate reductase (DHFR) and cdc6, and those involved in cell cycle

regulation include cyclin A, cyclin E, cyclin D1, p107, cdc2, E2F-1, E2F-4, E2F-5 and $p14/19^{ARF}$ (La Thangue, 1994; Adams and Kaelin, 1995; Kaelin, 1999).

The E2F family consists of six members all of which display different affinities for the pocket protein family members. Each member of the E2F family, except E2F-6, contains a domain for DNA binding, heterodimerisation and *trans*activation (Dyson, 1998; Helin, 1998). E2F's role as an S-phase promoting gene inducer is dependent on its DNA binding and *trans*activation domains. The *trans*activation domain in the E2F proteins is the region that mediates their binding to the regulatory pocket proteins. E2F-1, -2 and -3 have a high affinity for pRb in contrast, E2F-4 and E2F-5 preferentially bind to p107 and p130. The interaction of pocket proteins with the E2F or DP partners alone is week however the interaction with the heterodimer is highly stable (Bandara *et al.*, 1993; Helin *et al.*, 1993). p107/p130 E2F complexes are more evident in differentiated or quiescent cells while pRb/E2F complexes are most evident during cyclin cells, particularly at the G_1/S phase transition.

In addition to their role in the up-regulation of transcription E2F's also play a role in transcriptional repression. Promoter analysis demonstrated that during G_O and early G_1 E2F's are found on promoters in complex with pocket proteins, and that this complex is repressive (Tommasi and Pfeifer, 1995; Zwicker *et al.*, 1996; Yasuhiko *et al.*, 2000). Also the mutational analysis of a number of E2F responsive promoters, such as those encoding B-Myb, E2F-1, E2F-2 and cyclin E, demonstrated that E2F's absence actually leads to an increase in transcription (Dalton, 1992; Lam and Watson, 1993; Geng *et al.*, 1996; Helin, 1998). It therefore appears that the primary function

of E2F on certain promoters is to negatively regulate transcription, potentially by acting as a pocket protein recruiter.

As well as pRb's role in E2F mediated transcription it can also negatively regulate the Ets-family transcription factor members, Elf-1 and PU.1 and additionally through a RCE region (retinoblastoma control element) in the c-fos promoter can repress TK expression (Kim *et al.*, 1991; Pietenpol *et al.*, 1991; Hagemeier *et al.*, 1993; Wang *et al.*, 1993).

An interesting feature of pRb is its ability to interact with the transcription apparatus associated factor $TF_{II}D$ via the $TAF_{II}250$ component (Shao *et al.*, 1995; Shao *et al.*, 1997). pRb interacts with $TAF_{II}250$ through multiple domains and the interaction inhibits $TAF_{II}250$ kinase activity but not its cell cycle regulatory HAT activity (Siegert and Robbins, 1999; Dunphy *et al.*, 2000). The ability of pRb to inhibit $TAF_{II}250$ enzymatic kinase activity points to an additional mechanism by which pRb can regulate the cell cycle by directly modulating the functions of the basal transcription machinery.

The ability of viral proteins such as SV40 large T antigen and adenoviral E1A to transform cells is to some extent dependent on their ability to overcome the growth suppressive activities of pRb (Hu *et al.*, 1990; Bandara and La Thangue, 1991; Chellappan *et al.*, 1991; Zamanian and La Thangue, 1992). The binding of pRb by viral proteins results in the appearance of free E2F and an increase in E2F transcriptional activity (Hu *et al.*, 1990; Vousden, 1995). Interestingly E1A can also

stimulate the binding of pRb to the transcriptional co-activator p300, but as yet the functional significance of the pRb/p300 interaction is unknown (Wang *et al.*, 1995a).

1-4.4. pRb regulation of E2F.

pRb in a hypophosphorylated form binds to E2F in a DNA bound context to regulate E2F transcriptional activity primarily during the G_O and G_1 stages of the cell cycle. The binding of pRb to E2F inactivates E2F *trans*activation, which prevents gene expression and cell cycle progression. Cyclin/cdk complexes release pRb repression of E2F, liberate free E2F and promote cell cycle progression (Figure 1.7b). Notably, pRb residues that are required for its binding to E2F and consequent inhibition of *trans*activation are frequently found mutated in human tumours (La Thangue, 1994).

E2F's transcriptional activity is potentially regulated in a number of ways by pRb (Dyson, 1998). The most direct way that pRb regulates E2F transcription is through its ability to bind and block the function of the transcriptional activation domain of E2F (Weintraub *et al.*, 1995). The pRb in the pRb/E2F complex also inhibits the recruitment of the transcriptional initiation complex by E2F (Ross *et al.*, 1999). In addition pRb can recruit enzymes that inhibit E2F's transcriptional activation. pRb binds to the histone deacetylase family of enzymes (HDAC's) which catalyses the deacetylation of histones and condensation of the nucleosome that leads to transcriptional repression (Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*,1998; Brehm and Kouzarides, 1999).

The ability of pRb to bind the chromosome remodelling complex Swi/Snf is another potential mechanism by which it represses E2F mediated transcription (Zhang *et al.*,

2000). Interestingly the pRb/HDAC complex is also bound by the Swi/Snf complex, which presents an interesting model in which it is the component of the pRb complex and not simply pRb that mediates its repressive activity of target genes. In agreement the pRb/Swi/Snf complex can repress cyclin A and cdc2 gene expression but not cyclin E or E2F-1 expression (Zhang *et al.*, 2000). Furthermore pRb has a potential role in the recruitment of a co-repressor complex through its ability to bind RbAp48 and RbAp46. RbAp48 and RbAp46 are components of the SIN3 co-repressor complex (Hassig *et al.*, 1997; Hassig and Schrieber, 1997)

The pocket proteins are also able to regulate the level of E2F mediated transcription by indirectly effecting the level of E2F protein (Dyson, 1998). Un-phosphorylated pRb protects E2F from ubiquitin mediated SCF like proteolysis and thereby maintains a steady state level of E2F (Hateboer *et al.*, 1996; Dyson, 1998; Marti *et al.*, 1999).

Interestingly, pRb also regulates the apoptotic function of E2F. Specifically E2F-1, and not the other E2F family members, when overexpressed in a $p53^{-/-}$ or $p53^{+/+}$ background can induce apoptosis (Qin *et al.*, 1994; Hsieh *et al.*, 1997; Phillips *et al.*, 1997). The mechanism of E2F-1 induced apoptosis at least in part occurs through the death receptor pathway and involves the inactivation of anti-apoptotic signals such as NF-κB (Phillips *et al.*, 1999). More specifically, E2F-1 stimulates $p14/19^{ARF}$ expression that in turn releases MDM2's suppression of p53 apoptotic function (Sharpless and DePinho, 1998).

The cellular properties of E2F are dependent on its localisation, given that its nuclear localisation is vital for transcriptional activation. The localisation of E2F into the nucleus is in part controlled by its DP partner molecule and is cell cycle regulated (de La Luna *et al.*, 1996; Magae *et al.*, 1996; Linderman *et al.*, 1997). Conceivably the expression of the DP component may be vital in transcriptional control.

1-4.5. Transcriptional activation and pRb.

Under certain cellular circumstances pRb has actually been shown to augment the transcriptional activation of certain transcription factors (Sellers and Kaelin, 1996). pRb's role as an activator has been linked with ATF2's transcriptional activation of the TGF-β2 and pRb promoters (Kim *et al.*, 1992). NF-IL6 in co-operation with pRb shows an increase in DNA binding affinity and transcription directed from the Il-6 promoter (Chen *et al.*, 1996b). The pocket region of pRb has also been shown to mediate an interaction with c-Jun, and this interaction stimulates c-Jun transcriptional activity (Need *et al.*, 1998). Interestingly the complexes between pRb and c-Jun are only visible in terminally differentiated cells and those re-entering the cell cycle after serum starvation (Need *et al.*, 1998).

pRb also binds to the potential transcriptional co-activators and nucleosome remodelers BRM and BRG1 to activate promoters containing glucocorticoid responsive elements (Dunaief *et al.*, 1994; Singh *et al.*, 1995). The exact role of pRb in these interactions is not fully understood but potentially it may involve the stabilisation of the protein DNA binding interface (Sellers and Kaelin, 1996)

The ability of pRb to positively regulate transcription depends, in most cell systems, on the integrity of the pRb pocket. Given that the pocket region of pRb is frequently mutated in human tumours it is conceivable that pRb's up-regulation of transcription is also required for its suppression of tumourigenesis (Sellers and Kaelin, 1996).

1-4.6. pRb phosphorylation.

The cell cycle repressive activity of pRb is directly regulated by its phosphorylation status (Mittnacht, 1998). In the active repressive state, such as in G_O, pRb is Un- or hypophosphorylated and during cell cycle progression pRb is successively phosphorylated to become inactive. The phosphorylation of pRb is a highly orchestrated process and corresponds with an increase in the expression of cyclins and a up-regulation of cyclin dependent kinase (cdk's) activity (Hatakeyama *et al.*, 1994; Lundberg and Weinberg, 1998). Dephosphorylation of pRb is carried out by phosphatases such as PP-1 and occurs in mitosis (Mittnacht, 1998) (Figure 1.7b).

pRb is phosphorylated on a number of potential S/T-P motifs throughout its sequence (Figure 1.7a). Interestingly, the *in vivo* phosphorylation of pRb by individual cyclin/cdk complexes helps confer the specific functions of pRb. Cell cycle progression and loss of pRb's repressive function involves the sequential activation of cyclin D/cdk4, cyclin D/cdk6, cyclin E/cdk2 and cyclin A/cdk2 complex during late G_1 and S phases (Hatakeyama *et al.*, 1994; Sherr and Roberts, 1995; Pines, 1995; Whyte, 1995; Lundberg and Weinberg, 1998) (Figure 1.7b). The phosphorylation of pRb during G_1 in response to mitogenic signals involves the activation of cyclin D/cdk4 and cyclinD/cdk6. The peak in cyclin E observed at the G_1/S phase transition

corresponds to a hyperphosphorylated and non-repressive inactive pRb molecule (Sherr and Roberts, 1995) (Figure 1.7b).

pRb's phosphorylation controls the repressive activity of pRb by disrupting its tertiary structure and therefore its ability to interact with its partner molecules such as HDAC's and E2F (Knusden and Wang, 1996; Vivette *et al.*, 1999). In particular the C-terminus of pRb is believed to interfere with the interaction capacity of the pocket region of pRb through a lysine rich patch (Harbour *et al.*, 1999). Several studies have demonstrated the existence of pRb/E2F complexes in both the S-phase and G_2/M phase of the cell cycle although the functional significance of such complexes is unknown (Schwarz *et al.*, 1993)

The phosphorylation of pRb is negatively regulated by the cyclin dependent kinase inhibitors, $p15^{INK4b}$, $p16^{INK4a}$, $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ (Harper *et al.*, 1993; Serrano *et al.*, 1993; Xiong *et al.*, 1993; Sherr and Roberts, 1995; Weinberg, 1995). The expression of the cyclin dependent kinases inhibitors correlates with the repressive function of pRb and is associated with their ability to directly influence pRb phosphorylation status. The high frequency of mutation in the *INK4a* and *INK4b* gene products helps highlight pRb's role in the suppression of tumourigenesis (Sharpless and Depinho, 1998).

The cell cycle promoting molecules c-Myc and Ras also impart their functions on the pRb pathway. Over-expression of Ras and c-Myc promotes the accumulation of active cyclin/cdk complexes that coincides with a loss of $p27^{Kip1}$ function (Alevizopoulos *et al.*, 1997; Leone *et al.*, 1997). It therefore appears that the

inactivation of pRb function by growth promoting signals is an important intermediary step in cell cycle progression.

1-4.7. pRb as an anti-apoptotic protein.

E2F-1's introduction into $rb^{-/-}$ cells induces inappropriate S-phase entry and apoptosis via both p53-dependent and independent mechanisms (Qin *et al.*, 1994; Almansan *et al.*, 1995; Hsieh *et al.*, 1997; Phillips *et al.*, 1997). The induction of apoptosis by E2F-1 is overcome by the co-expression of pRb or MDM2 and is dependent on pRb ability to bind E2F-1 (Hsieh *et al.*, 1997; Wang, 1997; Loughran and La Thangue, 2000). The ability of E2F-1 to induce apoptosis is transcriptionally independent as mutant forms of E2F-1 that are devoid of the transcriptional activation domain retain the ability to drive apoptosis (Hsieh *et al.*, 1997). However the DNA binding function of E2F-1 is an absolute requirement for apoptosis (Phillips *et al.*, 1997). The regulation of E2F-1 apoptotic function by pRb is confirmed by the high levels of apoptosis observed in $rb^{-/-}$ mice tissues and by the resistance of $e2f^{-/-};rb^{-/-}$ cells to apoptotic inducing signals (Yamasaki *et al.*, 1996; Yamasaki *et al.*, 1998). In addition E1A, which functionally inactivates pRb also induces apoptosis.

Conversely pRb has also been shown to promote p53's apoptotic function by the inhibition of MDM2 function (Hsieh *et al.*, 1999). Given that the C-terminus of pRb, which binds MDM2, is cleaved by an Interleukin 1β-converting enzyme like protease (ICE-like) in response to TNF induced apoptosis, the role of pRb in apoptosis is potentially regulated by its interaction with MDM2 (Reinder *et al.*, 1996). In support, the cleavage of the C-terminus of pRb is blocked by Ced-3/ICE inhibitors that

prevent apoptosis (Bing and Dou, 1996). It is interesting to speculate that MDM2 regulates apoptosis through its ability, at least in part, to modulate pRb function.

1-4.8. Terminal differentiation and pRb.

A role for pRb in terminal differentiation was initially suspected given that $rb^{-/-}$ mice display defects in erythriod, neuronal and lens development (Mulligan and Jacks, 1998; Lipinski and Jacks, 1999). Interestingly the loss of pRb does not affect the induction of differentiation but rather its completion and termination, indicating that pRb is important in late development. The transcriptional activation of some myogenic genes by MyoD also requires the presence of pRb and in addition pRb can cooperate with C/EBP to promote adipocyte differentiation (Chen *et al.*, 1996a; Sellers and Kaelin, 1996). Given that mutations in pRb that reduce its ability to bind E2F still retain an ability to augment transcription of MyoD dependent genes it is presumed that pRb role as a tumour suppressor stems also, in part, from its role in differentiation (Gu *et al.*, 1993; Sellers *et al.*, 1998)

1-4.9. Conclusions.

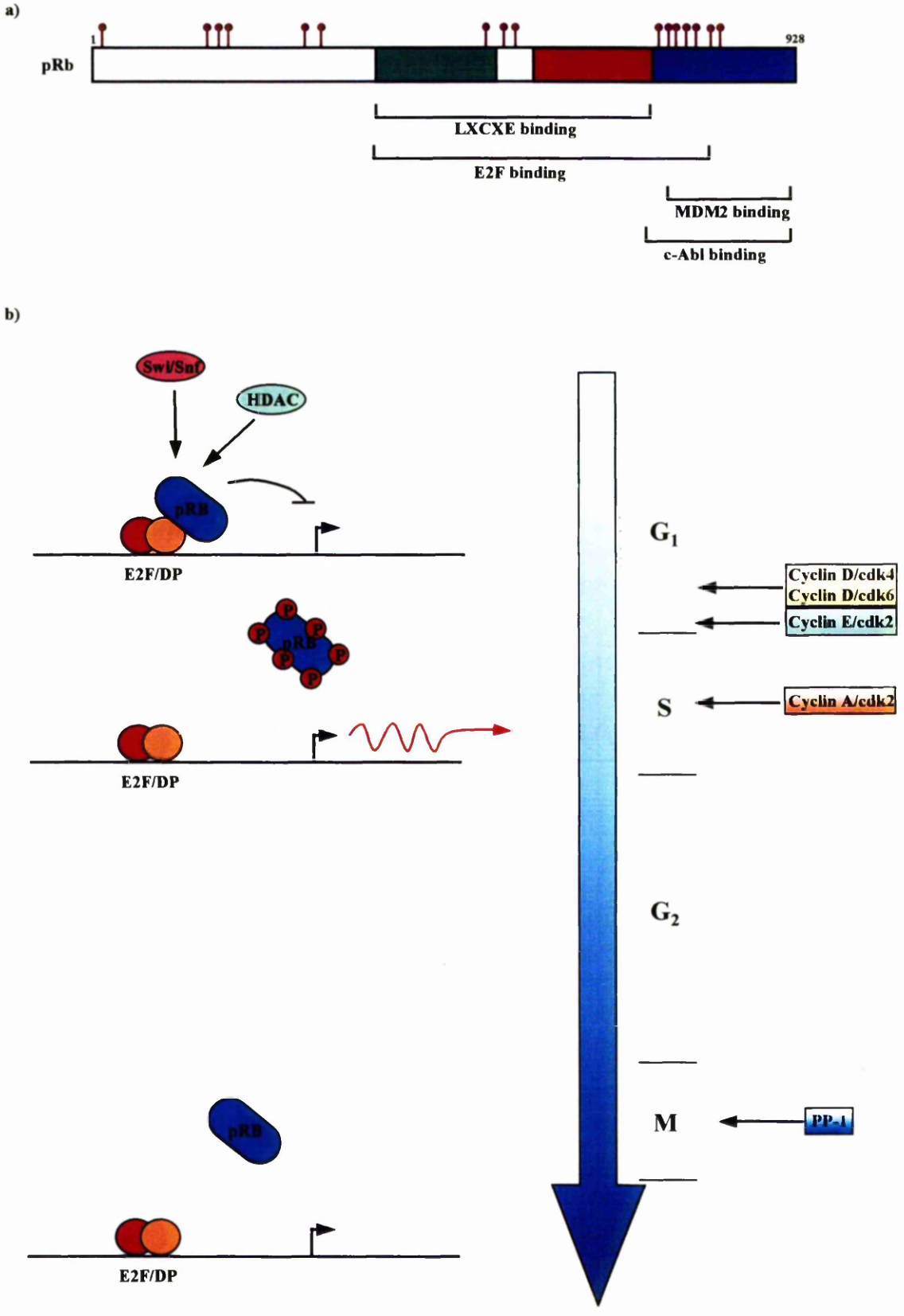
The pRb tumour suppressor is clearly a vital mediator of cell cycle control, that responds to both positive and negative signals to regulate proliferation, differentiation and apoptosis. pRb modulates the function of a number of transcription factors by nature of its ability to assemble an active repressor complex. In addition the ability of pRb to bind to chromatin modulators is potentially an important mechanism that helps initiate gene transcription.

The importance of pRb in gene transcription is underlined by its ability to regulate Pol I, Pol II and Pol III mediated transcription (La Thangue, 1994; Dyson, 1998; Sellers and Kealin, 1996). Indeed the regulation of the components of the pRb pathway are the source of numerous studies into the design of anti-proliferative drugs.

Figure 1.7. Structure and function of pRb.

a). Structure of the retinoblastoma protein (pRb). The 16 potential S/T-P phosphorylation sites are indicated together with the large pocket domain that contains the LXCXE binding motif (green and red regions). The C-terminal pocket region is also highlighted (blue). The minimal binding region required for E2F, MDM2 and c-Abl are indicated.

b). Model of pRb regulation. pRb is unphosphorylated in G_O and G_1 and forms stable complexes with the E2F/DP heterodimers. The activities of pRb are controlled at least in part by its ability to recruit histone deacetylases and chromatin remodelling complexes. In late G_1 pRb is hyperphosphorylated by cyclin/cdk complexes. hyperphosphorylated pRb is unable to associate with E2F/DP and so is unable to repress transcription. Transcription driven by E2F helps drive the cell cycle and in the M phase pRb is dephosphorylated by PP1.


a)
pRb
1
928
LXCXE binding
E2F binding
MDM2 binding
c-Abl binding
b)
Swi/Snf
HDAC
pRB
E2F/DP
P
pRB
E2F/DP
pRB
E2F/DP
G1
Cyclin D/cdk4
Cyclin D/cdk6
Cyclin E/cdk2
S
Cyclin A/cdk2
G2
M
PP-1

1-5. Objectives.

The role of co-activators and their associated molecules in the regulation of transcription is poorly understood. It is becoming evident that the co-activator complex is not simply a collection of proteins that carry out one definite function but that the exact composition of the complex determines its function. Specifically, subtle changes in the composition of the co-activator complex are likely to be responsible for dictating its promoter specific activities and are a vitally important mechanism of cellular growth control.

It is clear that the p300 transcriptional co-activator plays an important role in cell cycle control. In addition it appears likely that p300/CBP allows cross-talk between different controlling pathways in order to bring about a given physiological outcome. An important cellular target of p300's molecular action is the p53 tumour suppressor protein.

A definitive question in cell cycle research relates to the physiological role of p300 in the regulation of the p53 response and the mechanism that controls the switch between the cell cycle arrest and apoptotic functions of p53. In this respect the role of a novel protein, JMY that co-operates with p300 in the p53 apoptotic response was investigated. Of particular interest was the C-terminal proline rich region of JMY that is alternatively spliced to generate a JMY variant that selectively activates the cell cycle arrest function of p53.

The cell cycle affect of p53 is regulated by the actions of the $p14/19^{ARF}$ tumour suppressor proteins. $p14/19^{ARF}$ controls the function of the oncogene MDM2 in order to release p53 and promote a cellular effect. As JMY is involved in the p53 response another objective of this study was to determine if JMY influenced the $p14/19^{ARF}$ pathway.

Finally, an additional objective of this research was to dissect the repressive and transcriptional activating functions of pRb. Loss of pRb function is believed to be an important step in the formation of tumours and significantly pRb tumour mutants posses the ability to co-operate with JMY in the *trans*activation of p53. This suggests a novel mechanism of protection against tumourigenesis that cells have developed in circumstances when the crucial pRb growth regulatory pathway has been disabled.

Understanding the nature and function of the JMY/p300 co-activator complex response will ultimately help dissect the role of p300 in the p53 response and consequently is likely to yield important answers in the understanding of tumourigenesis.

2. Materials and Methods.

2.1. Plasmids.

The following plasmids have been previously described; pCMV-HA-JMY (Shikama *et al.*, 1998), pCMV-βgal (Zamanian and La Thangue, 1992), pCMV-p14^{ARF} (Scott *et al.*, 1998), pCMV-MDM2 (Loughran and La Thangue, 2000), Exon 1β-luc (Bates *et al.*, 1998), pBax-luc (Haupt *et al.*, 1995; Friedlander *et al.*, 1996), pWWP-luc (El-Deiry *et al.*, 1993), pGADD45-luc (Chen *et al.*, 1995), pMDM2-luc (Haupt *et al.*, 1995), pG5E1b-luc (Lee *et al.*, 1998) pCMV-p300 (Eckner *et al.*, 1996a), pCMV-JMY$^{502-983}$ (Shikama *et al.*, 1999), pCMV-JMY$^{683-983}$ (Shikama *et al.*, 1999), pCMV-JMY$^{1-504}$ (Shikama *et al.*, 1999), pCMV-JMY$^{118-403}$ (Shikama *et al.*, 1999), pCMV-CD20 (Lee *et al.*, 1998), pCMV-p53 (Lee *et al.*, 1998), pCMV-pRb (Zamanian and La Thangue, 1992), pCMV-E2F1 (Kaelin *et al.*, 1992), pCMV-JMY$^{1-403}$ (Shikama *et al.*, 1999), pCMV-RbΔ22 (Zamanian and La Thangue, 1992), pCMV-Gal4-E2F-1$^{380-437}$ (Lee *et al.*, 1998).

The following plasmids were gratefully received as gifts; pGEXKG-p14^{ARF} from Sarah Mason, pPIG3-luc and pCMV-p53ΔP from Dr Dobblestein, pCMV-HDAC-I from Dr Schreiber, pTG$_{13}$-luc from Chang-Woo Lee, pCMV-JMY^{NLS} from Laurent DeLavaine, pCMV-RB$^{372-972}$ from Ho-Man Chan and JMYΔC from Noriko Shikama. The N-terminal JMY specific antibody and polypeptide were a generous gift from Noriko Shikama. Dr N. Shikama isolated and provided the sequencing information described for the splice variant clones, JV$_2$Δ$^{766-840}$, JV$_4$FS^{+2} and JV$_6$FS^{+1B}.

To construct pCMV-HA-JMYΔP the PCR products generated by 3' RT-PCR were cloned into the pCR 2.1 vector (Invitrogen). The clone corresponding to the JMYΔP variant was digested with Kpn1 (site within JMY) and Xba1 (site from within pCR 2.1). The fragment was ligated in frame to a pCMV-2X HA-JMY$^{1-983}$ coding vector previously cut with Kpn1 and Xba1.

2.2. Transfection.

For transfections all cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% foetal calf serum (FCS) and the antibiotics streptomycin (10mg/ml) and penicillin (100U/ml). Cell cultures were maintained in a water saturated 5% CO_2 atmosphere at 37°C.

Cells were plated out 24 hours prior to transfection at $5x10^5$ for reporter assays, $1x10^6$ for flow cytometry and immuno-precipitation or $2x10^5$ for immuno-staining. Plating densities were optimized at 70% surface coverage in all cases. Three hours prior to transfection, the media was removed and replaced with fresh growth medium. In all cases calcium phosphate-DNA precipitation was used. The indicated concentrations of plasmid DNA were mixed with a 2M $CaCl_2$ solution to give a final salt concentration of 200mM in a volume appropriate to the plating density. The Solution was drop wise added, with agitation, to an equal volume of 2XHBS (50mM HEPES pH 7.1, 280mM NaCl and 1.5mM Na_2HPO_4) and the mixture incubated at room temperature for 30 minutes before addition to cells. The total volume of precipitate added was maintained at a constant ratio of 1ml total volume precipitate per $1x10^6$ cells.

Whenever required pcDNA-3, or pSG-5 backbone vector was used to maintain a constant concentration of DNA. All transfections included CMV-βgal as an internal control for transfection efficiency.

12-17 hours post transfection cells were washed three times with 37°C PBS to remove excess precipitate and fresh media added. Cells were harvested 30-40 hours post transfection.

In cases where trichostatin A was used it was added to cells in fresh growth media at a final concentration of 150nM 10 hours before harvesting. Non-treated cells were incubated with an equal volume of the solvent used to dissolve the trichostatin A (100% ethanol).

2.3. Luciferase and β-galactosidase assays.

For luciferase assays, cells were washed three times with PBS prior to lysis in 500μl reporter lysis buffer (25mM Tris-H_3PO_4 pH 7.8, 2mM 1,2 diaminocyclohexane tetra acetic acid, 2mM DTT, 10% glycerol and 1% Triton X-100). The lysed extract was centrifuged at 13000rpm for 15 minutes to remove cell debris before luciferase activity was measured. Luciferase activity was measured by the addition of 300μl of luciferase assay reagent (Promega) to 100μl cell extract in a luminometer (Berthold Lumant).

Internal control β-galactosidase activity was determined by mixing 100μl of cell extract with an equal volume of 2X β-galactosidase buffer (200mM sodium phosphate buffer pH 7.3, 2mM $MgCl_2$, 100mM β-mercaptoethanol and 1.33mg/ml O-

nitrophenyl-β-D-galactopyranoside). The reaction mixtures were incubated at 37°C until faintly yellow in colour at which point enzyme activity was quantified by measuring the optical density of the mixture at 420nm.

In cases where fold activation and fold repression were calculated the following formulas were used:-

[1] Fold activation (rel. reporter) = $\frac{\text{Activity of reporter. + Activator.}}{\text{Activity of reporter.}}$

[2] Fold repression by $p14^{ARF}$ = $\frac{\text{Activity of reporter + Activator.}}{\text{Activity of reporter + activity of activator + } p14^{ARF}}$

2.4. Immunofluorescence.

Cells transfected, on 35mm glass coverslips, were washed three times in PBS prior to fixation in a solution of 4% paraformaldehyde at room temperature for 15 minutes. Cells were then washed in PBS and permeabilised in a PBS solution containing 0.2% Triton X-100 for 10 minutes at room temperature. Fixed cells were then blocked in a 10% FCS PBS solution for 10 minutes at room temperature. Primary antibodies in a 5% FCS PBS solution were then incubated with the cells for 30 minutes at room temperature. After washing three times in a PBS 10% FCS solution the secondary antibody diluted in a PBS 5% FCS solution was added for 30 minutes at room temperature. Anti-mouse, anti-goat, or anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) was used for detection (Southern Biotechnology Associates Inc). Finally, coverslips were washed three times in PBS and once in PBS containing DAPI (4,6-Diamidino-2-

phenylindole) before mounting on slides with Citifluor (Citifluor Ltd). Immunofluorescence was viewed under a fluorescent microscope (Olympus).

In cases where cells were doubly transfected either anti-mouse, anti-goat or anti-rabbit conjugated to either fluorescein isothiocyanate or rhodamine isothiocyanate were used accordingly. Where cellular compartmentalisation was quantified the percentage of cells in each cellular localisation was defined relative to the whole population examined. Whole cell staining was defined as cells expressing equal quantities of protein in the cytoplasm and nucleus. Nucleolar staining was defined as dense bodies within the nucleus that correspond to region of highly condensed DNA.

2.5. Flow cytometry.

Flow cytometry analysis was carried out on cells transfected with the indicated amounts of plasmid DNA. In all cases cells were transfected with 5μg of CD20 expression vector and captured by monitoring the expression of the cell surface protein, CD20. After transfection cells were harvested by treatment with cell dissociation solution (Sigma) for 15 minutes at 37°C. Cell were washed in DMEM by centrifugation at 2000rpm for 3 minutes and re-suspended in 200μl of DMEM containing 20μl of a mouse anti-CD20, leu 16, antibody (Becton Dickinson) coupled to fluorescein isothiocyanate (FITC). The cell suspension was incubated on ice for 30 minutes followed by washing in ice cold PBS. Cell pellets were then fixed by the dropwise addition of a 50% ethanol/PBS solution and left overnight at 4°C.

Cells were then washed in PBS and re-suspended in 500μl of PBS containing propidium iodide (20μg/ml) and RNase (125U/ml). Flow cytometry was performed

on a Becton Dickinson fluorescence activated cell sorter. All cell populations were analyzed at a rate of 100-200 cells/second to prevent mis-read. To determine the cell cycle profiles of transfected populations the intensity of propidium iodine staining was analyzed in cell populations that were positive for FITC staining. Using CellQuest software, the cell cycle profiles of 10000 transfected cells was ascertained. The data presented show a representative example from multiple assays.

2.6. Immuno-precipitation.

Transfected cells were washed twice in PBS and harvested by scraping in TNN buffer on ice (50mM Tris-HCl pH 7.4, 120mM NaCl, 5mM EDTA, 0.5% NP-40, 50mM NaF, 1mM DTT, 1mM PMSF, 0.2 mM sodium orthovanedate, leupeptin (0.5 μg/ml), bestatin (40μg/ml), protease inhibitor (0.5μg/ml), trypsin inhibitor (1.0μg/ml) and aprotinin (0.5μg/ml)). The cell extract was centrifuged for 10 minutes at 10000rpm and pre-cleared by incubating with protein-G agarose for 30 minutes at 4°C with agitation. The supernatant was harvested and incubated at 4^0C with agitation for 1 hour after the addition of 4μl of primary antibody. The reaction was continued for a further 30 minutes at 4°C following the addition of 50μl of protein-A agarose (50w/v slurry). The agarose beads were collected by centrifugation for 30 seconds at 5000rpm and were subsequently washed three times in TNN reaction buffer. Bound proteins were released into 2X SDS loading buffer (250mM Tris-HCl pH 6.8, 20% glycerol (v/v), 4% SDS (w/v), 0.1% bromophenol blue (w/v), 200mM DTT, and 5% β-mercaptoethanol). The sample was denatured and separated by SDS-PAGE and the protein of interest detected by Western blot using a specific antibody.

2.7. Apoptosis assays.

Transfected SAOS-2 cells were washed and grown in DMEM supplemented with 0.2% FCS, streptomycin 10mg/ml and penicillin 100U/ml for 17 hours before analysis. Transfected populations of cells were washed and fixed in a 4% paraformaldehyde solution for 15 minutes at room temperature, rinsed thoroughly and permeabilised in a PBS solution containing 0.1 % Trition X-100 and 0.1% sodium citrate for 5 minutes. Cells were then incubated with primary antibody for 30 minutes in order to assay transfected cells. Cells were then incubated in a Ca^{2+} reaction buffer containing fluorescein-dUTP, dNTP and terminal deoxynucleotidyl transferase at 37°C for one hour. In addition a secondary antibody was included that was tetramethyl rhodamine isothiocyanate (TRITC) labeled in order to allow the visualization of transfected cells. Following incubation cells were washed three times in PBS and once in PBS containing DAPI (2μg/ml) mounted and viewed under a fluorescent microscope (Olympus).

Apoptotic cells were counted in transfected staining cells in order to ensure a correlation between transfection and apoptosis. Transfected cells were visualized under a red filter and apoptotic cells under a green filter. At least 1000 transfected cells were counted in order to obtain representative data. Background apoptosis was determined by TUNEL assay alone in the whole population. Percentage stimulation was calculated relative to the level of apoptosis in p53 transfected cells.

2.8. Gluthione S-tranferase recombinant proteins.

BL21(DE3)pLysS (Promega) bacterial cultures transformed with the plasmid of interest were grown to a mid-logarithmic stage in LB-broth (Sigma). Protein

expression was induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) at a final concentration of 0.5mM for 4 hours at 37°C. During protein induction the protease inhibitor PMSF was added to the cultures every 30 minutes at a final concentration of 0.5mM. Bacterial pellets re-suspended in 10ml's of PBS containing 1% Triton X-100, 0.5 mM PMSF and 50mM DTT were sonicated on ice for two 30 second bursts. Bacterial debris was pelletted by centrifugation and re-centrifugation at 12000 rpm for 30 minutes at 4°C. 250µl of a 50% v/v suspension of gluthathione-agarose beads were added to the supernatant and the mixture incubated, with rotation, at 4°C for one hour. The suspension was washed twice with PBS containing 0.5% Np-40 and once with PBS by centrifugation at 4000rpm for 5 minutes at 4°C. Beads were stored at 4°C in PBS-azide and protein expression monitored by coomassie staining after SDS-PAGE.

2.9. *In Vitro* protein expression.

In vitro protein transcription and translation was carried out using a TNT T7 coupled Reticulocyte lysate system (Promega). Luciferase and MDM2 were expressed as recommended by the manufacturer. All the JMY constructs were expressed to an optimized reaction condition. Briefly; 30µl of rabbit reticulocyte lysate, 3µl reaction buffer, 3µl T7 RNA polymerase and 2µl of RNase inhibitor were added to 1µg of purified plasmid DNA. Expression was initiated by the addition of 6µl amino acids minus methionine mixture and 4 µl of ^{35}S labeled amino acid mixture (Amersham). The reaction volume was maintained at 50µl by the addition of water and allowed to proceed at 30°C for 3 hours. Protein expression was examined by loading the indicated amounts of crude inputs onto a SDS-PAGE gel and the level of ^{35}S signal examined by autoradiography.

2.10. Western blot analysis.

Protein expression levels were measured by immobilization of crude extract onto nitrocellulose membrane (Inverclyde Biochemicals) followed by detection with specific antibodies. Specific goat, mouse or rabbit primary antibodies were used for detection of the protein of interest and the signal amplified either by the addition of a specific horseradish peroxidase conjugated secondary antibody (1;7500) or a calf alkaline phosphatase conjugated secondary antibody (1;5000). Detection was achieved using substrate tablets for alkaline phosphatase (Sigma Fast™ 5-Bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium) or enhanced chemiluminescent (ECL) for horseradish peroxidase (Pierce).

2.11. *In vitro* binding assays.

In vitro binding reactions were carried out by using approximately 10μg of GST or GST fusion protein bound to glutathione agarose beads (Amersham Inc). Beads were added to equal quantities of *in vitro* translated product. The reaction was carried out in a constant 200μl volume of reaction buffer (100mM Tris-HCl pH 8.0, 150mM $NaCl_2$, 0.5% Np-40, 1mM PMSF, 1mM DTT and a recommended dilution of protease inhibitor cocktail). After incubation at 4°C for 3 hours the beads were collected and washed four times in reaction buffer and once in PBS. Proteins were released in SDS-sample buffer, electrophoresed and the assay monitored by autoradiography. In all cases equal loading was confirmed by coomassie staining of GST and GST fusion proteins.

2.12. RNA isolation.

Whole cell F9 mRNA was isolated from asynchronously growing cell cultures. Cell cultures were lysed in RNA extraction buffer (100mM NaCl, 1.5mM $MgCl_2$, 10mM Tris-HCl pH 8.6, 0.5% Np-40, 1mM DTT, 1000 U RNasin, 2% SDS, 25mM EDTA). Proteinase K was then added at a final concentration of 200μg/ml and the reaction left at 37°C for 1 hour. Cellular RNA was then extracted using phenol:chloroform. DNA digestion was completed by DNAase I (final concentration 2μg/ml) treatment of the RNA at 37°C for 1 hour. RNA was then extracted by phenol:chloroform treatment and the RNA was ethanol precipitated and stored at –80°C in TE.

2.13. RT-PCR.

RT-PCR was carried out on whole cell RNA isolated from F9 cells or on RNA kindly provided by Dr Shikama. Whole cell RNA was isolated as described and subjected to 3' RT-PCR using a specific oligonucleotide directed against a non-coding RNA sequence of the C-terminal JMY message.

F9 cellular mRNA (500ng) was incubated at 90°C for 4 minutes with 50ρmol of specific oligonucleotide (O_2) primer directed against the 3' non-coding sequence of JMY as determined by Dr Shikama. The primer sequence was $^{+3271}$5'-CTCTGCCAACCCAGTGTTCTTCC-3'$^{+3249}$. The reaction was cooled on ice and AMV reverse transcriptase (Avian myeloblastosis virus) added (5U) together with DTT to a final concentration of 10mM, RNase inhibitor (5U), 0.5mM final concentration of dNTP mixture, and 1X reaction buffer (50mM Tris-HCl pH 8.3, 50mM KCl, 10mM $MgCl_2$, 0.5mM Spermidine, 10mM DTT) to give a final volume of 50μl. cDNA synthesis was allowed to proceed at 45°C for 1 hour. The cDNA was

then ethanol precipitated and stored in TE (10mM Tris-HCl pH 8.0, 1mM EDTA) at –80°C.

2.14. cDNA amplification (PCR).

Primer pairs of 3' JMY specific sequence were used to amplify the cDNA signal. The wild-type JMY sequence is encoded by bases 1-+2949 and the oligonucleotide sequences and positions in base pairs from the first ATG in the wild-type JMY coding sequence are indicated. The primer pairs used were;

PCR $T_M = 68°C$

O_1 $^{+2096}$5'-AGAGCAGAGGACACTGGATAGAC-3'$^{+2119}$

O_2 $^{+3271}$5'-CTCTGCCAACCCAGTGTTCTTCC-3'$^{+3249}$

The PCR reactions were carried out using $^{G}/_{C}$ rich *Taq* DNA polymerase (5U) (Clontech). PCR was performed by the addition of 1:10th of the cDNA form the RT-PCR reaction to a solution containing a 0.2mM dNTP mixture, 1X reaction buffer, 100pmol of each 5' and 3' specific primer pair, 5U $^{G}/_{C}$ rich *taq* DNA polymerase to give a final working volume of 20µl. Standard PCR reaction cycles was performed using the stated annealing temperatures of the oligonucleotide pairs.

Nested primers from sequences within the predicted PCR products were then used to further amplify DNA species isolated from the 3' of JMY. The nested primer pair used were;

PCR $T_M = 68°C$

O_1 $^{+2096}$5'-AGAGCAGAGGACACTGGATAGAC-3'$^{+2119}$

O_3 $^{+3112}$5'-TAGTAACCTCCCGTTTGTGCTTCC-3'$^{+3089}$

PCR was carried out as for the initial amplification only 10% of the first PCR reaction was used as template. PCR reactions were carried out as standard and the oligonucleotide annealing temperature are indicated. PCR reactions (5% v/v) were cloned into the A/T topo cloning vector system (Novagen).

2.15. Hybridisation screening.

In order to detect JMY specific clones from 3' RT-PCR the A/T cloning reaction was probed using a ^{32}P-α-GTP radio labeled oligonucleotide probe. The probe sequence was (O_4) $^{+2259}$5'-GAACAACTTGAATCC-3'$^{+2273}$. Ligation reactions were transformed into XL1 blue *E. coli* and colonies transferred to nitocellulose filters (Hybound), lysed and DNA fixed by UV crosslinking. Filters were then examined by hybridisation.

Hybridisation was carried out overnight at 40°C in Denhardts solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin) containing 6XSSC (900mM NaCl, 90mM sodium citrate pH 7.2) and 0.4% SDS. Non specific binding was removed by washing filters in a 6XSSC, 0.2% SDS solution three times for 30 minutes at 45°C. Filters were examined by autoradiography.

3. Isoforms of JMY.

3.1. Introduction.

Eukaryotic transcription directed by RNA polymerase I, II, and III produces RNA that is composed of both coding and non-coding sequences. The protein coding sequences of genes, called exons, are spatially jumbled by non-coding intron sequences that range in size depending on the gene. The processing of the intron and exon sequences is known as splicing, and leads to the construction of transcripts which encode physiologically expressed proteins.

The product of gene transcription, the precursor mRNA molecule, is composed of both intron and exon sequences. Precursor mRNA is structurally bound by a large protein complex, the spliceosome, which contains of a variety of small ribonucleo-proteins, called snRNPs (small nuclear ribonucleoproteins). snRNP protein complexes are associated with a number of stable RNA molecules designated U1 to U12. snRNP's specifically function in the recognition of specific donor and acceptor sites in the precursor mRNA molecule from where they promote the formation of the spliceosome. snRNP's induce RNA strand breaks in the precursor mRNA and promote exon joining. Specifically the spliceosome uses ATP energy to carry out the accurate removal of introns (Anraku, 1997; Hartel *et al.*, 1997).

The splicing of mRNA is an intricate process dependent on many molecular events and if these events are not carried out with precision then functional coding mRNA is not produced. Consequently numerous studies have demonstrated that genetic mutations at exon and intron boundaries leads to the production of mutant non-

functional proteins that in turn disrupt the normal pattern of cell physiology (Philips and Cooper, 2000).

A major evolutionary reason for the existence of splicing is the diversity of protein expression it allows. Alternative splicing allows the production of functionally distinct members of the same protein from the same gene to generate genetic flexibility in the cell. Interestingly, many of the key cell cycle regulatory proteins appear to undergo extensive splicing which increases their diversity of function.

3.2. Isolation and characteristics of JMY.

Using a yeast two-hybrid approach with pLexA-p300$^{611-2284}$ as bait a new p300 binding protein, JMY was isolated (Junction-mediating and regulatory protein) (Shikama *et al.*, 1999). JMY is a 110kDa proteins that lacks any significant similarity to other known proteins. The protein sequence possesses a number of interesting features, including a central region that resembles a motif in the adenovirus E1A protein, a C-terminal domain rich in proline residues and an N-terminal region that contains a number of potential S/T-P phosphorylation sites (Figure 3.1a and 3.1b).

JMY is a particularly interesting protein given that its gene, *jmy*, is located on chromosome 5 in band 5q 13.2. Interestingly, the long arm of chromosome 5 is often disrupted in a wide range of malignancies, in particular, leukaemia (Shikama *et al.*, 1999). Potentially, the disruption of *jmy* may be a prerequisite or modifier of tumour formation, and consequently its function may be vital in the maintenance of an archetypal cell. However to date no specific deletion in the mRNA of JMY has been detected in tumour cells (Shikama, personal communication).

Two transcripts, a 9.5kb and less abundant 6kb species, encode the JMY message. Both transcripts are expressed in a wide variety of mouse tissues apart from testis where a smaller 4kb transcript exists (Shikama *et al.*, 1999).

3.3. 3' splicing of JMY.

As JMY is potentially a target of inactivation in malignancies it was of interest to determine if the transcripts encoding JMY are subject to alternative splicing. The analysis of the C-terminus of JMY lead to the observation that JMY was extensively alternatively spliced in the 3' region. Indeed endogenous protein expression analysis carried out in SAOS-2 cells confirmed the presence of multiple forms of JMY protein (Shikama, personal communication).

Using RNA derived from F9 embryonal carcinoma cells, a number of 3' splicing variant transcripts of JMY were isolated and examined. An RT-PCR approach was used to amplify mRNA from a region of JMY encompassing the C-terminus. Reverse transcription was used to synthesis cDNA, from a primer directed from a previously identified 3' untranslated region of the *jmy* gene. Nested PCR, using oligonucleotides directed against the C-terminal sequence of JMY, was then performed to allow detection of JMY specific C-terminal encoded sequences (Figure 3.2a). After multiple rounds of PCR specific JMY cDNA's were isolated, by A/T cloning, and their base sequence analysed and compared to wild-type JMY coding sequence (Figure 3.2b). Given this strategy, no variant could be isolated before the aspartic acid (D) residue at position 650 (Figure 3.1b).

Sequence comparisons, of the 3' isolated sequences with the wild-type JMY sequence, demonstrated not only that JMY was potentially extensively spliced but also that the splicing sites, donor and acceptor, were diverse (Figure 3.3a). Of the potential 3' splice variants examined, three resulted from frame shifts in the reading frame of wild-type sequence. Interestingly no wild-type JMY sequence was detected in the amplified mRNA splice sequences, although its expression in F9 cells can not be excluded.

Notably, the detection of multiple forms of JMY 3'RNA species from F9 cells although suggestive of splicing in the JMY message dose not confirm such a conclusion. Potentially the relevance of such RNA species to the endogenous RNA population is merely suggestive and as such is a consideration when interpreting the following results. Herein the isolated RNA species and their potential protein products are refereed to as either splicing variants or isoforms although their endogenous presence requires conformation by RNA protection assays.

3.4. Splice junction of JMY

Sequence analysis of a population of JMY 3' mRNA variants allowed the recognition of a number of potential splice junctions. The JMYΔP isoform lacks 18 residues located in the C-terminal region, from residue 795 to 813, which in the wild-type protein contains a high proportion of proline residues (83%) (Figure 3.3b). JMYΔP is derived as a consequence of splicing bases encoding amino acid 794P to 814P and remarkably possesses an almost perfect deletion of the proline rich domain (Figure 3.3ai and 3.3ci). The in frame splicing of the JMY message also results in the generation of two other isoforms that are devoid of the proline rich domain, $JV_2\Delta^{766-}$

^{840} and $JV_5\Delta^{740\text{-}827}$. The $JV_2\Delta^{766\text{-}840}$ variant is generated by splicing the bases encoding amino acid 765A (Alanine) to bases for 841G (Glycine) and the $JV_5\Delta^{740\text{-}827}$ isoform by splicing bases encoding amino acid 739T (Threonine) to those encoding 828T (Threonine) (Figure 3.3aii, cii and 3.3av, cv).

In addition to the in frame splicing events detected, three other splicing variants were isolated that were generated as a consequence of a change in the reading frame of the wild-type JMY message. A splice in the JMY message from the bases encoding amino acid 759T (Threonine) to the bases of amino acid 981W (Tryptophan) generated a C-terminally deleted JMY variant, JV_4FS^{+2}. In comparison to the wild-type JMY message the reading frame of JV_4FS^{+2} is altered by +2 bases at the amino acid encoding 981W (Tryptophan). This results in an isoform that is deleted in the C-terminus of JMY but contains additional sequence encoded for by the 3' un-translated region (Figure 3.3a iv and 3.3c iv).

The JV_3FS^{+1A} variant was generated by splicing the bases encoding amino acid 765A (Alanine) to those encoding 852A (Alanine). The splicing event results in a +1 change in the 3' reading frame relative to wild-type JMY that generates an internal stop codon (Figure 3.3avi and 3.3cvi). The JV_6FS^{+1B} splicing isoform is terminated in the same reading frame as JV_3FS^{+1A} but the splice sites are different. JV_6FS^{+1B} is generated by splicing the bases encoding amino acid 739T (Threonine) to those encoding 844R (Arginine) and is read in the +1 reading frame relative to wild-type JMY (Figure 3.3aiii, 3.3ciii).

3.5. Conclusions.

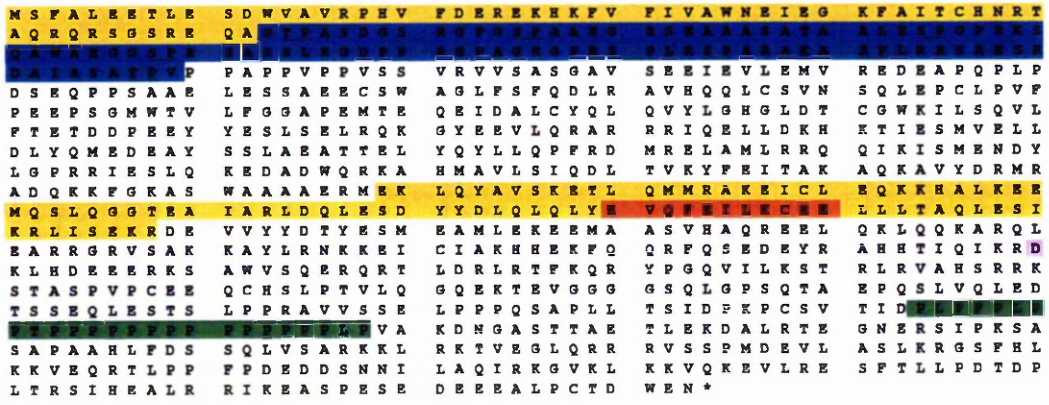
The complexity of 3' JMY mRNA splicing is undoubtedly an important mechanism that regulates the function of JMY. Indeed given the selective removal of the 3' proline rich region of JMY it is conceivable that the region is a major controlling domain in JMY function. The complexity of JMY is also further highlighted by the isolation of other JMY related family members, although their function is as yet unknown (Shikama, personal communication). Clearly the family status and extensive splicing of JMY point to its role as a highly regulated cellular molecule.

Figure 3.1. Characteristics of JMY.

a). The sequence of the 983 amino acid protein JMY. The S/T-P motifs in the N-terminus (blue) are underlined, and the p300 binding domains from amino acids 1-119 and 469-558 are highlighted (yellow). The adenovirus E1A CR2 like motif, **E**VQF**E**IL**K**C**E**E is indicated (red). The proline rich region is highlighted in green and the first potentially detectable 3' splice amino acid, 650D (Aspartic acid), is shown (pink).

b). Domain structure of JMY. The N- and C-terminal p300 binding domains are depicted (yellow), together with the N-terminal S/T-P rich region (blue), the E1A like motif (red) and the C-terminal proline rich domain (green). The 3' region studied for alternative splicing is indicated.

a)



b)

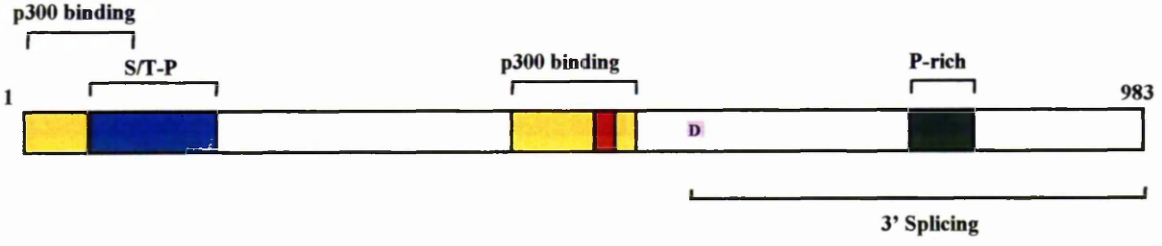


Figure 3.2. Isolation of 3' splicing variants of JMY.

a). Diagrammatic representation of JMY. Indicated are the N-terminal and central p300 binding domains (yellow), the N-terminal S/T-P rich region (blue) and the C-Terminal proline rich domain (green). The oligonucleotide used for cDNA synthesis is indicated (O_2) together with the nested oligonucleotides used for the amplification of cDNA (O_1, O_2 and O_3). Also indicated is the position of the oligonucleotide used for colony screening (O_4).

b). The products from the PCR reactions generated in 3.2a were loaded on an agarose gel and DNA levels examined by ethidium Bromide staining. PCR reactions were ligated into the Invitrogen A/T cloning vector, pCR 2.1, and the presence of positive JMY clones confirmed by probe hybridisation with ^{32}p-α-labelled olionucleotide 4.

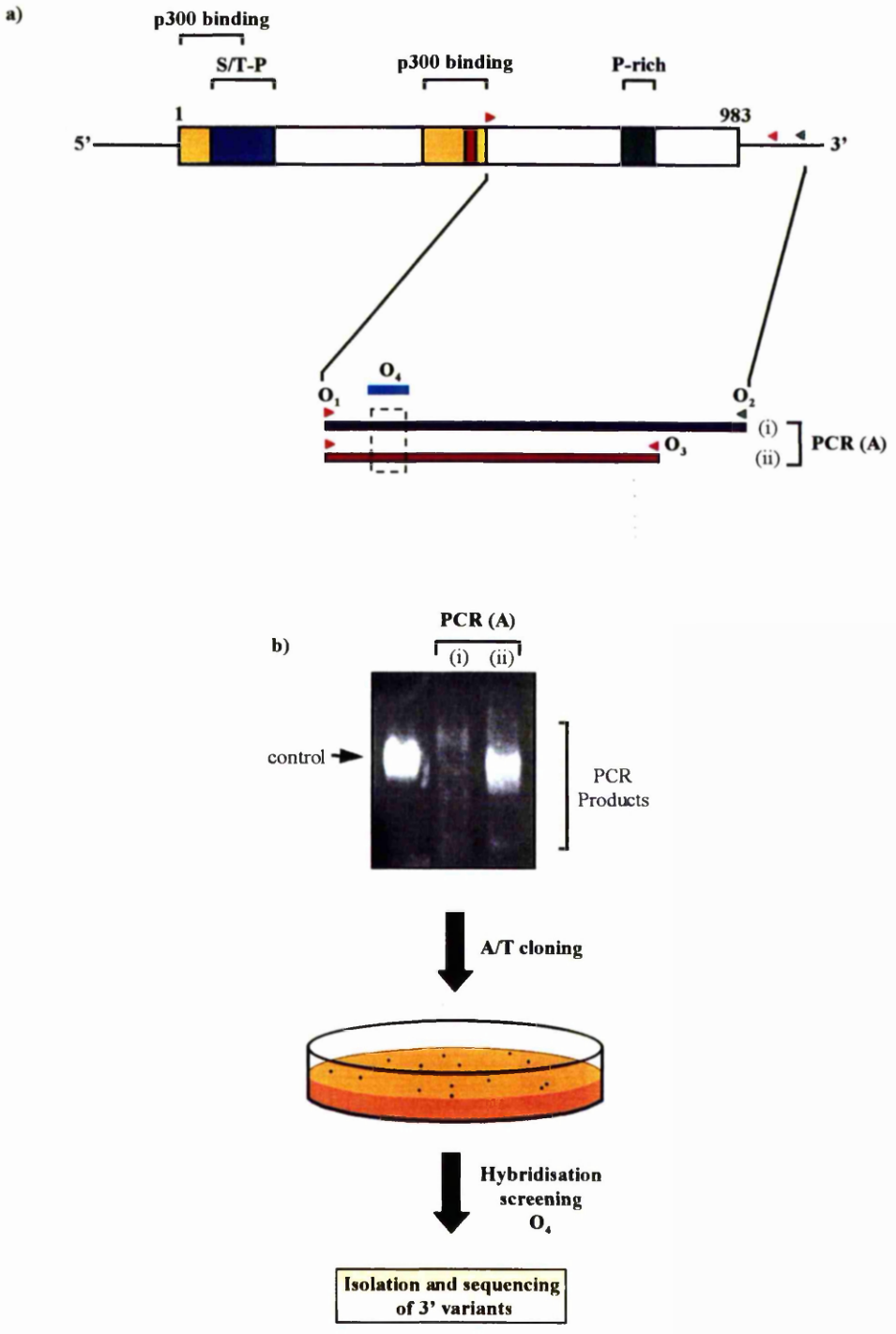

a)
p300 binding
S/T-P
p300 binding
P-rich
1
983
5'
3'
O4
O1
O2
O3
(i)
(ii)
PCR (A)
b)
PCR (A)
(i)
(ii)
control
PCR
Products
A/T cloning
Hybridisation
screening
O4
Isolation and sequencing
of 3' variants

Figure 3.3. JMY 3' splice variants.

a). Diagrammatic representation of JMY. Highlighted are the 3' splice variants isolated from F9 embryonic carcinoma cells. i) The JMYΔP variant that encompasses a splice event that deletes a proline rich region in the C-terminus of JMY (84%). Variant ii), iii), iv), v) and vi) are a representation of other 3' splicing events that give rise to 3' spliced JMY construct.

b). The variants shown in 3.3a all commonly lack the proline rich region from amino acids 794 to 812 of wild-type JMY. The sequence of the region is indicated.

c). Amino acid comparisons of the 3' splicing variants depicted in 3.3a. The wild-type JMY sequence form amino acids 721G (Glycine) to 983N (Asparagine) is indicated. The splicing events are indicated -:

i) JMYΔP. ^{794}P ^{814}P

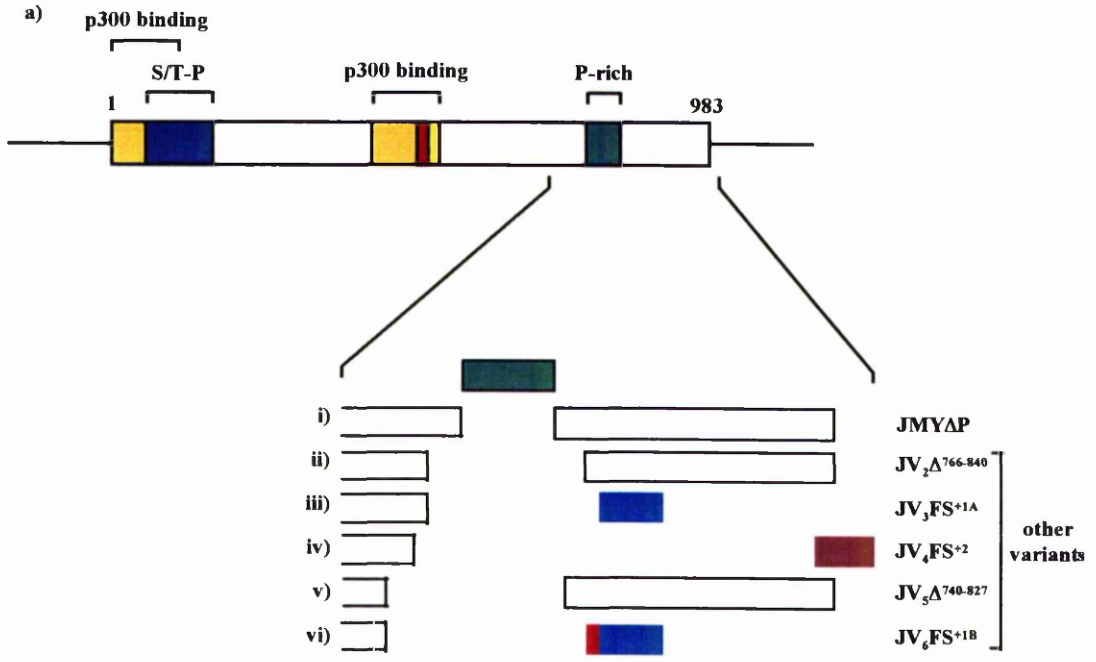
ii) $JV_2\Delta^{766\text{-}840}$. ^{765}A ^{841}G

iii) JV_3FS^{+1A}. ^{765}A ^{852}A ↓ AAG-ACT

iv) JV_4FS^{+2}. ^{759}T ^{981}W ↓ TGG-GAG

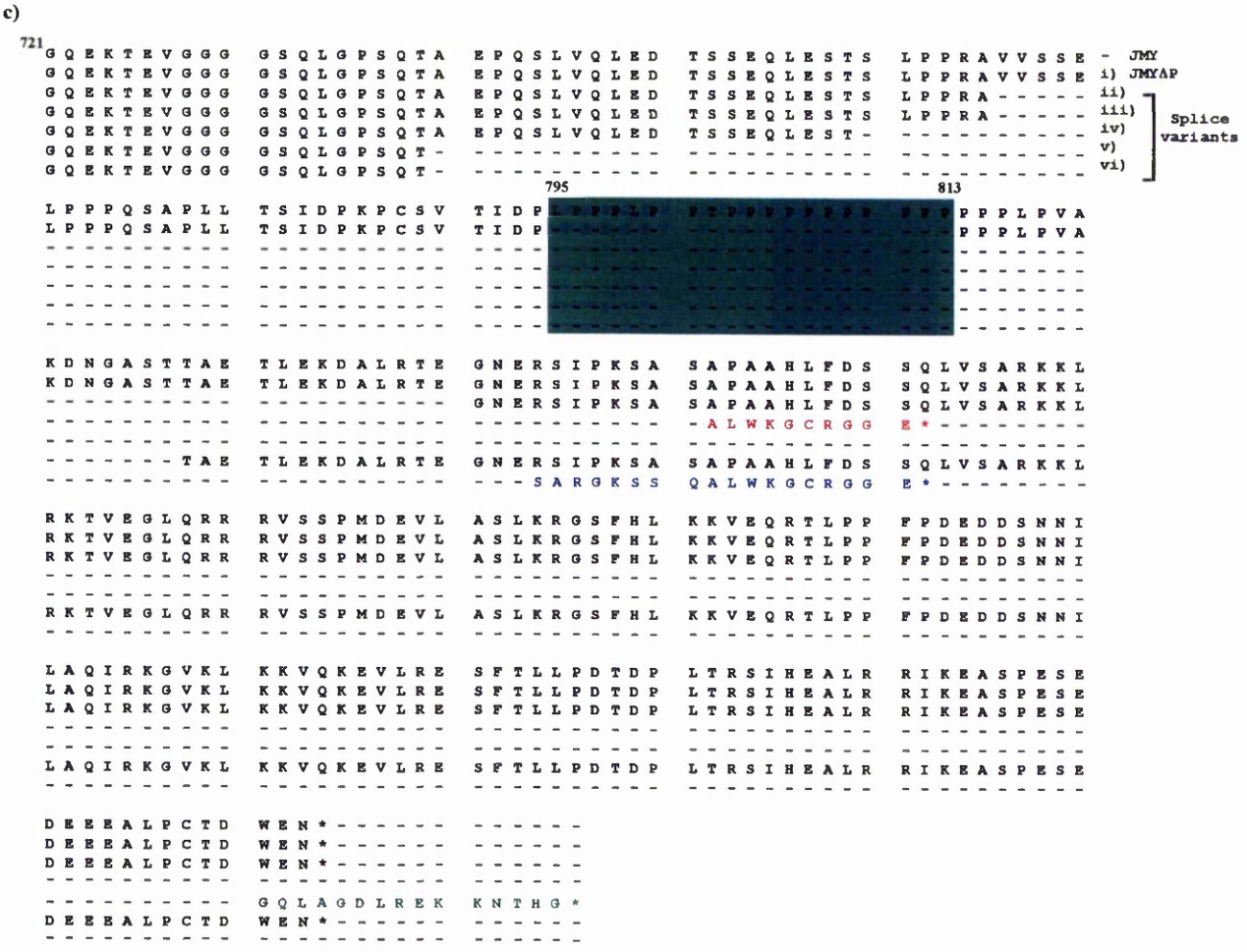
v) $JV_5\Delta^{740\text{-}827}$. ^{739}T ^{828}T

vi) JV_6FS^{+1B}. ^{739}T ^{844}R ↓ CTC-AGC



b) 5'-L-P-P-P-L-P-P-T-P-P-P-P-P-P-P-P-P-P-P-3'

c)



4. JMY, a new co-activator of the p53 response.

4.1. Introduction.

The p300/CBP transcriptional co-activator family regulates the cell cycle by co-ordinating the actions of transcription factors. The key cell regulatory transcription factor p53 is one such target of p300/CBP function (Avantaggiata *et al.*, 1997; Gu *et al.*, 1997; Lill *et al.*, 1997a). p300/CBP facilitates the transcription of p53 mediated gene expression to augment the p53 response.

The inactivation of the p53 pathway is a prerequisite for the formation of tumour cells and the maintenance of viral infections. p53's ability to stall genomic replication allows the repair of genetic lesions that maintains a healthy cell (Levine, 1997). In circumstances where the genetic shock is too severe for recovery, p53 can induce cell death (Gottlieb and Oren, 1996). The controlling mechanisms that discriminate between p53's role in cell cycle arrest and apoptosis are currently not well understood.

The ability of p300/CBP to co-operate with the p53 response provides a mechanistic control point for the p53 pathway. In addition to p300/CBP itself a number of molecules exist in complex with p300/CBP but their function in the regulation of p53 is unknown. Given that JMY is a p300/CBP associated molecule that has an unknown function it is of clear interest to elucidate the role, if any, of JMY in the p300/CBP-p53 molecular pathway.

4.2. JMY binds p300 *in vivo*.

Previous studies have suggested that p300/CBP forms a multi-meric complex *in vivo* and that the composition of the complex is ultimately responsible for its function (Eckner *et al.*, 1996b; Roeder, 1996; Chen *et al.*, 1997; Grossman *et al.*, 1998; Korzus *et al.*, 1998; Xu *et al.*, 1999). Consistent with the isolation of JMY as a p300 binding protein it was demonstrated that JMY contained at least two independent p300-binding domains (Shikama *et al.*, 1999) (Figure 4.1a). In addition p300 itself interacts with JMY through two separate binding domains (Shikama *et al.*, 1999) (Figure 4.1b). Shikama *et al.* (1999) also demonstrated that JMY and p300 directly interact *in vitro*. The ability of JMY, the natural splice JMYΔP and a C-terminal truncation mutant, JMYΔC, to bind p300 *in vivo* was therefore of interest and was assayed by immuno-precipitation.

Anti-HA immuno-precipitates from SAOS-2 cells overexpressing HA-JMY, HA-JMYΔP or HA-JMYΔC all contained a p300 specific component (Figure 4.1c). HA-JMY and HA-JMYΔP immuno-precipitates contained an equivalent level of p300 while HA-JMYΔC showed a reduction in the level of *in vivo* complexed p300. The ability of endogenous p300 to be immuno-precipitated with JMY clearly demonstrates that the two proteins exist in an *in vivo* complex and that physiological 3' splicing of JMY in relation to the proline rich domain does not effect this interaction. Given that deletion of the C-terminus of JMY reduces the level of *in vivo* bound p300, even through the region is outside the previously identified binding domains, it is conceivable that JMY's interaction with p300 is in part mediated by its C-terminus (Shikama *et al.*, 1999).

4.3. JMY co-activates p53 transcription.

Given that p300 and CBP are physiological co-activators of p53 and that this co-activation leads to the enhancement of p53's cellular function it was of interest to determine JMY's ability to influence p53 transcriptional activation function (Avantaggiati *et al.*, 1997; Gu *et al.*, 1997; Lill *et al.*, 1997a; Lee *et al.*, 1998). As previously shown p53 efficiently activates expression from the *bax*, *mdm2*, *waf1* and *gadd*45 promoters (Figure 4.2) (Lee *et al.*, 1998). The *bax*, *mdm2*, *gadd45* and *WWP* reporters consist of promoter regions, that contain p53 DNA binding consensus sites, fused upstream of a luciferase reporter gene (El-Deiry *et al.*, 1993; Chen *et al.*, 1995; Haupt *et al.*, 1995).

The *bax* promoter, consistent with its role in p53 mediated apoptosis, is activated upon the introduction of exogenous p53 expression plasmid into SAOS-2 cells and this transcription is in turn co-activated by the co-expression of HA-JMY (Miyashita *et al.*, 1994a; Miyashita and Reed, 1995). JMY induces a two-fold stimulation of p53 mediated *bax* promoter driven transcription (Figure 4.2a and 4.3a). Intriguingly the natural splice, JMYΔP, was unable to co-activate p53 driven transcription of the *bax* gene even through it retains p300 binding properties (Figure 4.2a). The JMYΔC mutant was unable to significantly co-stimulate p53 mediated transcription of the *bax* promoter (Figure 4.3a). As JMY is unable to bind p53 directly it was assumed that endogenous p300 was acting as a bridging molecule in JMY's co-activation of transcription (Shikama, personal communication). The reporter activity in the absence of p53 was unaffected by JMY, JMYΔP or JMYΔC indicating that JMY's ability to act as a co-activator was specific for p53 in the context of *bax*.

Consistent with the C-terminus of JMY being important for p53 transcriptional co-activation and p300 binding was the observation that a C-terminal JMY deletion mutant, JMYΔ$^{403-983}$, possessed dominant negative activity (Shikama *et al.*, 1999). The JMYΔ$^{403-983}$ mutant retains the N-terminal p300 binding domain and S/T-P rich cluster but has lost the central p300 binding domain and C-terminal region. The level of transcriptional activity by p53 was significantly compromised by the co-expression of JMYΔ$^{403-983}$, presumably as a result of its ability to interfere with the endogenous JMY/p300 and p53/p300 interactions (Figure 4.3a). Implying that endogenous JMY is required for p53 mediated transcription.

An N-terminal JMY deleted mutant that binds p300 *in vitro*, JMYΔ$^{1-502}$, still retained the ability to transcriptionally co-activation p53, although at a level significantly reduced relative to wild-type (Figure 4.3a). Implying that the C-terminus of JMY, at least in part, confers its ability to act as a transcriptional co-activator (Shikama *et al.*, 1999). Together these results suggest that JMY's ability to effectively co-activate p53 mediated transcription requires not only its p300 binding capacity but also its C-terminal region. Furthermore, it appears that the integrity of the C-terminus of JMY may influence co-activation as a result of its role in p300 binding.

JMY's ability to stimulate p53 mediated transcription in the context of G_1 arrest was examined using the cell cycle arrest associated promoters taken from the *gadd45* and *waf1* genes. The co-expression of JMY with p53 only minimally stimulated expression from the *WWP* and *gadd45* promoters above the level of p53 alone (Figure 4.2c and 4.2d). The lack of substantial co-activation can be attributed to the

responsiveness of the reporter constructs to the addition of p53 alone. Interestingly, although subtle, JMYΔP was repeatedly more active than JMY in the co-activation of p53's *trans*activation of the *WWP* promoter (Figure 4.2c). JMYΔP co-activation of p53-dependent transcription, driven from the *gadd45* promoter, was equivalent to wild-type JMY which was repeatedly similar to p53 alone (Figure 4.2d).

In order to examine further the enhanced activity of JMYΔP in *WWP* co-activation the artificial TG_{13}-luc reporter, that consists of thirteen p53 consensus sites taken from the $p21^{Waf1/Cip1}$ promoter fused upstream of a luciferase reporter gene, was studied (Lee *et al.*, 1998). p53 mediated transcription from the TG_{13}-luc promoter was co-stimulated efficiently by JMYΔP but JMY did not alter the level of p53 mediated transcription (Figure 4.3b). Given these findings it is possible that the proline rich domain of JMY, that is selectively spliced, functions as a promoter specific p53 co-activator domain.

The p53 regulated promoter taken from the *mdm2* gene was also marginally co-activated by the expression of JMY and JMYΔP in the presence of p53 (Figure 4.2b). It is of interest to note that transcription from the TG_{13}, *mdm2*, *WWP* and *gadd45* promoters was p53-dependent as JMY and JMYΔP did not alter the activity of the reporters constructs in the absence of p53 (data not shown and Figure 4.3b).

Together these results suggest that JMY is a physiological p53 co-activator, presumably as a consequence of its association with and recruitment by p300. The inability of JMYΔP to co-activate p53 mediated transcription driven from the *bax* promoter implies that 3' splicing of JMY produces functionally distinct variants. The

loss of the proline rich domain confers promoter specificity on JMY as only the *bax* and *WWP* promoters were affected. Indeed the entire C-terminus of JMY appears to influence JMY's ability to act as a transcription co-activator as some p53 promoters show a high level of co-activation by JMYΔC while others are compromised (Figure 4.4a and 4.12d), further strengthening the hypothesis that the C-terminus of JMY confers p53's transcriptional promoter specific properties.

Given that JMY's ability to co-activate p53 transcription presumably stems from its capacity to bind p300 it was of interest to determine if JMY and p300 would co-operate in the activation of p53 mediated transcription. The *bax*-luciferase promoter construct was activated by the addition of p53 and as previously shown p53 mediated transcription was co-activated by the addition of exogenous p300 (Lee *et al.*, 1998; Shikama *et al.*, 1999). JMY effectively co-activated p53-dependent transcription from the *bax*-luciferase reporter and transcription was further enhanced by the addition of p300 (Figure 4.4a). JMY and p300 therefore effectively co-operated in the co-activation of p53's transcription. JMYΔP was unable to co-activate the *bax* reporter and consequently did not significantly co-operate with p300 in p53 mediated transcription (Figure 4.4a). Interestingly, p300's ability to stimulate p53 mediated transcription was unaffected in the presence of JMYΔP which suggests that JMYΔP does not dominate p300's function as a co-activator for p53 in the context of apoptosis (Figure 4.4a).

As p53 mediated transcription is activated following its post-translational modification and stabilisation it was of significance to determine if JMY affected p53 stability. The addition of HA-JMY, HA-JMYΔP and HA-JMYΔC into SAOS-2 cells

along with p53 did not effect the expression level of p53 or p53's stability (Figure 4.4b). The lack of p53 stabilisation points to JMY acting as a co-activator and not post-translational modifier of p53.

In transient reporter assays JMY acts as a co-activator that helps stimulate p300/p53 mediated transcription. Significantly the 3' splicing of JMY appears to play a role in the promoter specificity of p53 mediated transcription and presumably p53's role in cell cycle arrest and apoptosis. It is clear that the p300/CBP *in vivo* complex components, of which JMY is one, enhance the transcriptional co-activator and hence cellular consequences of p300/CBP action.

4.4. JMY co-activates endogenous gene expression.

Given that p53 stimulates the transcription of artificial promoters *in vivo* and that JMY acts as a co-activator of p53 transcription it was of interest to determine if JMY would stimulate the transcription of endogenous genes.

SAOS-2 cells, which lack p53, when transfected with p53 begin to express both the upper and lower forms of MDM2 (Figure 4.5a). Consistent with JMY's role as a co-activator, the co-expression of JMY and p53 stimulated the level, although only slightly, of MDM2 expression compared to that seen with p53 alone (Figure 4.5aiii). The co-activator stimulation of MDM2 protein expression mirrored the reporter gene activation studies. Similarly, the JMYΔP 3' splice variant behaved as wild-type JMY in the co-activation of MDM2 expression (Figure 4.5aiii). The activation of MDM2 expression was p53-dependent as the over-expression of JMY or JMYΔP in the absence of exogenous p53 did not stimulate MDM2 expression. Surprisingly the

stimulation of MDM2 expression by JMY/p53 did not lead to a reduction in the level of p53 (Figure 4.5aii). The stability of p53 in the presence of high levels of MDM2 may be explained by the sensitivity and transient nature of the experiment or may a consequence of JMY influencing MDM2's function.

Given that JMY showed a preference over JMYΔP in the co-activation of p53 transcription from of the *bax* promoter it was of significance to determine the level of endogenous Bax expression. Cells transfected with p53 expressed an elevated level of Bax protein and JMY co-stimulated this transcription (Figure 4.5b). Interestingly and consistent with the inability of JMYΔP to activate p53 mediated transcription of the *bax* promoter construct, no increase in p53's induction of Bax protein was detected after the co-transfection of SAOS-2 cell with p53 and JMYΔP (Figure 4.5b). Bax protein induced expression by JMY is p53-dependent, as the over-expression of JMY or JMYΔP did not induce the co-activation of Bax expression in the absence of p53 (Figure 4.5b).

Strikingly, and in agreement with the reporter gene assays, the transfection and over-expression of JMYΔP in U2OS cells, which express endogenous p53, increased the level of $p21^{Waf1/Cip1}$ expressed (Figure 4.5c). Both JMY and JMYΔC however were unable to increase $p21^{Waf1/Cip1}$ protein levels (Figure 4.5c). These results confirm that 3' splicing of JMY bestows promoter specific properties on JMY. In addition it appears that JMY's role as a co-activator of p53-mediated transcription is gene specific as some genes display a higher degree of co-activation than others (Figure 4.2 and 4.5).

4.5. p53 translocates JMY to the nucleus.

The ability of p53 to act as a transcription factor is associated with its nuclear localisation. Nuclear localisation of p53 is dependent on its inherent nuclear localisation signal (NLS) as well as its interaction with MDM2 (Kubbutat *et al.*, 1997; Haupt *et al.*, 1997; Roth *et al.*, 1998). Given that JMY appears to participate in the p53 transcriptional response it was of significance to determine if JMY cellular localisation corresponded to its ability to act as a p53 transcriptional co-activator.

Initially in order to address JMY sub-cellar localisation a polycolonal antibody raised against the N-terminus of JMY was used in the detection of the endogenous JMY population. In non-transfected SAOS-2 cells fluorescent staining established that in the majority of cells JMY was localised throughout the cell (Figure 4.6ai and 4.6aii). Interestingly a small population of cells displayed exclusively nuclear or cytoplasmic staining for JMY.

As the localisation of the 3' splicing variant, JMYΔP, could not be observed or discriminated directly using the N-terminal antibody, cells were transfected with HA tagged JMY variants prior to immuno-florescence. SAOS-2 cells were transfected with expression plasmids for HA-JMY, HA-JMYΔP or HA-JMYΔC. HA-JMY cellular localisation mirrored that observed with the JMY polyclonal antibody as the largest population of cells stained simultaneously both nuclear and cytoplasmic (Figure 4.6bi and 4.6bii). Again a population of cells displayed exclusively cytoplasmic or nuclear staining. Under the same cellular conditions HA-JMY-ΔP and HA-JMYΔC displayed a similar staining pattern as wild-type JMY with the majority of cells staining whole cell (Figure 4.6ci, 4.6cii, 4.6di and 4.6dii). In embryonic

fibroblast taken from *p53*$^{-/-}$ and *p53*$^{-/-}$;*mdm2*$^{-/-}$ mice an identical staining pattern as for SAOS-2 cells was observed for HA-JMY, HA-JMYΔP and HA-JMYΔC (data not shown).

The cellular localisation of JMY in SAOS-2 cells implied that JMY's localisation was regulated. Interestingly, a large population of endogenous JMY must be in the transcriptionally dormant cellular compartment, the cytoplasm. Given that JMY activates p53 mediated transcription and that nuclear localisation is required for transcriptional co-activation it was of interest to determine if p53 affected JMY's cellular localisation. To this end SAOS-2 cells were co-transfected with p53 and either HA-JMY, HA-JMYΔP or HA-JMYΔC. The over-expression of p53 in SAOS-2 cells resulted in its almost exclusively, nuclear localisation (Figure 4.7). Presumably cells overexpressing p53 that displayed weak cytoplasmic staining were undergoing p53 degradation as a consequence of MDM2's over-expression.

The over-expression of HA-JMY and p53 lead to the observation that JMY localisation was altered in the presence of p53. The previously observed cytoplasmic population of JMY was replaced by a predominantly nuclear population (Figure 4.7a). In an identical manner the subcellular localisation of JMYΔP and JMYΔC was also altered, becoming nuclear in the presence of p53 (Figure 4.7b and 4.7c). Given that p53 does not alter the expression level of JMY it is conceivable that p53 traffics or stimulates the trafficking of JMY from the cytoplasm to the nucleus. The ability of p53 to stimulate the translocation of JMY from the cytoplasm to the nucleus presumably contributes to JMY's ability to stimulate p53 mediated transcription. Given that SAOS-2 cells contain endogenous p300 it is arguable that the translocation

of JMY is through a p300 mediated mechanism. Interestingly p300's intrinsic HAT activity appears to regulate nuclear import and export although in the context of JMY this mechanism of action has not been explored to date (Bannister *et al.*, 2000).

Although the shift in JMY's cellular localisation in the presence of p53 was reproducible, in a set of assays the extent of change in JMY's localisation in the presence of p53 was not uniform. It therefore appears that a feasible conclusion is that the JMY/p53 co-staining pattern is a cell cycle regulated event, although further studies are required to confirm such a hypothesis.

A N-terminal nuclear localisation signal (NLS) tagged JMY protein that is constitutively nuclear is less efficient than wild-type JMY in the co-activation of p53 mediated transcription (Figure 4.3a). This suggests that it is not JMY's nuclear localisation that is important for its function as a co-activator but the signals that trigger its localisation. In addition, p53 is not the only trigger for JMY's nuclear localisation as a population of endogenous and overexpressed JMY is exclusively nuclear in the absence of p53 (Figure 4.6 and 5.7).

4.6. JMY is present in a DNA damage induced p53 complex.

The induction of p53 stabilisation by DNA damage stimulates cell cycle arrest and promotes apoptosis (Levine, 1997) (Figure 4.8a). DNA damage affects cells by increasing the transcription of target genes that control the cell cycle and apoptotic pathways (Figure 4.8a). As JMY participates in the activation of p53 mediated transcription it was of interest to determine if JMY was found complexed with p53 *in vivo* under conditions of p53 activation.

Previously both JMY and p300 were shown not to be DNA damage-inducible genes (Shikama *et al.*, 1999). p53 is stabilised and activated by the treatment of cells with the DNA damaging agent actinomycin D. In order to study the composition of the JMY complex under damaging conditions U2OS cells treated with actinomycin D were examined for JMY binding polypeptides. As expected p53 was effectively induced following the DNA damage of U2OS cells (Figure 4.8b). Given that JMY stimulates p53 mediated transcription it was of interest to determine if p53 was present in the JMY complex following DNA damage. To this end both untreated and treated U2OS cells were immuno-precipitated with an anti-JMY N-terminal, polycolonal antibody and the level of p53 determined by western blot. As expected from the immuno-staining data, the JMY complex in asynchronous U20S cells contains p53 (Figure 4.8b). Interestingly, and in agreement with previously published data, DNA damaged U2OS cells contain a significantly higher level of p53 in the JMY immuno-complex (Shikama *et al.*, 1999) (Figure 4.8b). Furthermore the importance of the JMY/p53 *in vivo* complex is emphasised by the observation that quantitatively the JMY complex appears to contain the majority of the endogenous p53.

Given that JMY stimulates p53 mediated transcription it is conceivable the high level of p53 in the JMY complex under DNA damaging conditions corresponds to a p53 mediated cell cycle checkpoint arrest or apoptotic event that is associated with transcriptional up-regulation. As the level of JMY is unaffected following DNA damage it is possible that the increase in the level of p53 found in the JMY complex

corresponds to an activated complex. Clearly JMY is a component of the DNA damage response of p53.

4.7. JMY's role in p53 mediated cell cycle arrest.

The ability of p53 to induce a G_1 arrest phenotype as a result of $p21^{Waf1/Cip1}$ expression is well documented and understood (El-Diery *et al.*, 1993; Gu *et al.*, 1993). As JMY co-localises with p53, is present in the DNA damage induced p53 complex and is able to co-stimulate the transcription of $p21^{Waf1/Cip1}$ it was of interest to determine the effect if any of JMY on p53's role as a checkpoint protein.

To this end, SAOS-2 cells were transiently transfected with HA-JMY, HA-JMYΔP or HA-JMYΔC either alone or in combination with p53. Transfected populations were selected using CD20 staining and DNA content and cell cycle stage were assayed using propidium iodide (Figure 4.9).

As expected, p53's introduction into SAOS-2 cells caused an increase in the G_1 population of cells (Figure 4.9b and 4.10a). Under the conditions of the transient assay no detectable G_2/M arrest was observed in p53 overexpressing cells. The over-expression of JMY alone did not affect the cell cycle suggesting that JMY plays a dormant role in the unstressed cell (Figure 4.9c and 4.10b). However in the presence of p53, JMY stimulated an increase in the population of G_1 arrested cells by almost 2 fold relative to p53 alone (Figure 4.9d and 4.10b). The increase in G_1 arrest presumable correlates with an increase in p53 mediated transcription.

Given that JMY did not alter the level of the endogenous $p21^{Waf1/Cip1}$ protein but JMYΔP did, it was of merit to determine if 3' splicing could affect p53's ability to arrest cells. JMYΔP like wild-type JMY did not affect the cell cycle but in combination with p53, JMYΔP produced a striking increase in the G_1 population of cells relative to p53 alone (Figure 4.9e, 4.9f and 4.10c). JMYΔP stimulated a four-fold increase in the p53 mediated G_1 arrest population.

The over-expression of JMYΔC alone resulted in an increase in the G_1 population of cells (Figure 4.9g and 4.10d). In combination with p53, JMYΔC increased the G_1 population of cells but the p53 and JMYΔC cell cycle arrest phenotype was additive and so non-synergistic effects can not be ruled out (Figure 4.9h and 4.10d). Conceivably JMYΔC may be adversely effecting the normal cellular processes that mediates a p53 response and indirectly effecting the cell cycle profile.

Together these results suggest that JMY functions in mediating a p53 G_1 arrest, but more significantly point to 3' splicing of JMY as an important regulatory mechanism of p53 function. JMYΔP clearly has an enhanced ability over JMY in the stimulation of p53 mediated cell cycle arrest.

4.8. JMY regulates p53-dependent apoptosis.

In addition to its role in cell cycle arrest, p53 functions as an apoptotic promoting gene product (Gottlieb and Oren, 1996; Levine, 1997). As JMY is present in an activated p53 DNA damage induced complex it is conceivable that JMY may be involved in p53's ability to stimulate apoptosis. In addition JMY stimulates

transcription of the apoptotic promoting gene product Bax in a p53-dependent manner.

In order to study JMY's ability to regulate the p53 apoptotic responses cells were assayed for the characteristic, apoptotic, DNA fragmentation phenotype. Fluorescent labelled nucleotide incorporation into DNA was visualised directly by immuno-fluorescence using the TUNEL assay. As expected the transient transfection of p53 into SAOS-2 cells in conditions conducive to apoptosis, namely low serum, stimulated an apoptotic response (Figure 4.11). Populations of cells overexpressing p53 showed a 30% increase in the level of apoptosis relative to those not expressing p53. The over-expression of HA-JMY and HA-JMYΔP slightly induced apoptosis in the absence of p53 by 5.8% and 3.7% respectively (Figure 4.11). More interestingly, the simultaneous over-expression of HA-JMY and p53 dramatically increased the population of apoptotic cells in comparison to p53 alone (Figure 4.11). The over-expression of JMY and p53 induced a 41% stimulation in the level of apoptosis compared to p53 alone. However the level of apoptosis in JMYΔP and p53 overexpressing cells remained at levels equivalent to p53 alone (Figure 4.11). The over-expression of JMYΔC produced an apoptotic phenotype identical to that of JMYΔP in the presence of p53 (Simms, personal communication).

The ability of JMY to stimulate p53 dependent apoptosis clearly demonstrates that JMY can enhance p53 induced cell death. Interestingly, the inability of JMYΔP to stimulate p53 mediated apoptosis but its ability to enhance cell cycle arrest supports an attractive hypothesis. The possibility that 3' splicing of JMY generates functionally distinct species of JMY that confer specificity on the p53 response

supports an attractive mechanism for controlling the switch between cell cycle arrest and apoptosis.

4.9. Transcription and proline rich domains.

Although Bax is a well characterised gene involved in the p53 apoptotic response a number of studies have demonstrated that Bax expression is dispensable for p53 mediated apoptosis (Knudson *et al.*, 1995). The ability of p53 to stimulate the expression of the *PIG3* gene may correlate with the induction of p53 mediated apoptosis (Polyak *et al.*, 1996). A vitally important domain in p53 that mediates *PIG3* expression is the N-terminal proline rich region. The polyproline domain of p53 is essential for PIG3 expression but dispensable for MDM2, $p21^{Waf1/Cip1}$ and Bax expression (Walker and Levine, 1996; Venot *et al.*, 1998). In order to confirm that JMY is participating in p53's induction of cell death by co-activating the transcription of genes involved in apoptosis the transcription of *PIG3* was examined.

In transient transfection assays p53 effectively activated the *PIG3*-luciferase reporter construct in a dose-dependent fashion (Venot *et al.*, 1998) (Figure 4.12a). As previously shown the deletion of the polyproline domain of p53 resulted in the loss of transcriptional activation (Figure 4.12a). Furthermore, as expected, the co-expression of JMY with p53 co-stimulated p53 directed expression from the *PIG3* promoter and the transcriptional co-activation was dependent on the p53 polyproline domain (Figure 4.12b). JMYΔP's ability to stimulate the transcription of the *PIG3* promoter, as for *bax*, was severely compromised relative to JMY (Figure 4.12c). JMYΔC ability to stimulate p53 mediated transcription was also compromised relative to wild-type JMY (Figure 4.12d). Interestingly the C-terminal deletion of JMY still retained a

degree of transcriptional co-activation, indicating that the C-terminus of JMY is not absolutely essential for co-activation but a contributor. The inability of p53ΔP to *trans*activate the *PIG3* promoter was unaffected by JMY, JMYΔP or JMYΔC (Figure 4.12).

Taken together these results support the observation that JMY plays a role in p53 mediated apoptosis by promoting gene expression. Furthermore they support the observation that 3' splicing of JMY determines the switch between the cell cycle arrest and apoptotic functions of p53.

Previously the polyproline domain of p53 was shown to be dispensable for the transcription of MDM2 and $p21^{Waf1/Cip1}$. In agreement with these observation p53ΔP *trans*activated the *WWP* promoter at levels equivalent to wild-type p53 (Figure 4.13a). Both JMY and JMYΔP co-activated p53ΔP *trans*activation of the *pWWP* reporter at level equivalent to those for co-activation of wild-type p53 (Figure 4.13a). Significantly and consistent with earlier results JMYΔP was reproducibly more active than JMY in the co-activation of either p53 or p53ΔP *trans*activation of pWWP-luciferase. Indeed JMYΔP's co-operation with p53ΔP was repeatedly greater that that seen with wild-type p53, further strengthening the role of proline rich domains in cell cycle arrest. The *mdm2* promoter construct showed no transcriptional activation by p53ΔP however the addition of JMY or JMYΔP resulted in a co-activation of expression that was independent of the presence of the proline rich domain (Figure 4.13b). The co-activation of p53ΔP *mdm2* promoter driven expression by JMY and JMYΔP was equivalent to that seen with wild-type p53 (Figure 4.13b).

Mechanistically, the polyproline domain of p53 may be a regulated region that controls the function of p53 whether it be apoptosis or cell cycle arrest. Clearly the proline rich domain in JMY performs a similar function. Given that JMYΔP and p53ΔP are able to co-operate in the induction of *WWP* expression it is conceivable that proline rich domains in transcriptional regulatory proteins may mediate cell cycle arrest. The separation of cell cycle arrest and apoptotic phenotypes by proline rich domains may be the determining event in cell death or cell survival. The proline rich domains in p53 and JMY clearly perform similar synergistic functions in inducing cell death.

4.10. Conclusions.

The p53 protein utilises p300/CBP's co-activation properties in order to transcriptionally activate gene expression (Avantaggiati *et al.*, 1997; Lill *et al.*, 1997a; Gu *et al.*, 1997; Lee *et al.*, 1998). The results presented here suggest that JMY as a consequence of its association with p300 plays an important role in dictating p53 mediated transcription. Given that co-activators complexes are known to play an important role in regulating eukaryotic transcription it is of significance to note that JMY appears to specifically enhance p53 ability to induce apoptosis. JMY specifically can upregulate p53's *trans*activation of apoptotic promoting gene products such as Bax and PIG3.

Isoforms of JMY appear to distinguish between the cell cycle arrest and apoptotic properties of p53. The 3' splicing events in JMY that specifically remove the highly proline rich domain compromise JMY's ability to induce p53 mediated apoptosis. However JMYΔP in combination with p53 is able to increase the cell cycle arrest

phenotype induced by p53 mediated transcription. Potentially, therefore, the expression of a particular array of JMY isoforms could alter the characteristics of the p53 response via their ability to modulate gene specific transcription (Figure 4.14).

The study presented here also implies that JMY and its isoforms are themselves regulated proteins. Indeed p53 itself is able to stimulate the translocation of JMY and its isoforms into the nucleus of cells.

Figure 4.1. JMY interacts with p300.

a). Functional Domains of JMY.

Diagrammatic representation of JMY, JMYΔP and a C-terminal truncation mutant JMYΔC. The previously mapped p300 binding domains are indicated (in yellow) at positions JMY^{1-119} and $JMY^{469-558}$ (Shikama *et al.*, 1999). The N-terminus contains a cluster of potential phosphorylation sites for S/T-P directed kinases (blue) and the central region contains a motif that resembles conserved region 2 (CR2) in the adenovirus E1A protein (red) (**EVQFEILXCEE**). The C-terminal proline rich region from amino acids 776-785 is highlighted (green).

b). Domains in p300.

Diagrammatic representation of p300 illustrating the cysteine/histidine rich domains (red), CH1, CH2, and CH3 together with the central Bromodomain (grey). The previously described binding regions for JMY from amino acids 611 to 1257 and 1572 to 2283 are indicated (Shikama *et al.*, 1999). The amino acid residue numbers indicate region boundaries.

c). JMY, JMYΔP and JMYΔC interact with p300 *in vivo*.

SAOS-2 cells were transiently transfected with either a vector encoding HA-JMY (5μg), HA-JMYΔP (5μg) or HA-JMYΔC (5μg). Cell extracts were subjected to immuno-precipitation with mouse anti-HA antibody and the immuno-precipitates were blotted with a N-terminal anti-p300 specific antibody. A specific 300kDa polypeptide corresponding to p300 was detected in the immuno-precipitates of all the JMY transfected cells. No p300 binding was detected in empty vector (pcDNA3HA) transfected cells (lane 1).

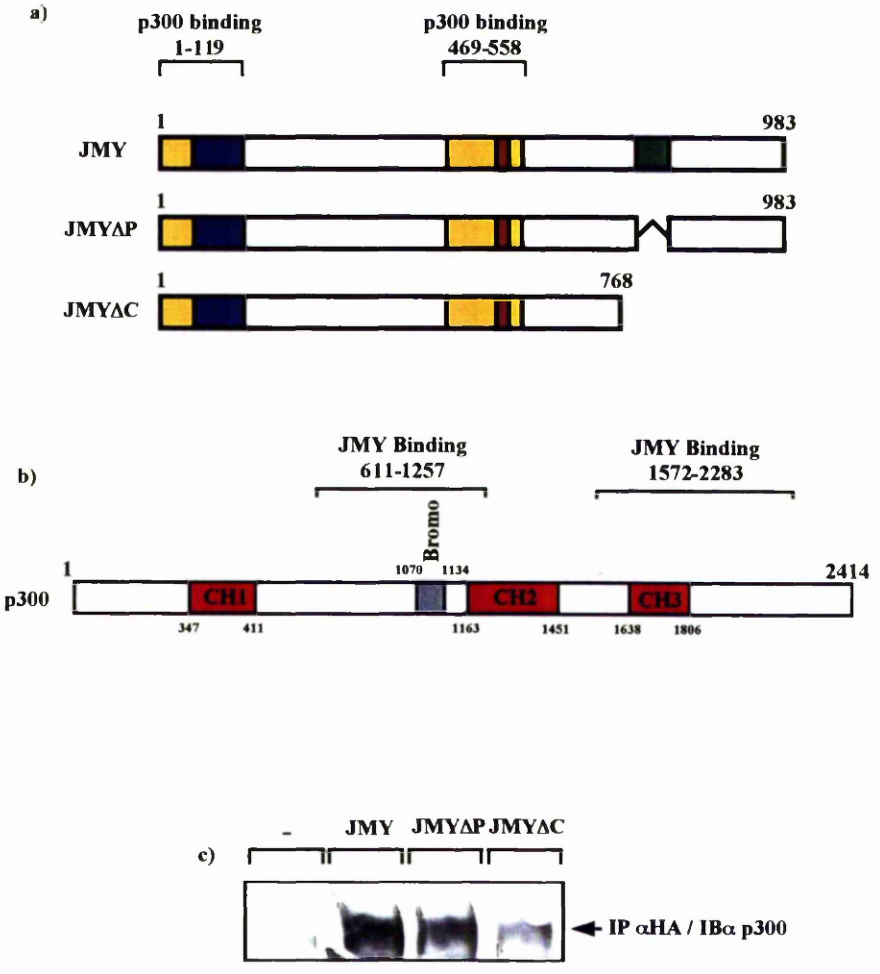

a)
p300 binding
1-119
p300 binding
469-558
JMY
1
983
JMYΔP
1
983
JMYΔC
1
768
b)
JMY Binding
611-1257
JMY Binding
1572-2283
Bromo
p300
1
1070
1134
2414
CH1
CH2
CH3
347
411
1163
1451
1638
1806
c)
-
JMY
JMYΔP
JMYΔC
IP αHA / IBα p300

Figure 4.2. JMY co-activates p53 transcription.

a). JMY drives *bax* expression.

SAOS-2 cells were transfected with 500ng of the p53 responsive promoter consisting of the *bax* gene promoter fused upstream of a luciferase reporter gene. Transcription factor dependent gene expression was achieved by the addition of 50ng of p53 expression plasmid. Co-activation of the p53 response was achieved by the addition of 5μg of HA-JMY. The effect of JMY 3' splicing on p53 co-activation was examined by the addition of 5μg of HA-JMYΔP. The values shown represent the average of three readings and depict the relative level of luciferase to β-galactosidase internal control expression.

b). *mdm2* expression.

SAOS-2 cells transfected with HA-JMY (5μg) and HA-JMYΔP (5μg) together with 50ng of p53 were assayed for their ability to drive expression of a luciferase reporter gene fused to the p53 responsive *mdm2* gene promoter (1μg). Relative activity is depicted by luciferase activity normalised relative to the level of a β-galactosidase internal control.

c). $p21^{Waf1/Cip1}$ promoter driven expression

The p53 responsive reporter gene construct, pWWP-luciferase (1μg) was introduced into SAOS-2 cells together with the expression vectors for p53 (50ng) and JMY (5μg) or JMYΔP (5μg). The expression of luciferase is depicted relative to that of a β-galastosidase internal control.

d). Regulation of the p53 target gene, *gadd45*.

pGADD45-luciferase (1μg) reporter gene construct, that contains a p53 responsive element, was introduced into SAOS-2 cells together with the expression vectors for p53 (50ng) and JMY (5μg) or JMYΔP (5μg). The expression of luciferase reporter gene is depicted relative to that of a β-galastosidase internal control.

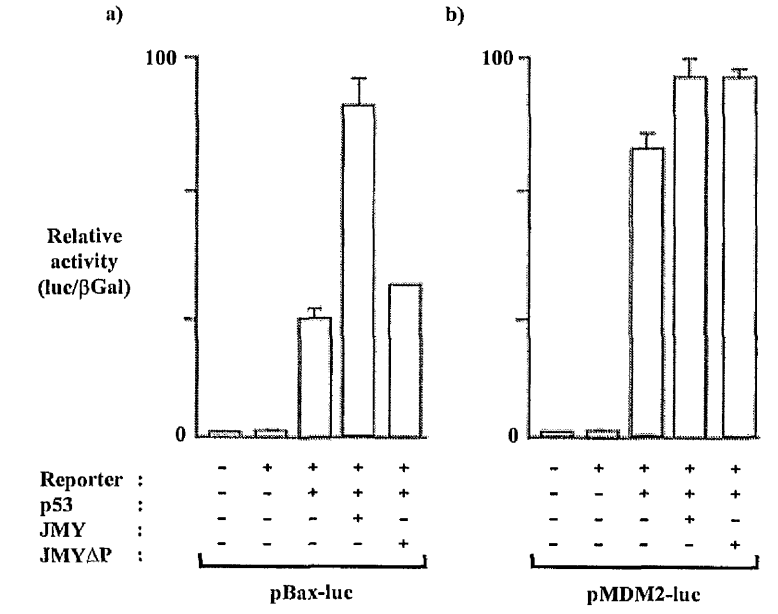

a)
b)
100
0
Relative activity (luc/βGal)
Reporter :
p53 :
JMY :
JMYΔP :
pBax-luc
pMDM2-luc
c)
d)
pWWP-luc
pGADD45-luc

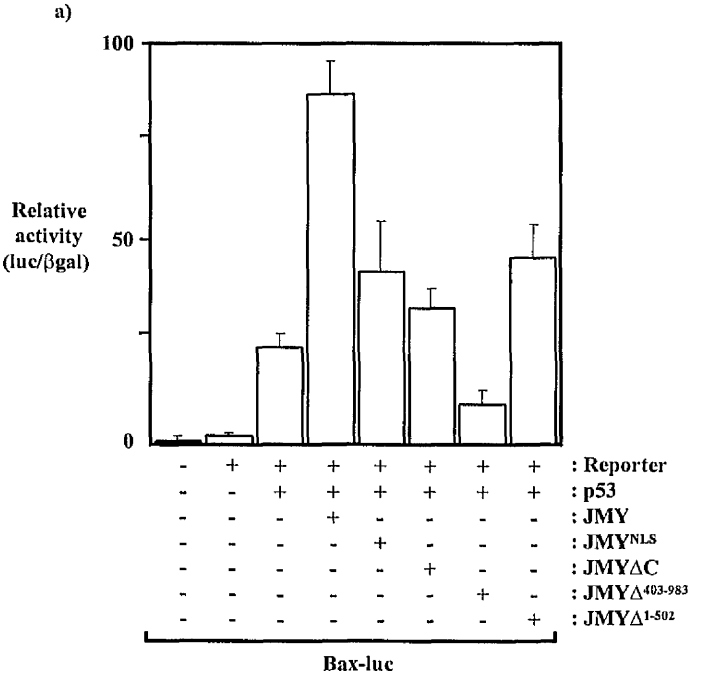
Figure 4.3. Analysis of JMY's co-activation properties.

a). Mutant analysis of JMY's ability to co-activate *bax* expression.

SAOS-2 cells were transfected with 500ng of the reporter vector, pBax-luciferase, together with 50ng of p53 expression vector. The co-activation of the p53 response was examined by the co-transfection of 5μg of expression vectors encoding JMY, JMY^{NLS}, JMYΔC, $JMY\Delta^{403\text{-}983}$ or $JMY\Delta^{1\text{-}502}$. The values shown represent the relative level of luciferase to internal control β-galactosidase expression.

b). Expression of the artificial TG_{13}-luciferase reporter.

The artificial TG_{13}-luciferase reporter (1μg) was introduced into SAOS-2 cells together with an expression vector for p53 (100ng). In addition 5μg of either JMY or JMYΔP expression vector were included. The expression of a luciferase reporter gene is depicted relative to that of a β-galastosidase internal control.


a)
100
50
0
Relative activity (luc/βgal)
- + + + + + + + : Reporter
- - + + + + + + : p53
- - - + - - - - : JMY
- - - - + - - - : JMY^NLS
- - - - - + - - : JMYΔC
- - - - - - + - : JMYΔ403-983
- - - - - - - + : JMYΔ1-502
Bax-luc

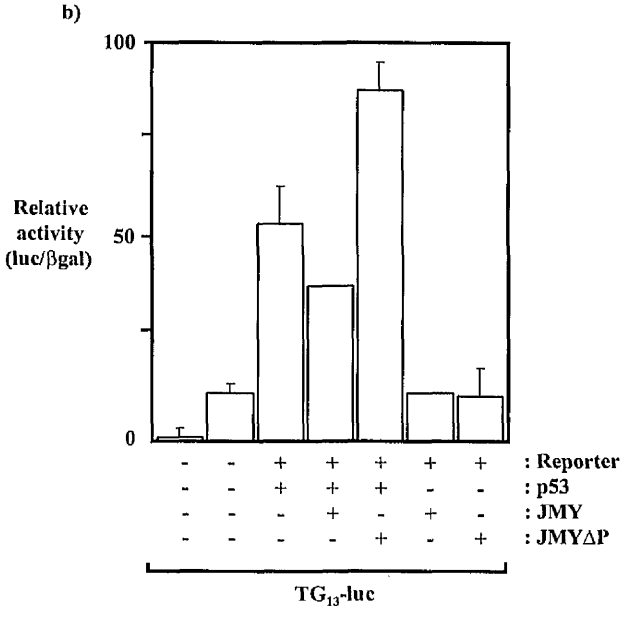

b)
100
50
0
Relative activity (luc/βgal)
- - + + + + + : Reporter
- - + + + - - : p53
- - - + - + - : JMY
- - - - + - + : JMYΔP
TG13-luc

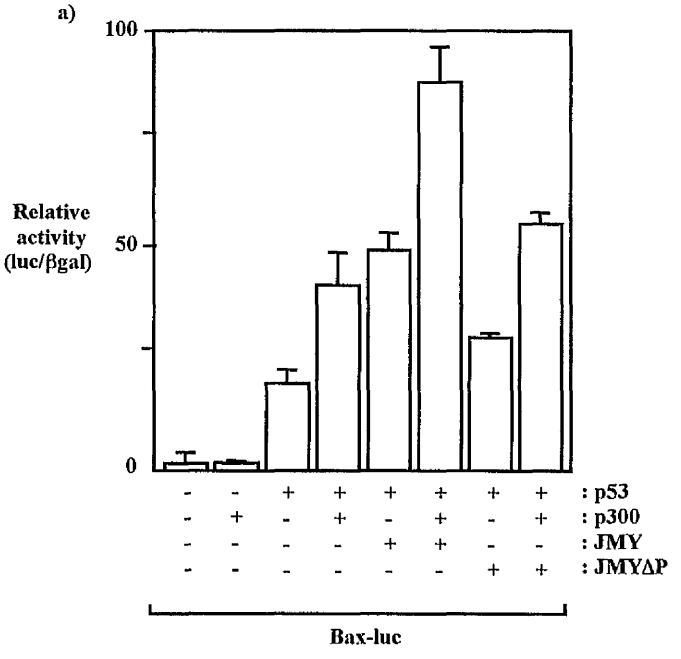
Figure 4.4. JMY co-operates with p300 in p53 mediated transcription.

a). Co-operation between p300 and JMY.

SAOS-2 cells transfected with 500ng of the p53 Bax-luc responsive reporter construct were assayed for the ability of JMY to co-operate with p300 in p53 mediated transcription. SAOS-2 cells were transfected with an activating amount of p53 (50ng) together with 5μg of either HA-JMY or HA-JMYΔP in the presence or absence of 5μg of p300 expression plasmid. The values shown represent the average of three readings and are the relative level of luciferase expression to that of a β-galactosidase internal control.

b). p53 stability.

SAOS-2 cells were transfected with 50ng of p53 alone or in the presence of HA-JMY (5μg), HA-JMYΔP (5μg), or HA-JMYΔC (5μg). Whole cell extracts were immunoblotted with anti-HA antibodies to detect the expression of the different JMY constructs (upper panel). The effect of JMY expression on p53's protein stability was determined by immunoblotting cell extracts with a anti-p53 antibody (lower panel).


a)
100
50
0
Relative activity (luc/βgal)
- - + + + + + + : p53
- + - + - + - + : p300
- - - - + + - - : JMY
- - - - - - + + : JMYΔP
Bax-luc

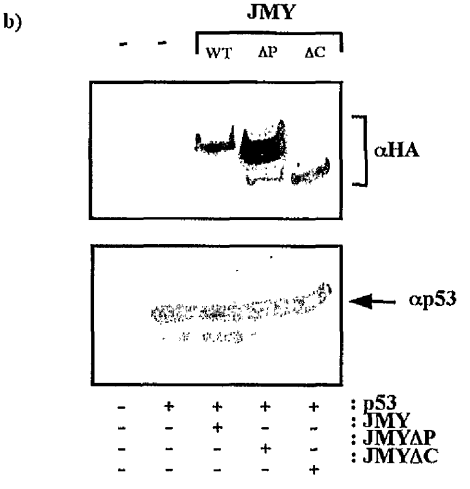

b)
JMY
- - WT ΔP ΔC
αHA
αp53
- + + + + : p53
- - + - - : JMY
- - - + - : JMYΔP
- - - - + : JMYΔC

Figure 4.5. Endogenous gene expression.

a). MDM2 expression.

To assess the induction of endogenous MDM2 protein, expression vectors for wild-type p53 (500ng) together with HA-JMY (5μg) or HA-JMYΔP (5μg) were transfected into SAOS-2 cells. Cell extracts from transfected cells were prepared and immunoblotted for exogenous HA (i) and p53 (ii) using anti-mouse HA and anti-mouse p53 antibodies respectfully. The level of MDM2 (iii) induced gene expression was determined by immunoblotting with an anti-MDM2 antibody. The treatment of each transfected extract is indicated together with the presence of the JMY, MDM2 and p53 specific polypeptides.

b). JMY stimulates Bax expression.

Endogenous Bax protein expression was analysed using an anti-Bax specific antibody on cell extracts prepared from SAOS-2 cells transfected with p53 (5μg) either alone or in the presence of HA-JMY (25μg) or HA-JMYΔP (25μg). Cell extract prepared from cells transfected with empty vector (pcDNA-3) is indicated and cell extract prepared from cells overexpressing HA-JMY and HA-JMYΔP in the absence of p53 is also shown.

c). JMYΔP upregulates $p21^{Waf1/Cip1}$ protein expression.

The $p53^{+/+}$ cell line, U2OS, was transfected with either 20μg of HA-JMY, HA-JMYΔP or JMYΔC. Cell extracts were examined for the level of endogenous $p21^{Waf1/Cip1}$ protein level by immunoblotting with an anti-$p21^{Waf1/Cip1}$ antibody. Equal protein loading was achieved by Bradford measurement and the position of the $p21^{Waf1/Cip1}$ specific polypeptide is indicated. Cell extract transfected with empty vector (pcDNA-3) is indicated.

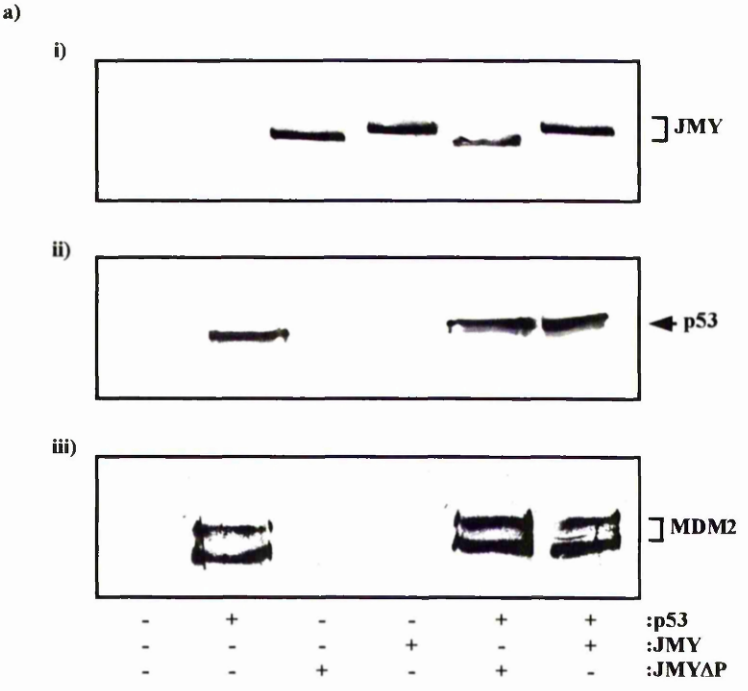

a)
i)
JMY
ii)
p53
iii)
MDM2
- + - - + + :p53
- - - + - + :JMY
- - + - + - :JMYΔP
b)
p53
- JMY JMYΔP
- - JMY JMYΔP
Bax
Bax
- JMY JMYΔP JMYΔC
c)
p21

Figure 4.6. Cellular localisation of JMY.

a). Endogenous JMY cellular localisation.

Asynchronous SAOS-2 cells were fixed and the cellular localisation of endogenously expressed JMY determined by immunofluorescence with an anti-JMY antibody. A rabbit polycolonal JMY antibody raised against the peptide sequence Try-Ala-Arg-Ser-Leu-Lys-Gly-Asp-Pro-Pro-Arg-Gly-Pro-Ala-Gly-Arg-Gly corresponding to an N-terminal region of JMY was used. Specific immuno-reactivity was visualised with rhodamine conjugated anti-rabbit immnuogloblin (i) and DAPI stain was included to aid nuclear identification (ii).

b). HA-JMY cellular localisation.

SAOS-2 cells were transfected with 5μg of a HA-JMY expression vector. Following fixation JMY's expression was determined by immuno-reactivity with an anti-mouse HA antibody and visualised by immuno-reactivity with an rhodamine conjugated anti-mouse immunogloblin (i). Nuclear stain DAPI was included to determine nuclei position (ii).

c). 3' splicing of JMY does not effect cellular localisation.

HA-JMYΔP (5μg) expression plasmid was transfected into SAOS-2 cells and the cellular localisation visualised using an anti-mouse HA antibody in combination with a rhodamine conjugated anti-mouse immunogloblin (i). DAPI stain was included to aid nuclear identification (ii)

d). Cellular localisation of a C-terminal truncation mutant of JMY.

SAOS-2 cells were transiently transfected with HA-JMYΔC (5μg) expression vector, fixed and assayed by immuno-reactivity with an anti-HA mouse antibody. Immuno-reactivity was visualised with rhodamine conjugated anti-mouse immunigloblin (i). DAPI nuclear stain was included (ii)

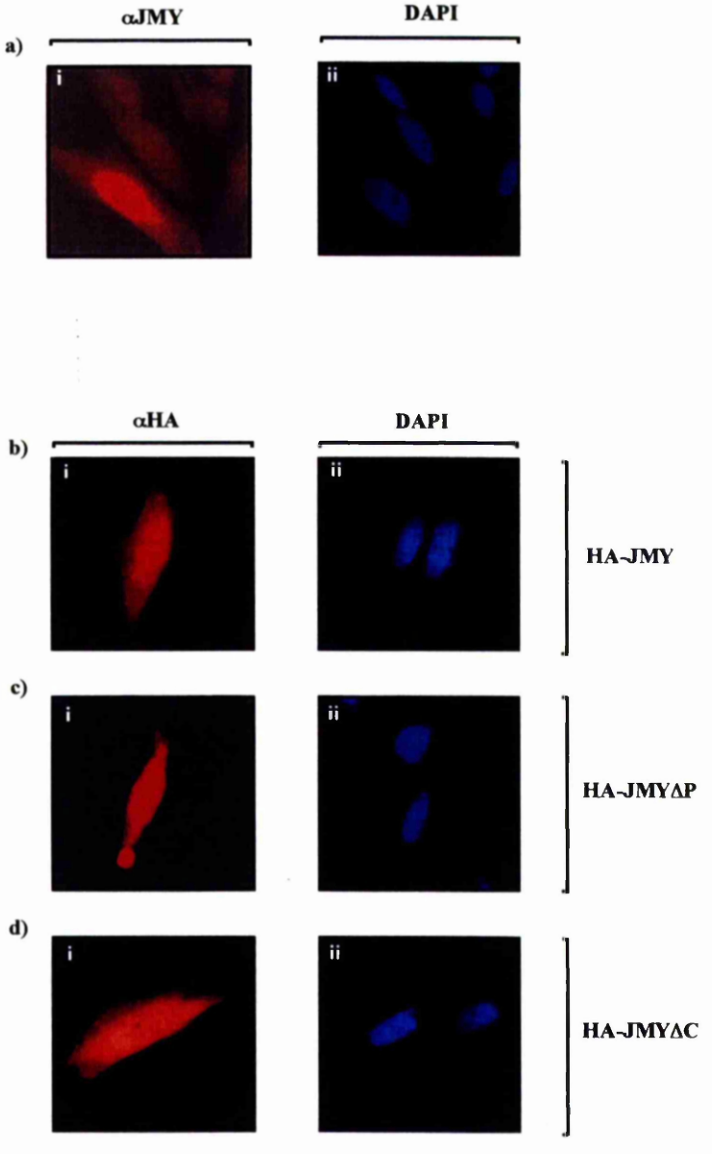

αJMY
DAPI
a)
i
ii
αHA
DAPI
b)
i
ii
HA-JMY
c)
i
ii
HA-JMYΔP
d)
i
ii
HA-JMYΔC

Figure 4.7. p53 mediates JMY's cellular localisation.

a). p53's affects JMY's cellular localisation.

The intracellular distribution of exogenous HA-JMY and p53 in SAOS-2 cells was assessed by direct immunoflorescence. 5μg of both p53 and HA-JMY were transfected into SAOS-2 cells and their expression visualised by staining with anti-rabbit HA for JMY and anti-mouse p53. Anti-HA was visualised with a rhodamine conjugated anti-rabbit immunogloblin (ii) and p53 with a fluorescein conjugated anti-mouse immunogloblin (ii). Nuclear stain DAPI was included to aid nuclear identification (iii).

b). p53 effects JMYΔP intracellular localisation.

SAOS-2 cells transfected with 5μg of HA-JMYΔP and 5μg of p53 were visualised for expression with anti-HA (i) and anti-p53 (ii) as in figure 4.7a. DAPI was included as a nuclear stain (iii).

c). p53 effects JMYΔC intracellular localisation.

SAOS-2 cells transfected with 5μg of HA-JMYΔC and 5μg of p53 were visualised for expression with anti-HA (i) and anti-p53 (ii) as in figure 4.7a. DAPI was included as a nuclear stain (iii).

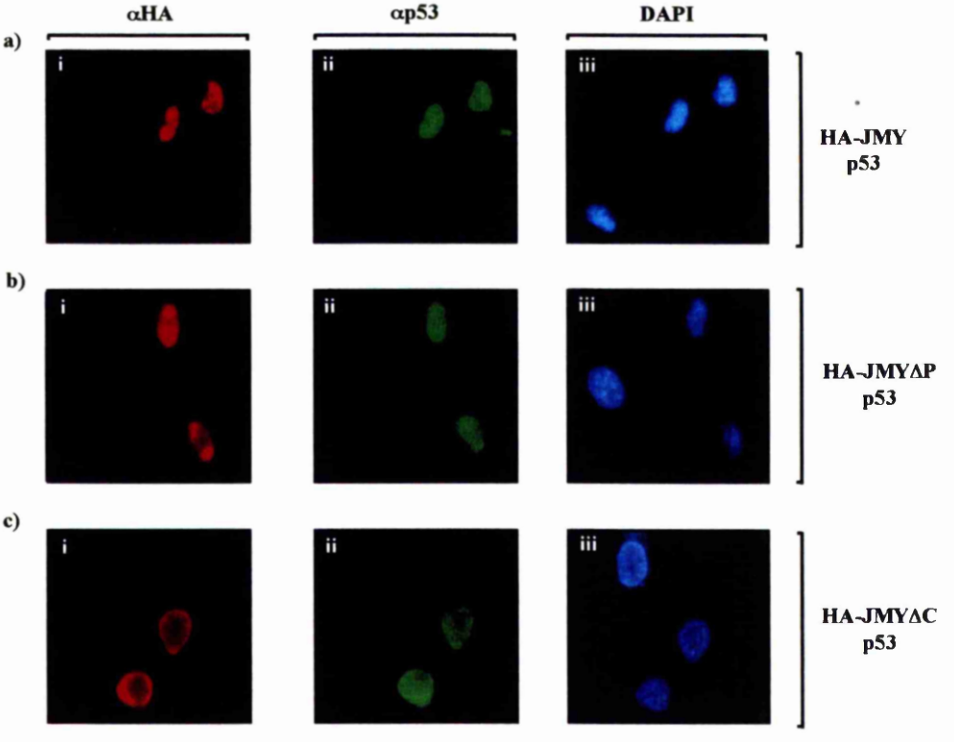

αHA
αp53
DAPI
a)
i
ii
iii
HA-JMY
p53
b)
i
ii
iii
HA-JMYΔP
p53
c)
i
ii
iii
HA-JMYΔC
p53

Figure 4.8. JMY functionally interacts with p53.

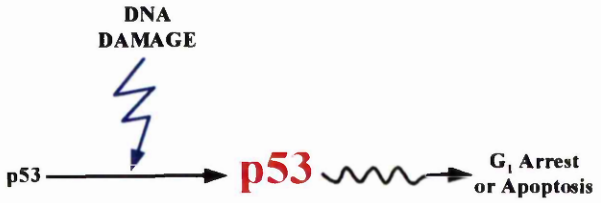
a). p53 DNA damage response.

Schematic diagram showing the response of p53 to DNA damage. Latent p53 is activated in response to DNA damage, the active p53 then induces the expression of genes which control G_1 arrest and apoptosis. The result of p53's response to DNA damage is either a G_1 arrest or apoptotic phenotype.

b). JMY's involvement in the p53 DNA damage response.

U2OS cells were treated with or without the DNA damaging agent actinomycin D (5nM). After 24hours whole cell extracts were prepared and subjected to immuno-precipitation. The induction of p53 gene expression was confirmed by blotting 15% of the cell extract for p53 with mouse anti-p53 antibody. The remaining extract was immuno-precipitated with the rabbit JMY specific N-terminal polycolonal antibody and subjected to immunoblotting with anti-p53 antibody. The presence of a p53 specific polypeptide in JMY immuno-precipitates and input extracts is indicated. Actinomycin D treated samples are labelled.

a)


DNA
DAMAGE
p53
p53
G1 Arrest
or Apoptosis

b)

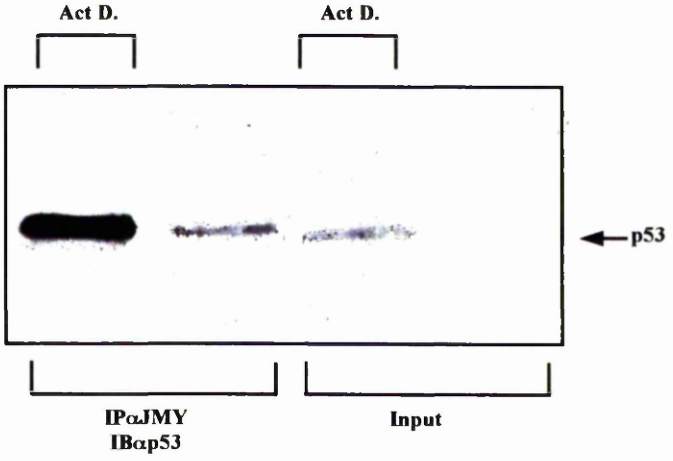

Act D.
Act D.
p53
IPαJMY
IBαp53
Input

Figure 4.9. Co-operation between JMY and p53 in cell cycle arrest.

a)-h). Cell cycle profiles of JMY over-expressing cells.

Asynchronous cultures of SAOS-2 cells were transfected with the 5μg of the cell surface expression marker CD20 and the indicated plasmids in the doses, p53 (3μg), JMY (5μg), JMYΔP (5μg), or JMYΔC (5μg). Total DNA content was equivalent to 20μg and was made up with pcDNA-3 empty vector. Transfected cells grown in 10% FCS were identified by staining with the anti-CD20 fluorescein conjugated immunogloblin and their DNA content observed using the DNA inter-chelater propidium iodide. Cell cycle profiles were assigned using CellQuest software.

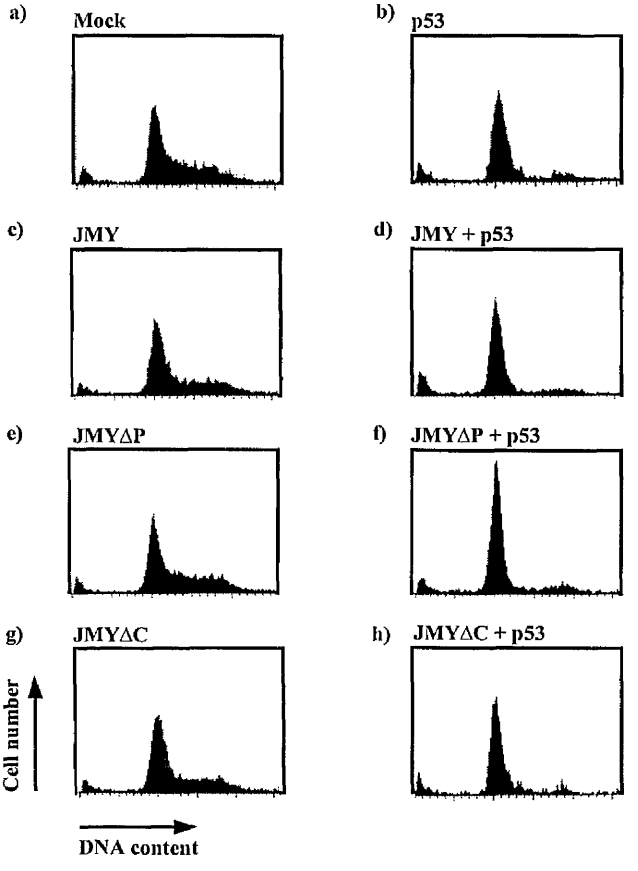

a)
Mock
b)
p53
c)
JMY
d)
JMY + p53
e)
JMYΔP
f)
JMYΔP + p53
g)
JMYΔC
h)
JMYΔC + p53
Cell number
DNA content

Figure 4.10. JMY 3' splicing induces an enhanced G_1 arrest phenotype.

a). p53 induces a G_1 cell cycle arrest.

The cell cycle profile for p53 from figure 4.9b was quantified using CellQuest software. The percentage change in cell number for G_1, S, and G_2M were calculated relative to mock transfected cells.

b). JMY co-operates with p53.

Profiles for JMY (Figure 4.9c) and JMY in combination with p53 (Figure 4.9d) were calculated as percentage change from mock (Figure 4.9a).

c). JMYΔP enhances a p53 G_1 arrest.

Profiles for JMYΔP (Figure 4.9e) and JMYΔP in combination with p53 (Figure 4.9f) were calculated as percentage change from mock (Figure 4.9a).

d). JMYΔC cell cycle phenotype

Profiles for JMYΔC (Figure 4.9g) and JMYΔC in combination with p53 (Figure 4.9h) were calculated as percentage change from mock (Figure 4.9a).

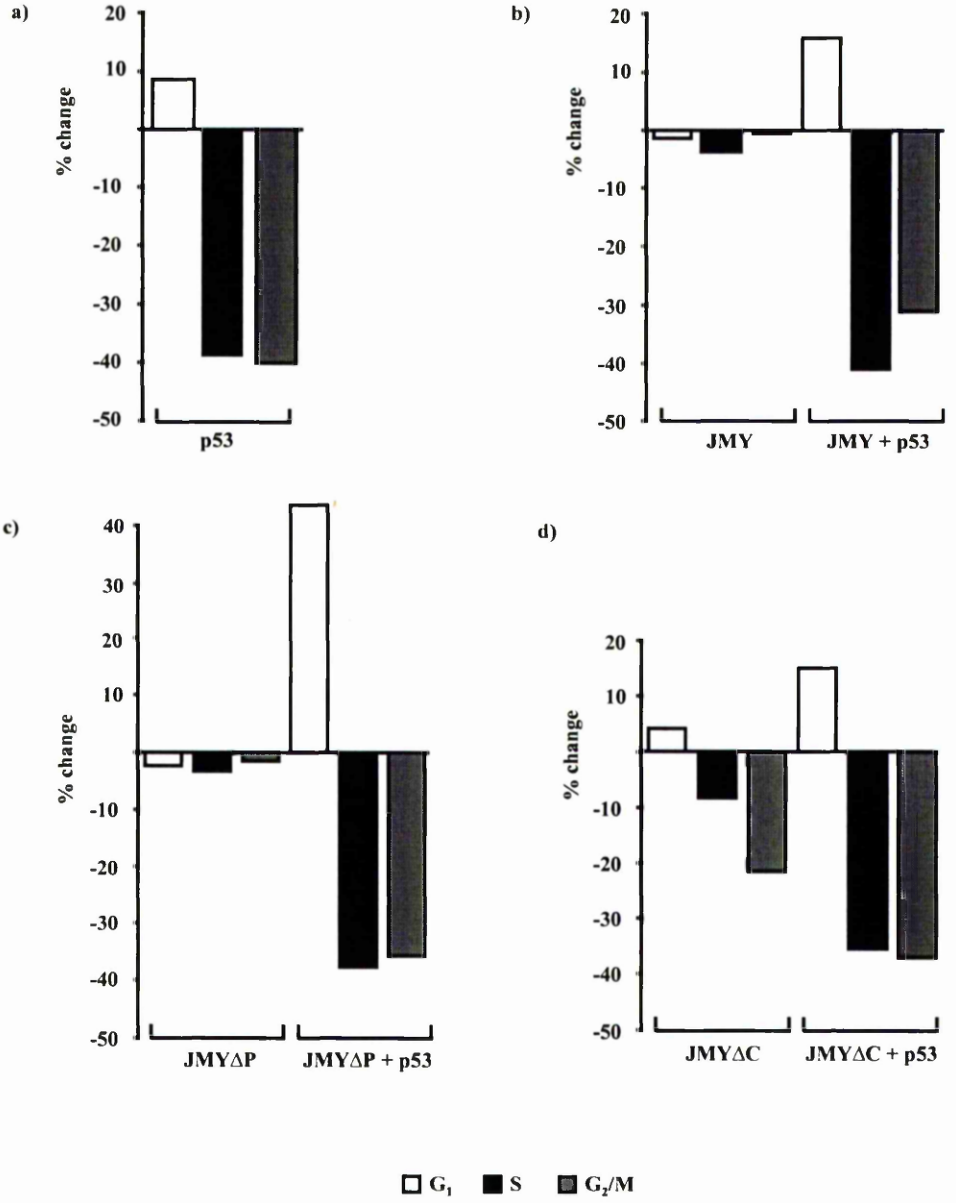

a)
b)
c)
d)
% change
20
10
-10
-20
-30
-40
-50
40
30
p53
JMY
JMY + p53
JMYΔP
JMYΔP + p53
JMYΔC
JMYΔC + p53
G1
S
G2/M

Figure 4.11. JMY participates in p53 mediated apoptosis.

a). p53 and JMY increase the apoptotic cell population.

Expression vectors for p53 (5μg) either alone or together with HA-JMY (5μg) or HA-JMYΔP (5μg) were introduced into SAOS-2 cells by transient transfection. Cells were fixed and analysed for p53 over-expression using a p53 anti-mouse antibody and visualised using anti-mouse rhodamine conjugated immunoglobulin. In cells overexpressing HA-JMY or HA-JMYΔP anti-mouse HA antibody in combination with an anti-mouse rhodamine conjugated immunogloblin were used to visualise transfected cells. Immuno-staining was performed in parallel with TUNEL (TdT mediated dUTP nick end labelling) in order to detect apoptotic cells. Background levels of apoptosis were determined using empty vector transfected cells.

Cells were visualised directly and the level of apoptosis in transfected cells quantified relative to empty vector transfected cells. The level of apoptosis for each treatment is indicated as a percentage change relative to mock. Percentage stimulation for JMY and JMYΔP in combination with p53 is calculated relative to p53 alone. The data represents the average of at least two independent observations.

a)

p53	Co-activator	Apoptosis (%)	Stimulation (%)
+	-	30.0	-
+	JMY	42.3	41.0
+	JMYΔP	28.6	-4.6
-	JMY	5.8	-
-	JMYΔP	3.7	-

Figure 4.12. JMY co-activates p53-dependent expression of *PIG3*.

a). p53's proline rich domain is required for *PIG3 trans*activation.

The 0.7kb *PIG3* gene promoter (300ng) cloned upstream of a luciferase reporter construct was introduced into SAOS-2 cells together with p53 and p53ΔP in the quantities (+ 100ng) and (++ 300ng) respectively. p53ΔP corresponds to a mutant p53 construct deleted in amino acids 62 to 91 that contains five PXXP motifs. Relative activity was calculated as luciferase expression relative to the expression of a β-galactosidase internal control. The expression of p53 and p53ΔP was confirmed by western blotting with an anti-p53 antibody (DO-1).

b). JMY co-activates *PIG3* expression by p53 but not p53ΔP.

SAOS-2 cells were transfected with the *PIG3*-luc reporter (300ng) together with either p53 or p53ΔP as in 4.12a. In addition the expression vector for HA-JMY was included (5μg). The expression of luciferase is calculated relative to an internal β-galastosidase control. JMY expression was confirmed by blotting transfected cell extracts with an anti-HA antibody. The specific 110kDa JMY specific polypeptide is indicated. Activity is plotted in scale with figure 4.12a, c, and d.

c). JMYΔP is impaired in *trans*activation of *PIG3* promoter driven expression.

SAOS-2 cells were transfected as in figure 4.12b except HA-JMYΔP (5μg) was included instead of HA-JMY. The values are the relative level of luciferase to β-galactosidase internal control expression. The expression level of HA-JMYΔP was confirmed by blotting with an anti-mouse HA antibody and the corresponding JMYΔP specific polypeptide is indicated. Activity is plotted in scale with figure 4.12a, b, and d.

d). JMYΔC activates *PIG3* expression.

SAOS-2 cells were transfected as in figure 4.12b except HA-JMYΔC (5μg) was included instead of HA-JMY. The values shown are the relative level of luciferase to β-galactosidase internal control expression. The expression level of HA-JMYΔC was confirmed by western blot with an anti-mouse HA antibody. Activity is plotted in scale with figure 4.12a, b, and c.

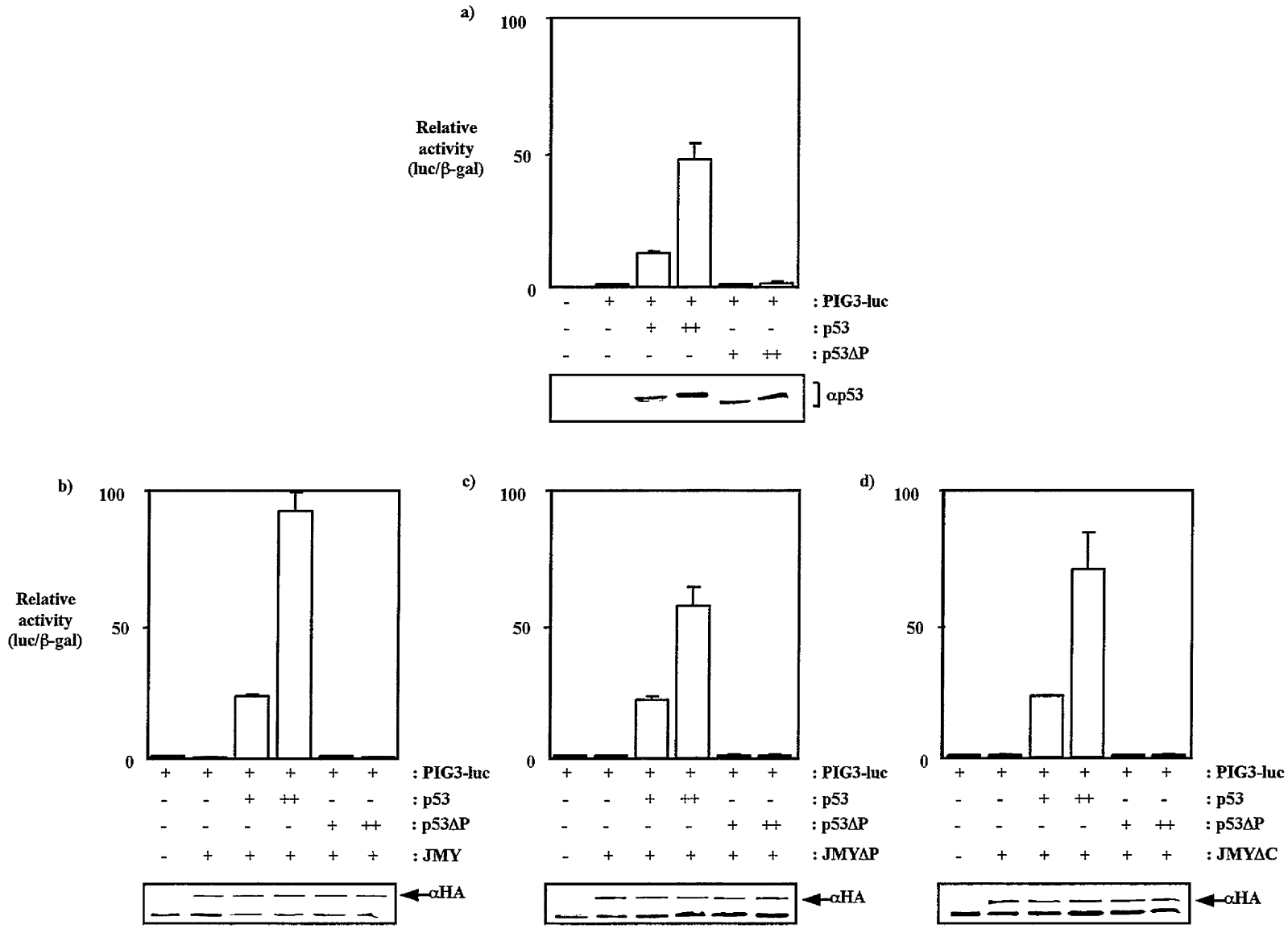

a)
100
50
0
Relative activity (luc/β-gal)
- + + + + + : PIG3-luc
- - + ++ - - : p53
- - - - + ++ : p53ΔP
αp53
b)
100
50
0
Relative activity (luc/β-gal)
+ + + + + + : PIG3-luc
- - + ++ - - : p53
- - - - + ++ : p53ΔP
- + + + + + : JMY
αHA
c)
100
50
0
+ + + + + + : PIG3-luc
- - + ++ - - : p53
- - - - + ++ : p53ΔP
- + + + + + : JMYΔP
αHA
d)
100
50
0
+ + + + + + : PIG3-luc
- - + ++ - - : p53
- - - - + ++ : p53ΔP
- + + + + + : JMYΔC
αHA

Figure 4.13. JMY co-activates p53 and p53ΔP *trans*activation.

a). p53 proline rich domain is dispensable for *WWP transactivation*.

SAOS-2 cells were transfected with 1μg of the pWWP-luciferase reporter together with 300ng of p53 or p53ΔP. In addition HA-JMY (5μg) and HA-JMYΔP (5μg) were included. Relative activity was calculated as luciferase expression relative to the expression of a β-galactosidase internal control.

b). JMY co-activates *mdm2* promoter driven expression driven by p53 and p53ΔP.

SAOS-2 cells were transfected with HA-JMY (5μg) and HA-JMYΔP (5μg) in addition to 300ng of p53 or p53ΔP and were assayed for their ability to drive expression of a luciferase reporter gene fused to the p53 responsive *mdm2* gene promoter (1μg). Relative activity is depicted by luciferase expression normalised relative to the level of a β-galactosidase internal control.

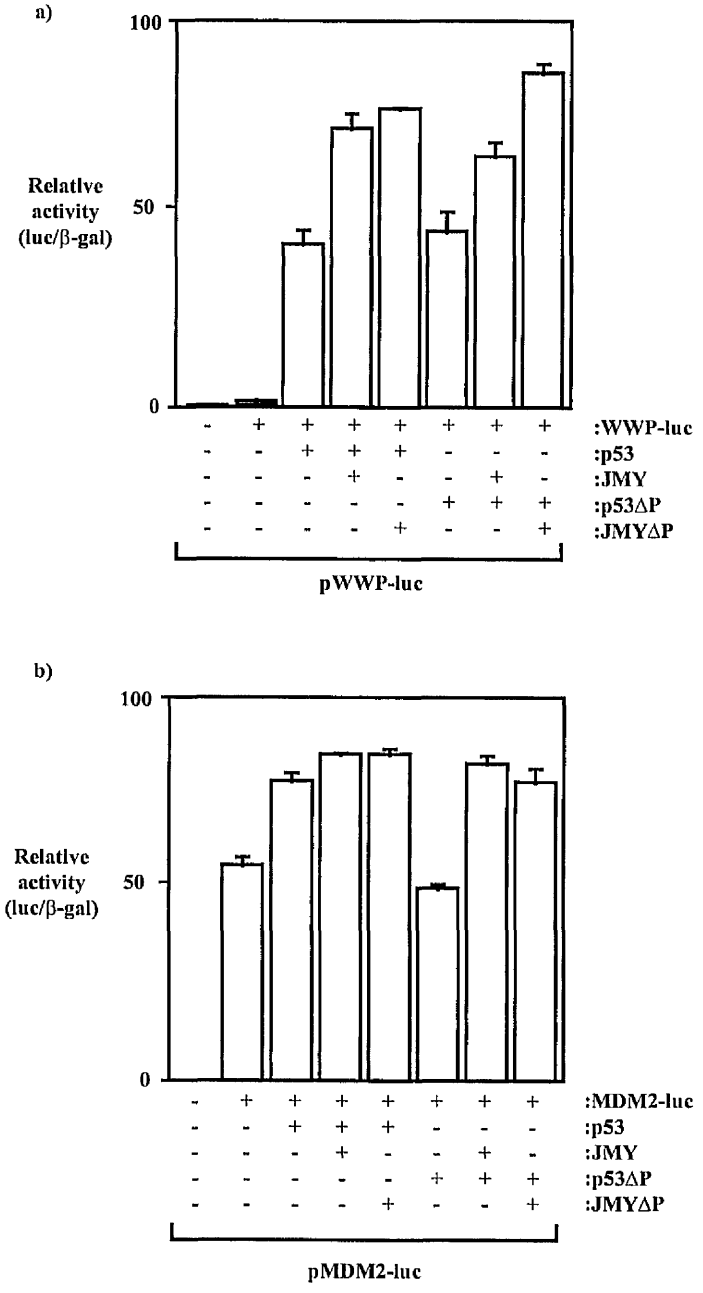
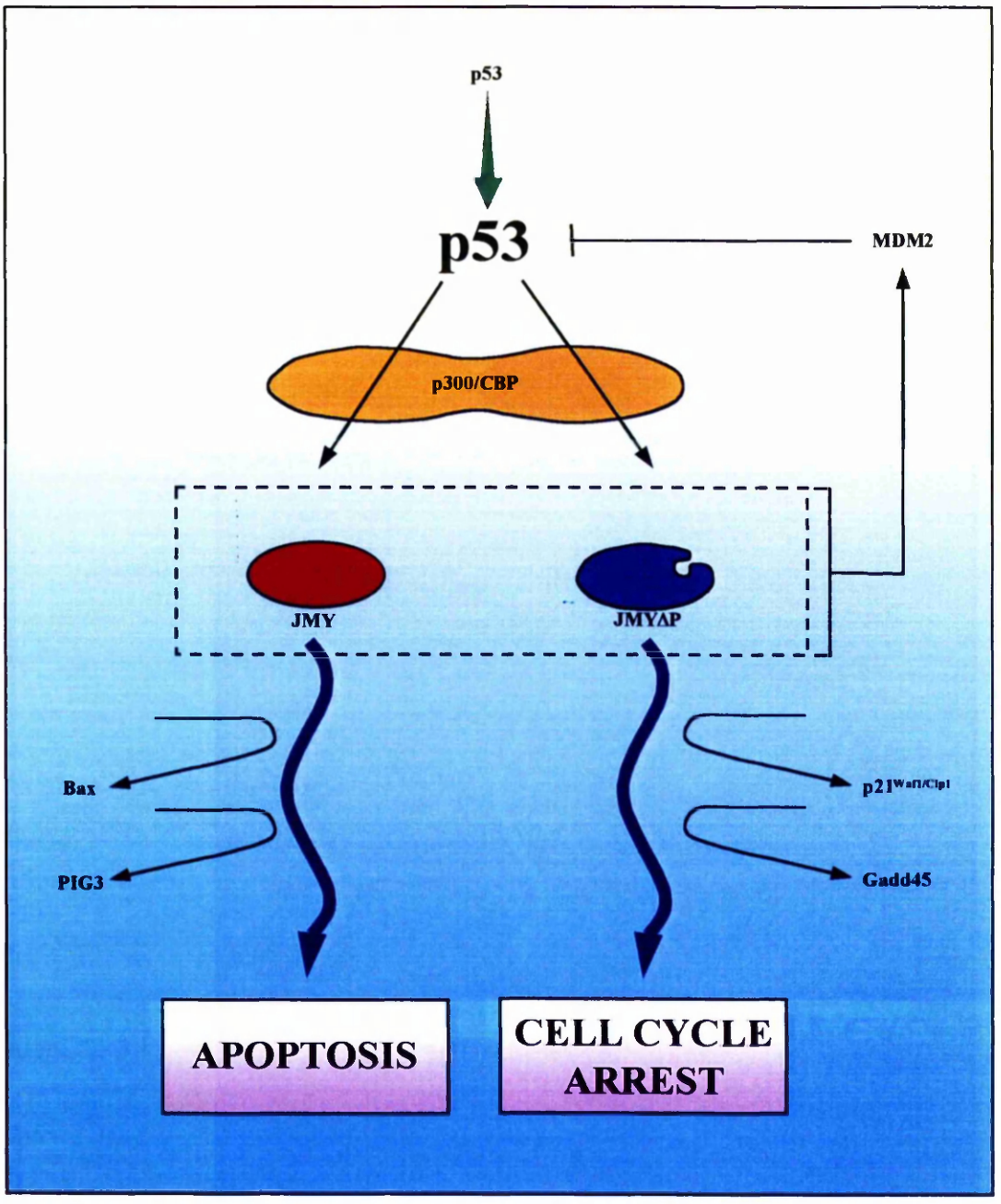

a)
100
50
0
Relative activity (luc/β-gal)
- + + + + + + + :WWP-luc
- - + + + - - - :p53
- - - + - - + - :JMY
- - - - - + + + :p53ΔP
- - - - + - - + :JMYΔP
pWWP-luc
b)
100
50
0
Relative activity (luc/β-gal)
- + + + + + + + :MDM2-luc
- - + + + - - - :p53
- - - + - - + - :JMY
- - - - - + + + :p53ΔP
- - - - + - - + :JMYΔP
pMDM2-luc

Figure 4.14. Summary diagram of JMY function.

p53's cellular activation has two cellular consequences, cell cycle arrest and apoptosis. Here I propose that JMY and the isoform, JMYΔP, acting through p300 are able to co-ordinate the p53 response, with JMY being specific for an apoptotic response and JMYΔP specific for cell cycle arrest. A potential role for the cellular JMY population is also in the regulation of the MDM2 pathway.


p53
p53
MDM2
p300/CBP
JMY
JMYΔP
Bax
PIG3
p21Waf1/Cip1
Gadd45
APOPTOSIS
CELL CYCLE ARREST

5. JMY, a new regulator of the $p14^{ARF}$ response.

5.1. Introduction.

The p300/CBP family of proteins function as pleiotrophic co-activators to facilitate activation by a wide variety of transcription factors that function as regulators of differentiation, signal transduction and cell cycle control (Eckner *et al.*, 1994; Shikama *et al.*, 1997). The key cell cycle regulators, E2F and p53, are both physiological targets of p300/CBP action and it is by the modulation of p300/CBP transcriptional regulation activities that viral proteins such as E1A and SV40 large T exert some of their cellular outcomes (Arany *et al.*, 1995; Avantaggiati *et al.*, 1996; Lill *et al.*, 1997a).

The functional inactivation, by cancerous cells, of the pRb and p53 tumour suppressor pathways is a major event in the disruption of normal cellular growth control and immortalisation. The *INK4a/ARF* locus is a central player in the regulation of these growth controlling pathways, by virtue of its ability to generate two functionally distant products, namely $p16^{INK4a}$ that functions upstream of pRb and $p14/19^{ARF}$ that regulates p53 stability. As the *INK4a/ARF* locus and its individual products are both frequently the target of mutational events that precede tumourigenesis their mechanisms of action are potentially of therapeutic interest.

Given that a major regulator of the $p14/19^{ARF}$ response is E2F and that E2F is a known target for p300/CBP, it is of interest to define a role for the p300/CBP

associated factors in p14/19^{ARF} function and regulation. As JMY is such a p300 associated co-activator molecule that participates in the p53 response to cellular stress it is of significance to elucidate the role, if any, that JMY plays in the p14/19^{ARF} pathway.

5.2. E2F stimulates transcription of the p14^{ARF} promoter.

Previous studies have demonstrated that the p14/19^{ARF} reporter is highly sensitive to E2F-1 over-expression (Bates *et al.*, 1998; Roberston and Jones, 1998; Inoue *et al.*, 1999). Consistent with these observations, the over-expression of E2F-1 effectively activated a p14^{ARF} promoter reporter construct in a dose-dependent, p53 negative SAOS-2 background (Figure 5.1b). The p14^{ARF} luciferase reporter construct consists of the exon 1β promoter region, from –805 to +59 which contains four potential anti-sense strand E2F consensus sites, fused to a luciferase reporter gene (Figure 5.1a) (Bates *et al.*, 1998; Robertson and Jones, 1998).

Given that a physiological co-activator for E2F-1 is p300/CBP it was of importance to determine the effectiveness of p300 in stimulating E2F-1 mediated transcription from the p14^{ARF} promoter. SAOS-2 cells transfected with the p14^{ARF} luciferase reporter and an activating level of E2F-1 were effectively co-activated by the addition of exogenous p300 expression vector (Figure 5.1c). The p300 specific two-fold activation of E2F-1 mediated transcription from the p14^{ARF} reporter supports the previous observations that a physiological mediator of E2F-1 transcription is p300 and furthermore suggests that p14^{ARF} expression is triggered by an E2F-1/p300 mechanism (Trouche *et al.*, 1996) (compare Figure 5.1b and 5.1c).

5.3. JMY transcriptionally enhances $p14^{ARF}$ expression.

The ability of JMY to co-stimulate E2F-1 transcription from the $p14^{ARF}$ luciferase promoter was tested by co-transfection of HA-JMY with E2F-1 in the presence and absence of exogenous p300. JMY effectively co-activated, by up to eight fold, E2F-1 dependent transcription from the $p14^{ARF}$ luciferase reporter in SAOS-2 cells, with the activity being further enhanced by the addition of exogenous p300 (Figure 5.1b and 5.1c). The ability of p300 to co-stimulate the activation of E2F-1 in the context of JMY is consistent with its function as a bridging transcriptional co-activator. In addition JMY's role as a transcriptional co-activator is also consistent with these results.

Given the ability of JMY to undergo 3' splicing, and that the splicing variants commonly are devoid of the C-terminal proline rich region, it was of interest to note that the co-stimulation of E2F-1 mediated $p14^{ARF}$ transcription by JMYΔP, although reduced relative to JMY, was still significant (Figure 5.1b). The ability of p300 to further enhance JMYΔP stimulation was only slightly compromised compared to JMY (Figure 5.1c). Notably, the transcription from the $p14^{ARF}$ promoter was E2F-1 dependent as p300, JMY and JMYΔP did not alter the activity of the reporter in the absence of E2F-1 (data not shown and Figure 5.2a).

Together these results support the role of E2F-1 as a transcription factor that drives the expression of $p14^{ARF}$. Two potential physiological regulators of $p14^{ARF}$ expression have also been identified by nature of their ability to efficiently co-activate E2F-1 transcription, namely p300 and JMY.

5.4. $p14^{ARF}$ transcription auto-regulation.

Given that E2F-1 activates $p14^{ARF}$ expression and that this expression is effectively co-activated by the addition of JMY and p300 it was of interest to dissect the transcriptional regulation of the $p14^{ARF}$ promoter further. Previous observations have shown that E2F-1 is essential for G_1 cell cycle progression, and that E2F's ability to act as a transcriptional co-activator is efficiently blocked by hypophosphorylated pRb. E2F-1 over-expression studies demonstrated that E2F-1 can induce apoptosis in co-operation with p53, although studies performed on $p53^{-/-}$ and $Rb^{-/-}$ mice embryos have more recently demonstrated that E2F-1 can induce a p53 independent mechanism of apoptosis (Hass-Kogan *et al.*, 1995; Macleod *et al.*, 1996). Given that a potential mechanism of p53 activation by E2F may be via $p14^{ARF}$ and that this activation may be the trigger that predisposes cells to apoptosis it is of interest to determine the effect of $p14^{ARF}$ on activated transcription. The observation that $p14^{ARF}$ can induce pRb hypophosphorylation indicates a potential autoregulatory mechanism whereby $p14^{ARF}$ may regulate its own expression (Labaer *et al.*, 1997; Kurokawa *et al.*, 1999).

$p14^{ARF}$ over expression in SAOS-2 and U2OS cells did not alter the basal transcription of the exon 1β-luc reporter nor of the internal control derived from β-galactosidase (data not shown, Figures 5.2a and 5.2b). However transcription of the exon 1β reporter by E2F-1 in SAOS-2 cells was effectively repressed by $p14^{ARF}$ in a pRb and p53 independent manner (Figure 5.2a). The level of expression driven from the E2F site within the exon 1β promoter was reduced by 2.5 fold in the presence of $p14^{ARF}$ (Figure 5.2c). Similarly the co-activated expression of exon 1β by JMY was reduced by 2.7 fold in the presence of $p14^{ARF}$ (Figure 5.2a and 5.2c).

In order to determine the specificity of the transcriptional repression ability of $p14^{ARF}$ the transcriptional activity of p53 was studied. In order to study p53 transcription the promoter taken from the *bax* gene that encodes a protein that facilitates apoptosis and responds to p53 was used (Miyashita and Reed, 1995; Friedlander *et al.*, 1996). The *bax* promoter was effectively induced in the presence of exogenous p53 in SAOS-2 cells (Figure 5.2b). As expected p53-dependent transcription of the *bax* promoter was increased by the co-expression of JMY. The addition of $p14^{ARF}$ into the activated *bax* promoter driven transcription although repressive was not as great as that seen for E2F-1, with transcription of both the p53 and p53/JMY mediated transcription being reduced by only 1.3 and 1.4 fold respectively (Figure 5.2c). Interestingly unlike previous studies $p14^{ARF}$ was unable to stabilise p53 under these cellular condition, presumably as a consequence of the transient nature of the assay (Figure 5.2d).

The ability of $p14^{ARF}$ to repress transcription in a transcription factor dependent fashion clearly indicates a new mechanism of $p14^{ARF}$ regulation. Significantly, the preference of $p14^{ARF}$ for E2F-1 was demonstrated by the increased repression seen over p53. Potentially the ability of $p14^{ARF}$ to auto-regulate its own expression provides a possible insight into a novel function of $p14^{ARF}$.

5.5. $p14^{ARF}$ interacts with JMY.

The ability of $p14^{ARF}$ to interact directly with MDM2 has previously been shown to be a determining factor in $p14^{ARF}$ ability to influence the p53 pathway (Zhang *et al.*, 1998; Weber *et al.*, 1999; Zhang and Xiong, 1999). To examine the possibility that $p14^{ARF}$ transcription and function is regulated by JMY it was of interest to determine the ability of the two proteins to interact. Thus using a pull-down approach in a cell

free system the ability of *in vitro* translated JMY, the natural splice JMYΔP and a C-terminal truncation mutant, JMYΔC, to interact with a bacterially purified GST-$p14^{ARF}$ protein was examined (Figure 5.3a, 5.3b and 5.3c).

JMY and JMYΔP bound weekly to bacterially expressed and purified GST-$p14^{ARF}$ whereas JMYΔC, a mutant devoid of the C-terminal 215 amino acids of JMY, bound with a similar efficiency as MDM2 (Figure 3c, upper panel). The specificity of JMY and JMYΔP binding to GST-$p14^{ARF}$ 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 products (Figure 5.3c, lower panel). Confirming that, although weekly, JMY and its natural splice JMYΔP were capable of interacting with $p14^{ARF}$ *in vitro*. Given that a C-terminal deletion, JMYΔC, showed an enhanced affinity for GST-$p14^{ARF}$ it is possible that the C-terminus of JMY plays an inhibitory role in the $p14^{ARF}$ interaction.

In order to further map the interaction region of JMY with $p14^{ARF}$ a panel of JMY deletion mutants were used (Figure 5.4a). Initial mapping results indicated that the C-terminal region of JMY played an inhibitory role in the interaction of $p14^{ARF}$ with JMY. A construct encompassing the C-terminus, $JMY^{683-983}$, that is devoid of a p300 binding domain failed to bind $p14^{ARF}$ (Figure 5.4aii and 5.4b). However a mutant of $JMY^{502-983}$ which retained the central p300 binding capacity was capable of interacting with $p14^{ARF}$ (Figure 5.4ai and 5.4b). As p300 is a potential bridging mediator in the interaction seen between $p14^{ARF}$ and JMY it was of relevance to determine the ability of the two previously identified p300 binding domains in JMY to influence $p14^{ARF}$ binding. An N-terminal construct of JMY^{1-504} that is devoid of the

central p300 binding domain, but contains the N-terminal binding domain, still retained the ability to bind $p14^{ARF}$ (Figure 5.4aiii and 5.4b). Given the link between MDM2 and p300 it was then of curiosity to determine whether the interaction of $p14^{ARF}$ was solely confined to the regions in JMY responsible for p300 binding. Using a mutant devoid of p300 binding namely, $JMY^{118-403}$ it was determined that $p14^{ARF}$, although weakly, was still capable of binding to JMY in the absence of p300 (Figure 5.4a iv and 5.4b).

Taken together these results point to at least two $p14^{ARF}$ interaction domains in JMY, one encompassed from amino acids 118 to 403 and a second from amino acids 502 to 683 that overlap's the central p300 binding domain in JMY. These results also confirm that the $p14^{ARF}$ interaction with JMY is at least in part outside the p300 binding function of JMY (Figure 5.4a).

5.6. $p14^{ARF}$ and JMY are present in a complex *in vivo*.

The ability of JMY to interact with $p14^{ARF}$ *in vitro* strongly suggests that such a physical complex may occur in mammalian cells. It was therefore of relevance to determine whether JMY and $p14^{ARF}$ were present in the same complex *in vivo*. Given that $p14^{ARF}$ physiologically targets MDM2 it is also of interest to determine if JMY influences the ability of MDM2 to associate with $p14^{ARF}$.

Previously $p14^{ARF}$ was shown to physically associate with p53 in the absence of MDM2 so in order to rule out a p53/p300 dependent mechanism of interaction $p53^{-/-}$;$Rb^{-/-}$ SAOS-2 cells were used (Kamijo *et al.*, 1998). HA-JMY and HA-JMYΔP together with $p14^{ARF}$ were transiently transfected into SAOS-2 cells. $p14^{ARF}$, HA-

JMY and HA-JMYΔP were all overexpressed in SAOS-2 cells (Figure 5.5a, upper and lower panels). The immuno-precipitation of cell extracts with anti-HA followed by immunoblotting with anti-$p14^{ARF}$ revealed that a specific 14 kDa polypeptide corresponding to $p14^{ARF}$ was present in the JMY *in vivo* complex (Figure 5.5b). Strangely, given its ability to interact *in vitro*, JMYΔP failed to immuno-precipitate $p14^{ARF}$ (Figure 5.5b). However the significance of the *in vivo* interaction assay in relation to JMYΔP is undermined, potentially due to its sensitivity, given that JMYΔP behaved as wild-type in all the other assays performed.

In order to determine the functional significance of the $p14^{ARF}$/JMY interaction on the previously described $p14^{ARF}$/MDM2 interaction, SAOS-2 cells transfected with $p14^{ARF}$, MDM2 and either HA-JMY or HA-JMYΔP were assayed for MDM2 binding to $p14^{ARF}$. In cells overexpressing either JMY or JMYΔP no detectable $p14^{ARF}$ polypeptide was present in the MDM2 immuno-precipitates, whereas cells, not overexpressing JMY, efficiently immuno-precipitated $p14^{ARF}$ with an MDM2 antibody (Figure 5.5c and 5.5d). Given that JMY and JMYΔP over-expression did not affect the stability of either MDM2 or $p14^{ARF}$ a loss in interaction due to stability can be ruled out (Figure 5c and 5d). Interestingly cells overexpressing MDM2 and $p14^{ARF}$ showed a higher molecular weight polypeptide corresponding to $p14^{ARF}$ in MDM2 immuno-precipitates (Figure 5.5d).

Taken together these results suggest a physiological interaction occurs between JMY and $p14^{ARF}$, and that the splicing of the 3' of JMY may affects this interaction. The inability of $p14^{ARF}$ to bind to MDM2 under conditions of JMY, or JMYΔP over-expression raises the possibility that JMY influences the cellular interactions of

$p14^{ARF}$. It is also evident that the MDM2 in complex with $p14^{ARF}$ affects $p14^{ARF}$ mobility and presumably either stability or post translation modification.

5.7. JMY regulates $p14^{ARF}$ nuclear localisation.

The ability of $p14^{ARF}$ to act as a regulator of p53 is closely associated with its characteristic nucleolar localisation pattern (Tao and Levine, 1999b; Zhang and Xiong, 1999). Tumour mutations mapping to the nucleolar localisation signal in exon 2 of $p14^{ARF}$ are known to prevent the $p14^{ARF}$ mediated stabilisation of p53 (Soufir *et al.*, 1998; Holland *et al.*, 1999). Potentially, $p14^{ARF}$ ability to regulate the cell cycle may invoke its capability to be localised in the nucleolus and given that JMY influences $p14^{ARF}$'s capacity to interact with MDM2 it was of interest to determine the influence of JMY on $p14^{ARF}$ cellular localisation.

To address the role of JMY on the intracellular localisation of $p14^{ARF}$ heamagglutin (HA) tagged JMY, JMYΔP and JMYΔC were expressed together with $p14^{ARF}$ by transient transfection in SAOS-2 cells. JMY, JMYΔP and JMYΔC in the absence of p53, as previously shown, are predominantly localised throughout the cell with both the cytoplasm and nucleus showing an equal intensity of staining (Figure 5.7 b, d, and f). In cells overexpressing $p14^{ARF}$ the characteristic nucleolar localisation pattern previously observed was visible, with a small population expressing nuclear $p14^{ARF}$ (Figure 5.6a and Figure5.7a). Under the conditions of the assay endogenous $p14^{ARF}$, which is expressed in SAOS-2 cells, could be occasionally visualised. Given that SAOS-2 lack p53 and pRb it can be assumed that a major regulatory pathway involved in $p14^{ARF}$ expression has been lost in these cells (Stott *et al.*, 1998). In order

to focus on the effects of exogenous JMY and $p14^{ARF}$ the protein sub-cellular localisation of only the transfected population of cells was examined.

The over-expression of JMY in SAOS-2 cells produced a characteristic whole cell-staining pattern, with 66 % of cells showing both nuclear and cytoplasmic staining (Figure 5.7b). In JMY and $p14^{ARF}$ overexpressing cells the subcellular localisation of JMY remained unaffected, however $p14^{ARF}$ localisation shifted (Figure 5.6b). Only 40% of JMY/$p14^{ARF}$ overexpressing cells displayed the characteristic nucleolar $p14^{ARF}$ localisation, with 40% now displaying a nuclear pattern for $p14^{ARF}$. Interestingly in these cells a population of $p14^{ARF}$ was also relocalised to the cytoplasm (7%) and a group of cells showed the characteristic whole cell localisation (13%) predominately visualised with JMY alone (Figure 5.6b and Figure5.7c).

Even though an *in vivo* interaction between JMYΔP and $p14^{ARF}$ was undetected a similar relocalisation pattern of $p14^{ARF}$ staining was observed with JMYΔP over-expression (Figure 5.6c). Although the retention of $p14^{ARF}$ in the nucleolar speckles (52%) was greater in the presence of JMYΔP, a shift in 48% of cells was observed. Over-expression of JMYΔP shifted 32% of $p14^{ARF}$ nuclear and 6% cytoplasmic (Figure 5.6c and Figure 7e). Similarly to JMY no detectable shift in JMYΔP subcellular localisation was observed in the presence of $p14^{ARF}$ (Figure 5.6c).

JMYΔC was more effective in the translocation of the $p14^{ARF}$ nucleolar signal (Figure 5.6d). As JMYΔC was more effective at binding $p14^{ARF}$ *in vitro* it is interesting that over 68% of cells observed a shift in $p14^{ARF}$ signal upon JMYΔC introduction into cells. JMYΔC shifted the $p14^{ARF}$ signal with 41% of cells staining nuclear, 5%

cytoplasmic and 22% whole cell (Figure 5.6d and Figures 5.7g). Again in the presence of $p14^{ARF}$ the cellular localisation of JMYΔC remained unchanged (Figure 5.6d).

Given that the ability of $p14^{ARF}$ to act as a cell cycle regulator is closely linked to its ability to translocate into nucleolar speckles it is interesting to observe that JMY, JMYΔP and JMYΔC significantly disrupted $p14^{ARF}$ nucleolar staining (Figure 5.6 and Figure 5.7). Translocation of $p14^{ARF}$ by JMY resulted in an increase in $p14^{ARF}$ stability, transcription or translation as cells in which $p14^{ARF}$ localisation had been altered showed a definite increase in the level of the $p14^{ARF}$ signal (Figure 5.1 and 5.6). Together these observations provide a clue as to a potential physiological role of JMY in $p14^{ARF}$ function.

5.8. $p14^{ARF}$ in cell cycle regulation.

The ability of $p14^{ARF}$ to induce a p53-dependent cell cycle G_1 arrest is well documented. As previously shown JMY is known to participate in the cellular response to DNA damage by inducing p53-dependent transcription and apoptosis. The ability of JMY to disrupt $p14^{ARF}$ nucleolar localisation suggests that JMY may function as a regulator of $p14^{ARF}$. Given these observations a fundamental question is the effect of JMY on $p14^{ARF}$ mediated cell cycle arrest.

To this end, SAOS-2 cells were transiently transfected with a combination of $p14^{ARF}$, JMY and p53. The cell cycle stages of transfected SAOS-2 cells were monitored by FACS analysis in cells showing positive expression of a transfected cell surface CD20 marker. Propidium Iodide was used to monitor the cellular DNA content.

As previously shown the introduction of p53 into SAOS-2 cells caused a G_1 arrest (Figure 5.8a). Introduction of JMY produced a slight increase in the number of cells in G_1 (Figure 5.8c). In agreement with others the introduction of $p14^{ARF}$ into SAOS-2 cells, in a p53 negative background, had little or no effect on the population of G_1 cells (Stott *et al.*, 1998) (Figure 5.8b). As previously shown JMY and p53 co-operated in p53 mediated G_1 arrest (Figure 5.8d). $p14^{ARF}$ and p53 co-operated in the induction of G_1 arrest presumably as a result of increased p53 stabilisation (Figure 5.8f). $p14^{ARF}$ did not alter the effect of JMY on the level of G_1 arrested cells as the same level of cells were observed in the presence of JMY/$p14^{ARF}$ as with JMY alone (Figure 5.8e). The re-introduction of p53 into the JMY/$p14^{ARF}$ expressing cells restored the dominant p53/JMY G_1 arrest phenotype (Figure 5.8g).

Notably, cells overexpressing p53, $p14^{ARF}$ and JMY simultaneously, displayed a higher proportion of cells in S-phase than those expressing p53 and $p14^{ARF}$, $p14^{ARF}$ and JMY or p53 and JMY (Figure 5.8). The level of S-phase seen in p53, $p14^{ARF}$ and JMY overexpressing cells was equivalent to that observed in p53 overexpressing cells (Figure 5.8a). However the in my hands the measurement of S-phase by flow cytometry proved inconsistent and variable and so although notable, no clear conclusions can be drawn from the levels of S-phase at present (Figure 4.10 and Figure 5.8).

Together these results confirm earlier findings that JMY's cell cycle regulatory effect is dominant over $p14^{ARF}$. Interestingly JMY and $p14^{ARF}$ were unable to co-operate in

the p53 induction of G_1 arrest implying that the JMY's and $p14^{ARF}$ mechanisms of inducing a p53 response are similar.

5.9. Conclusions.

$p14^{ARF}$ stands at the nexus of the MDM2/p53 regulatory pathway. The ability of $p14^{ARF}$ to bind and sequester MDM2 into nuclear bodies is believed to be a major cellular controlling event in the release of p53 regulation (Zhang and Xiong, 1999). Evidence presented here supports that of others in that E2F transcriptionally regulates $p14^{ARF}$ expression (Bates *et al.*, 1998; Roberston and Jones, 1998). In addition $p14^{ARF}$ itself appears to down-regulate E2F mediated transcription and hence it's own transcription.

The results presented here implicate JMY in the transcriptional up-regulation of E2F responsive genes and specifically $p14^{ARF}$, potentially through its ability to bind p300. Functionally JMY can physically interact with $p14^{ARF}$ and is present in the $p14^{ARF}$ *in vivo* complex. Over-expression studies have demonstrated that JMY can affect $p14^{ARF}$ function as a consequence of its ability to sequester $p14^{ARF}$ from nuclear bodies in to the nucleus. A potential consequence of JMY's actions on $p14^{ARF}$ function is the abolishment of $p14^{ARF}$ ability to bind MDM2. In addition MDM2 specifically binds to as yet unknown forms of $p14^{ARF}$ (Figure 5.9).

Figure 5.1. Activation of the Exon1β reporter.

a). Organisation of the human $p14^{ARF}$ exon 1β luciferase reporter promoter construct. Putative transcription factor binding sites for Sp1 (•) and the four potential anti-sense strand E2F(—) sites within the exon $p14^{ARF}$ 1β promoter region from +59 to –805 are indicated. The E2F sites at –265 and +27 are good matches to the binding consensus site for E2F (-TTTCCCGCC$^{A}/_{T}$$^{A}/_{T}$-) whereas the sites at –249 and –69 are poor matches. The indicated initiation codon for luciferase expression is located at +59 and the previously mapped transcriptional start site within the $p14^{ARF}$ reporter 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). The promoter has an observed CpG content of 0.85 over a 2400bp region downstream from the Initiator element (+1) (Robertson and Jones, 1998).

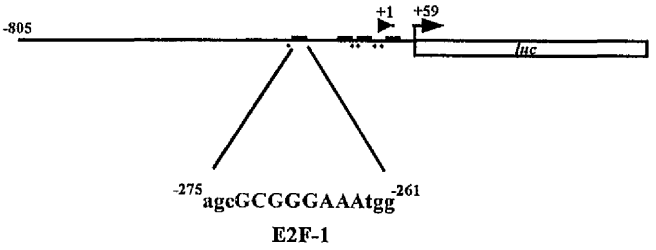
b). JMY *trans*activation of the $p14^{ARF}$ promoter.

SAOS-2 cells were transfected with 1ug of exon 1β luciferase reporter vector together with 100ng of the E2F-1 transcription factor expression vector. Co-activation of expression was achieved with the addition of increasing amounts of JMY and JMYΔP expression vectors in the quantities 1μg (+), 3μg (++) and 5μg (+++) respectively. Relative activity is depicted by luciferase normalised relative to the level of a β-galactosidase internal control.

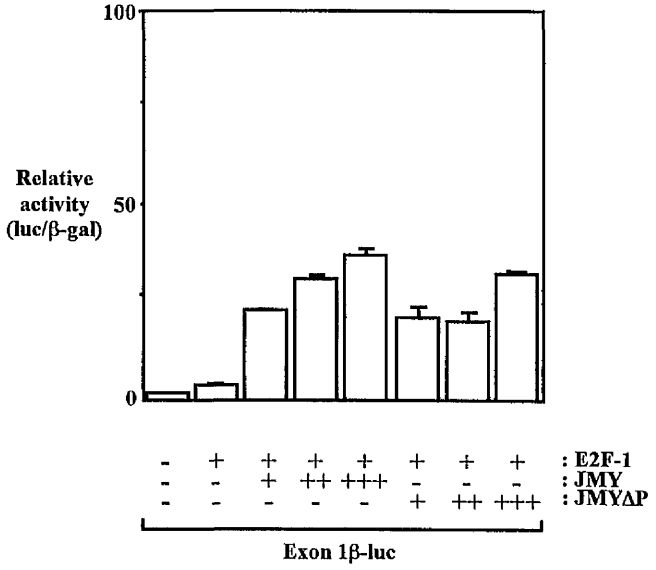
c). Co-activation of $p14^{ARF}$ transcription by p300.

SAOS-2 cells were transfected with exon 1β luciferase (1ug) together with the activator E2F-1 (100ng) and increasing quantities of JMY or JMYΔP vectors as described in figure 5.1b. In addition 5μg of the expression vector for p300 was included. The values shown are the average of duplicate readings and represent the relative level of luciferase to a β-galactosidase internal control. The relative expression seen in figure 5.1b is plotted in scale with figure 5.1c.

a)


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

b)


100
Relative activity (luc/β-gal)
50
0
- + + + + + + + : E2F-1
- - + ++ +++ - - - : JMY
- - - - - + ++ +++ : JMYΔP
Exon 1β-luc

c)

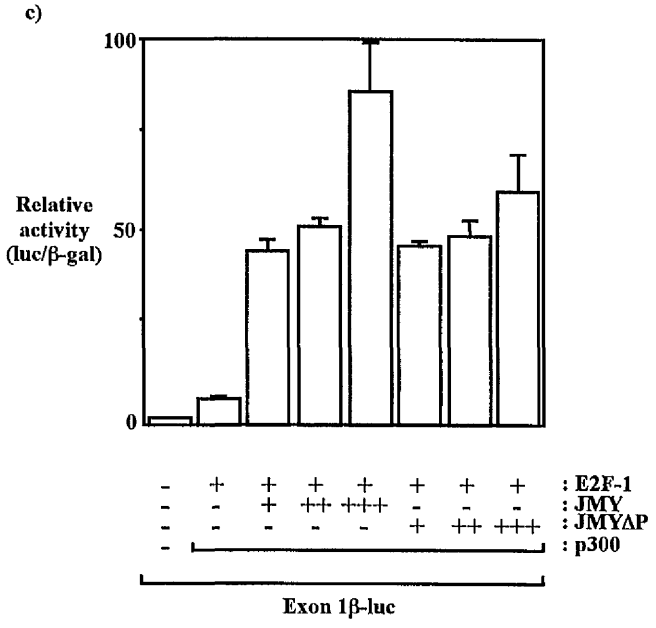

100
Relative activity (luc/β-gal)
50
0
- + + + + + + + : E2F-1
- - + ++ +++ - - - : JMY
- - - - - + ++ +++ : JMYΔP
- : p300
Exon 1β-luc

Figure 5.2. Self regulation of p14*arf* expression.

a). $p14^{ARF}$ transcriptional regulation.

Exon 1β luciferase reporter (1μg), E2F-1 (100ng), and JMY (2μg) expression vectors were introduced separately and in combination as indicated into SAOS-2 cells by transient transfection. The regulatory effect of $p14^{ARF}$ was studied by the introduction of a $p14^{ARF}$ expression vector at 5μg into each treatment. Relative activity represents the level of transcription of luciferase to a β-galactosidase internal control.

b). Specificity of $p14^{ARF}$ transcription regulation.

The p53 responsive reporter construct, pBax-luciferase (500ng) was introduced into SAOS-2 cells together with expression vectors for p53 (50ng) and JMY (2μg). Relative transcriptional activity of the reporter was measured in the presence and absence of 5μg of $p14^{ARF}$ expression vector. The expression of luciferase was normalised to an internal β-galactosidase control gene.

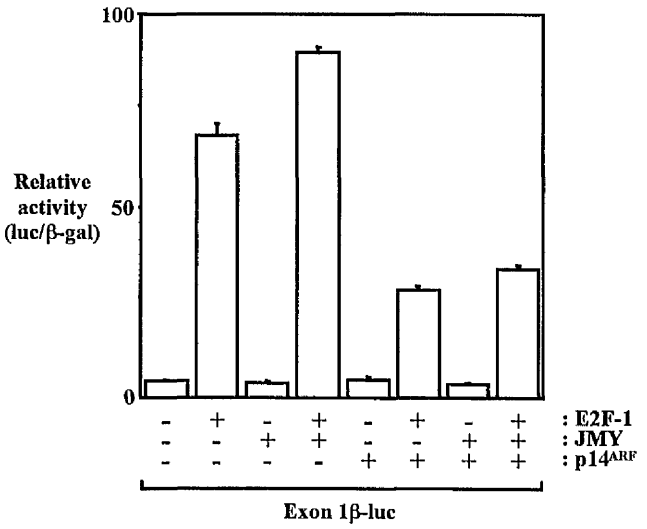
c). Quantification of $p14^{ARF}$ transcriptional effect.

Activity levels from figure 5.2a and figure 5.2b were quantified relative to basal reporter activity and calculated as a fold induction or repression. Fold activity represents the increase in expression relative to the unstimulated reporter, where reporter alone equals one. Fold repressions by $p14^{ARF}$ were calculated relative to the addition of the relevant component to the reporter.

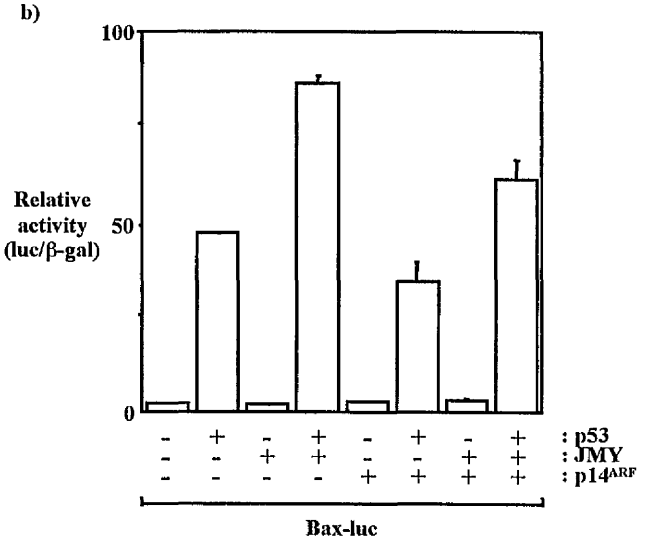
d). $p14^{ARF}$ effect on p53 stability.

SAOS-2 cells were transfected with the expression vectors for p53 (50ng) and $p14^{ARF}$ (5μg) as indicated. Cell extracts were immunoblotted with an anti-p53 antibody and a specific 53kDa polypeptide was detected as indicated.

a)



b)



c)

	Exon 1β-luc			Bax-luc		
	[1]Fold Activity (rel. reporter)		[2]Fold repression By $p14^{ARF}$	[1]Fold Activity (rel. reporter)		[2]Fold repression By $p14^{ARF}$
E2F-1	:	16.6	-	p53	:	22.4	-
JMY	:	0	-	JMY	:	0	-
E2F-1 / JMY	:	22.1	-	p53 / JMY	:	47.9	-
$p14^{ARF}$	:	0	-	$p14^{ARF}$	:	0	-
E2F-1 / $p14^{ARF}$	:	5.9	2.5	p53 / $p14^{ARF}$	:	13.3	1.3
JMY / $p14^{ARF}$	:	0.1	1.1	JMY / $p14^{ARF}$	:	0	0.5
E2F-1 / JMY / $p14^{ARF}$	:	9.4	2.7	p53 / JMY / $p14^{ARF}$	:	19.2	1.4

d)

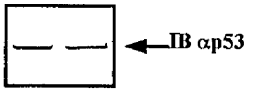


Figure 5.3. JMY interacts with p14^{ARF}.

a). GST-p14^{ARF} purification.

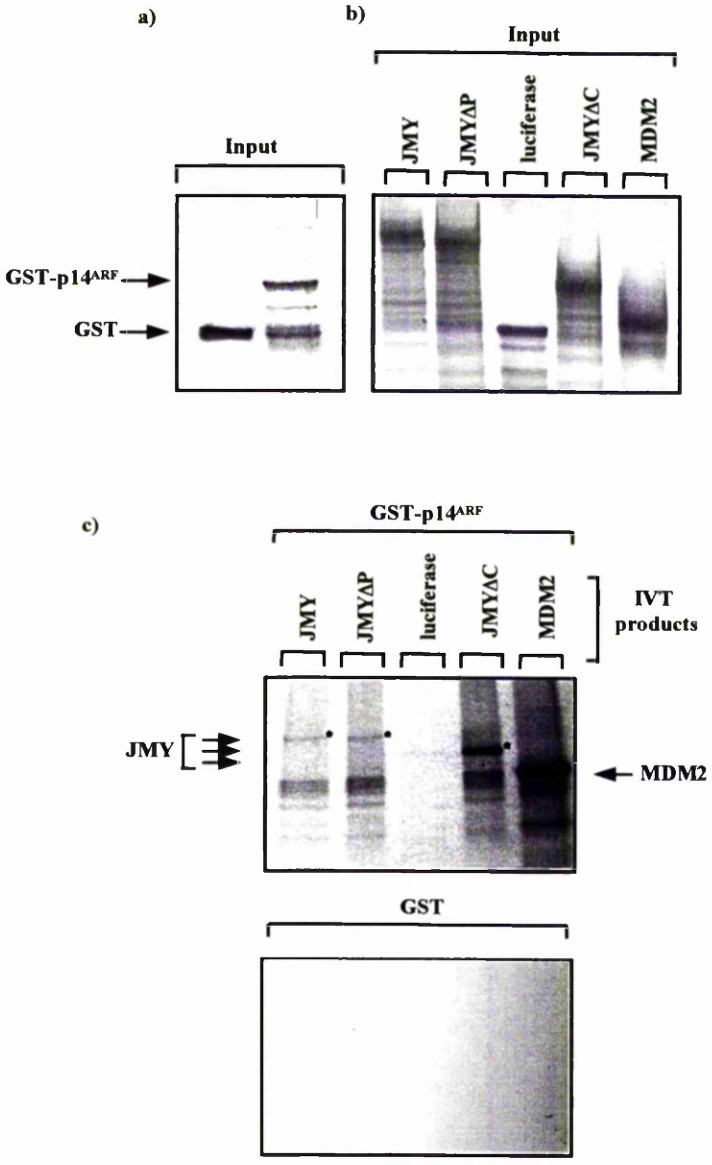
10% SDS PAGE coomassie stained gel of bacterially expressed and purified GST (500ng) and GST-p14^{ARF} (500ng). As indicated, pull-down assays were performed with 500ng of GST or GST-p14^{ARF}.

b). Translation products.

JMY, JMYΔP, JMYΔC, luciferase, and MDM2 were all expressed and ^{35}S methionine labelled *in vitro* from a promoter containing a T7 polymerase site (Promega). A 10 % SDS PAGE gel shows 20% of the input level of each protein used for binding assays to GST and GST-p14^{ARF}.

c). p14^{ARF} specifically binds JMY.

Either GST-p14^{ARF}, upper panel or GST protein alone, lower panel were incubated with *in vitro* translated JMY, JMYΔP, JMYΔC, MDM2, or luciferase from figure 5.3b. The bound products were separated and loaded on a 10% SDS PAGE gel. After separation the binding was assayed by autoradiography. The presence of JMY specific *in vitro* products is represented by (•). The binding of MDM2, which acts as a known p14^{ARF} partner is indicated



• JMY specific bands

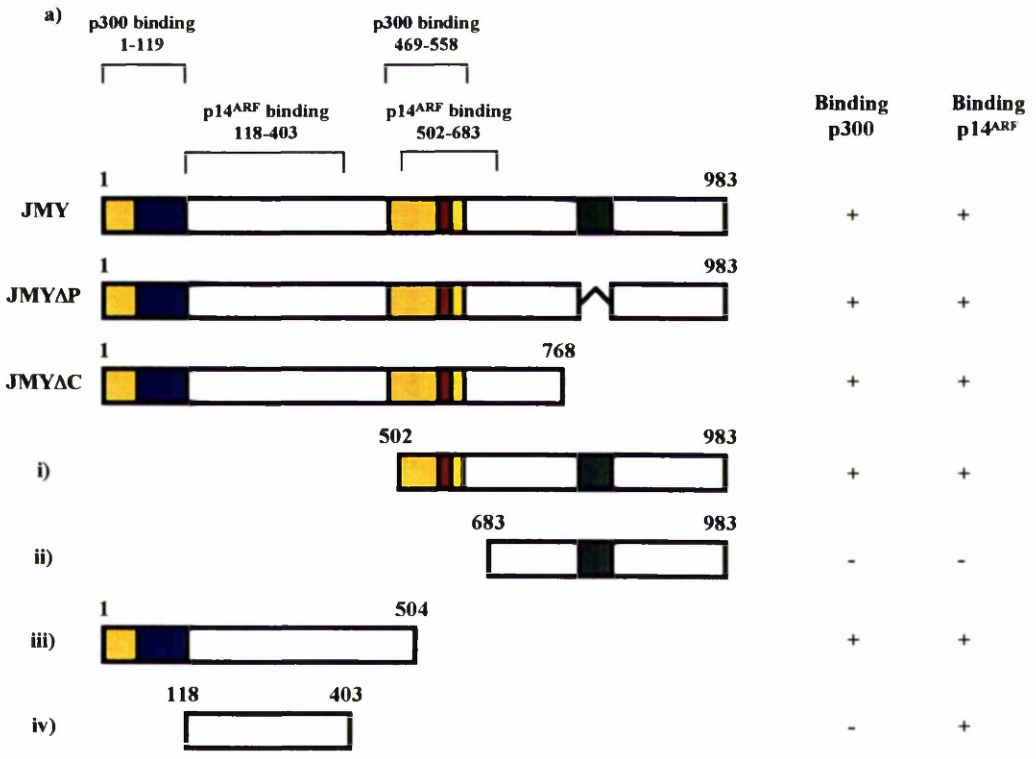
Figure 5.4. Region of JMY required for the interaction with $p14^{ARF}$.

a). Diagrammatic summary of functional domains in JMY.

Diagrammatic representation of JMY, JMYΔP and the truncation mutants used for *in vitro* domain mapping. The proline rich region in the C-terminus is highlighted (green) together with the location of potential N-terminal S/T-P motif (blue). The E1A CR2 like central motif is indicated (red) as well as the two, N-terminal$^{1-119}$ and central$^{469-558}$, p300 binding domains (yellow). Indicated is the p300 binding capacity of each of the constructs determined previously (Shikama *et al.*, 1999) together with a representation of the $p14^{ARF}$ binding data. The two identified $p14^{ARF}$ binding regions in JMY are highlighted, at $JMY^{118-403}$ and $JMY^{502-683}$.

b). Specific domains in JMY mediates its $p14^{ARF}$ interaction.

JMY constructs corresponding to i, ii, iii and iv from figure 5.4a were *in vitro* translated using the Promega T7 TNT coupled system. 20 % of each construct was loaded as a control (IN). GST-$p14^{ARF}$ (500ng) was used to determine the binding efficiency of each of the JMY truncation mutants and GST alone (500ng) was used to control for non-specific binding. $JMY^{502-983}$ and JMY^{1-504} bound to GST-$p14^{ARF}$ with similar efficiencies, at a level corresponding to 20% of input, while $JMY^{118-403}$ showed a low affinity for GST-$p14^{ARF}$ at a level of approximately 5% input.


a)
p300 binding
1-119
p300 binding
469-558
p14ARF binding
118-403
p14ARF binding
502-683
Binding
p300
Binding
p14ARF
1
983
JMY
+
+
1
983
JMYΔP
+
+
1
768
JMYΔC
+
+
502
983
i)
+
+
683
983
ii)
-
-
1
504
iii)
+
+
118
403
iv)
-
+

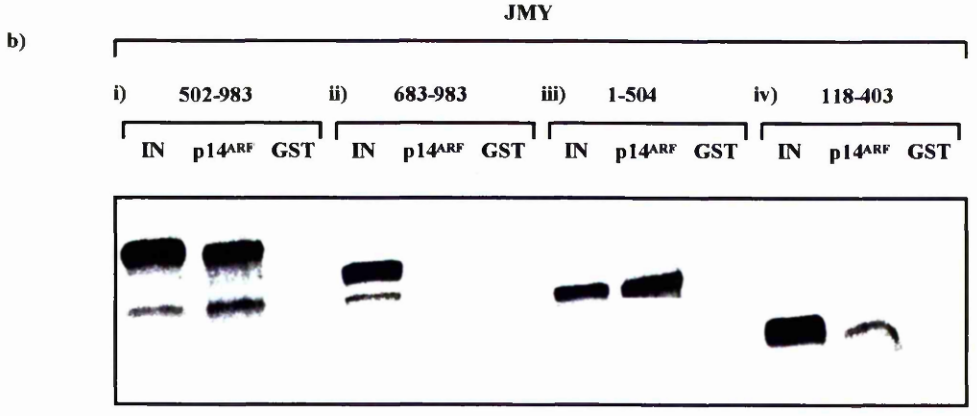

b)
JMY
i) 502-983
ii) 683-983
iii) 1-504
iv) 118-403
IN
p14ARF
GST
IN
p14ARF
GST
IN
p14ARF
GST
IN
p14ARF
GST

Figure 5.5. JMY physically associates with $p14^{ARF}$ in mammalian cells.

a). Over-expression of JMY and $p14^{ARF}$

SAOS-2 cells were transiently transfected with vectors encoding $p14^{ARF}$ (5μg) and either HA-JMY (5μg) or HA-JMYΔP (5μg). Immunoblot analysis was performed with mouse anti-HA(11) and mouse anti-$p14^{ARF}$. The position of the 110kDa HA-JMY and 105kDa HA-JMYΔP polypeptides is indicated together with the 14kDa $p14^{ARF}$ specific polypeptide. The level of endogenous $p14^{ARF}$ was too low for detection.

b). $p14^{ARF}$ interacts with JMY *in vivo*

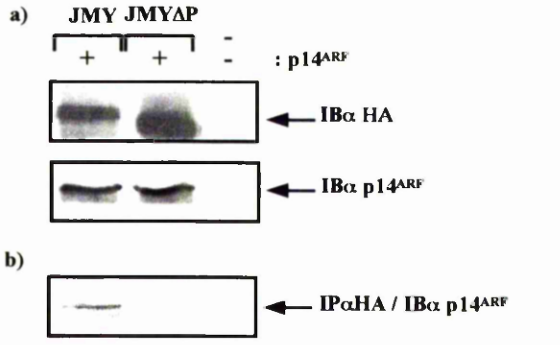
Protein extracts were immuno-precipitated with mouse anti-HA antibody. Following separation the immuno-precipitates were analysed for $p14^{ARF}$ binding using a mouse anti-$p14^{ARF}$ specific antibody. A specific 14kDa $p14^{ARF}$ polypeptide is indicated in the immuno-precipitate from JMY overexpressing cells. No $p14^{ARF}$ was detected in the immuno-precipitates from non-transfected or JMYΔP overexpressing cells.

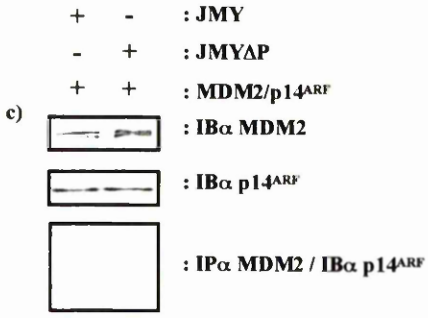
c). JMY prevents the $p14^{ARF}$/MDM2 interaction *in vivo*.

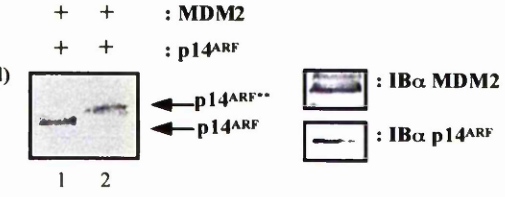
SAOS-2 cells were transfected with 5μg of $p14^{ARF}$ and 5μg MDM2 together with either 5μg of JMY or JMYΔP. The levels of MDM2 and $p14^{ARF}$ expression were monitored using mouse anti-MDM2 and mouse anti-$p14^{ARF}$ antibodies. The immuno-precipitation of $p14^{ARF}$ with a mouse anti-MDM2 antibody was performed and the level of $p14^{ARF}$ detected by immunoblotting with an anti-$p14^{ARF}$ antibody.

d). MDM2 binds a distinct form of $p14^{ARF}$.

SAOS-2 cells transfected with 5ug of $p14^{ARF}$ and 5μg of MDM2 were immuno-precipitated with either 1: anti-$p14^{ARF}$ or 2: anti-MDM2 antibodies. The immuno-precipitates were resolved on a 10% SDS gel and subjected to immunoblotting with an anti-$p14^{ARF}$ antibody. A higher mobility form of $p14^{ARF}$ was resolved in MDM2 immuno-precipitates and is indicated by **. The level of MDM2 and $p14^{ARF}$ over-expression was detected using anti-MDM2 and anti-$p14^{ARF}$ specific antibodies







1 :IPα p14ARF / IBα p14ARF

2 :IPα MDM2 / IBα p14ARF

Figure 5.6. JMY mediates $p14^{ARF}$ cellular localisation.

a). The cellular localisation of $p14^{ARF}$ in SAOS-2 cells.

SAOS-2 cells were transiently transfected with 5μg of $p14^{ARF}$ expression vector, fixed and assayed by immuno-reactivity with an anti-$p14^{ARF}$ antibody. Immuno-reactivity was visualised with a fluorescein conjugated anti-mouse immunogloblin (i). DAPI staining was included in order to confirm the position of nuclei (ii).

b). JMY effects $p14^{ARF}$ intracellular localisation.

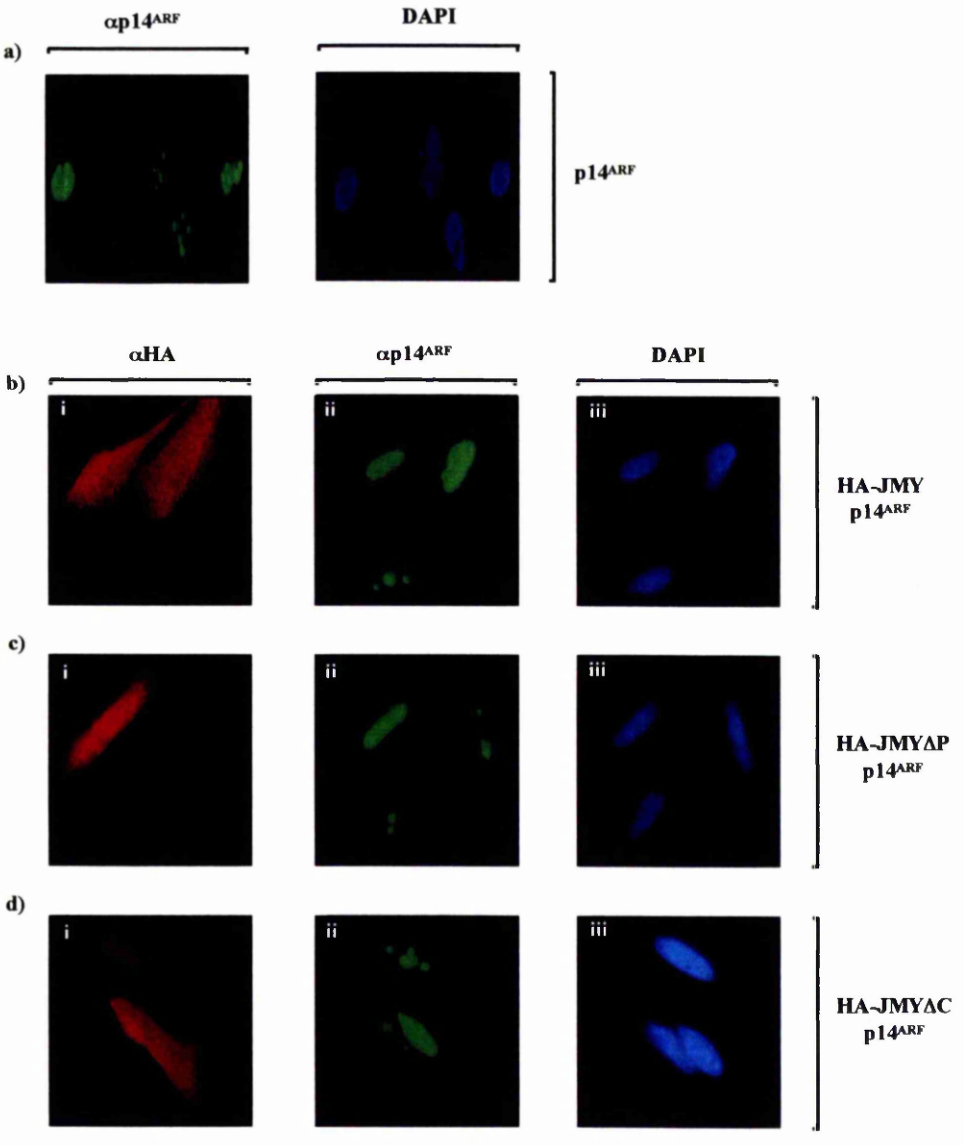
The intracellular distribution of exogenous HA-JMY and $p14^{ARF}$ in SAOS-2 cells was assessed by direct immunoflorescence. 5μg of both $p14^{ARF}$ and HA-JMY were transfected into SAOS-2 cells and their expression visualised by staining with anti-rabbit HA for JMY and anti-mouse $p14^{ARF}$. Anti-HA was visualised with a rhodamine conjugated anti-rabbit immunogloblin (ii) and $p14^{ARF}$ with a fluorescein conjugated anti-mouse immunogloblin (ii). Nuclear stain DAPI was included to confirm the position of nuclei (iii).

c). Effect of JMYΔP on $p14^{ARF}$ intracellular localisation.

SAOS-2 cells transfected with 5μg of HA-JMYΔP and 5μg of $p14^{ARF}$ were visualised for expression with anti-HA (i) and anti-$p14^{ARF}$ (ii) as in figure 5.6b. DAPI was included as a nuclear stain (iii).

d). Effect of JMYΔC on $p14^{ARF}$ intracellular localisation.

The distribution of $p14^{ARF}$ was monitored as above with the exception that 5μg of JMYΔC (i) expression vector was transfected into SAOS-2 cells with $p14^{ARF}$ (ii). Again DAPI was included for nuclear visualisation (iii).


αp14ARF
DAPI
a)
p14ARF
αHA
αp14ARF
DAPI
b)
i
ii
iii
HA-JMY
p14ARF
c)
i
ii
iii
HA-JMYΔP
p14ARF
d)
i
ii
iii
HA-JMYΔC
p14ARF

Treatment	Sample size	Nuclear		Cytoplasmic		Nucleolar		Whole Cell		
		Cell number	%	Cell number	%	Cell number	%	Cell number	%	
a) p14^{ARF}	(365)	4	1	-	-	361	99	-	-	*
b) JMY	(407)	55	14	52	20	-	-	270	66	
c) JMY / p14^{ARF}	(482)	192	40	35	7	191	40	64	13	*
d) JMYΔP	(352)	48	12	54	15	-	-	256	73	
e) JMYΔP / p14^{ARF}	(507)	162	32	33	6	262	52	50	10	*
f) JMYΔC	(412)	32	8	70	17	-	-	310	75	
g) JMYΔC / p14^{ARF}	(621)	254	41	32	5	200	32	135	22	*

Figure 5.7. Quantification of JMY's effect on p14^{ARF} localisation.
Data derived from the direct visualisation of transfected SAOS-2 cells as in figure 5.6 was quantified and expressed as percentage numbers. * indicates p14^{ARF} staining.

Figure 5.8. Effect of $p14^{ARF}$ and JMY on cell cycle arrest.

a)-g). Cell cycle effects of JMY, p53 and $p14^{ARF}$.

Flow cytometry was performed on asynchronous cultures of SAOS-2 cells transfected with the CD20 expression vector (5μg) together with p53 (3μg), JMY (5μg), $p14^{ARF}$ (5μg) or mixtures therein. Total DNA content was equivalent to 23μg and excess was made up with pcDNA-3. Transfected cells grown in 10% FCS were identified by staining with the anti-CD20 fluorescein conjugated immnuogloblin. The treatment of each cell cycle profile is indicated. Cell cycle events were quantified using CellQuest software. The percentage change in cell number for G_1, S and G_2/M were calculated as percentage change against mock transfected cells.

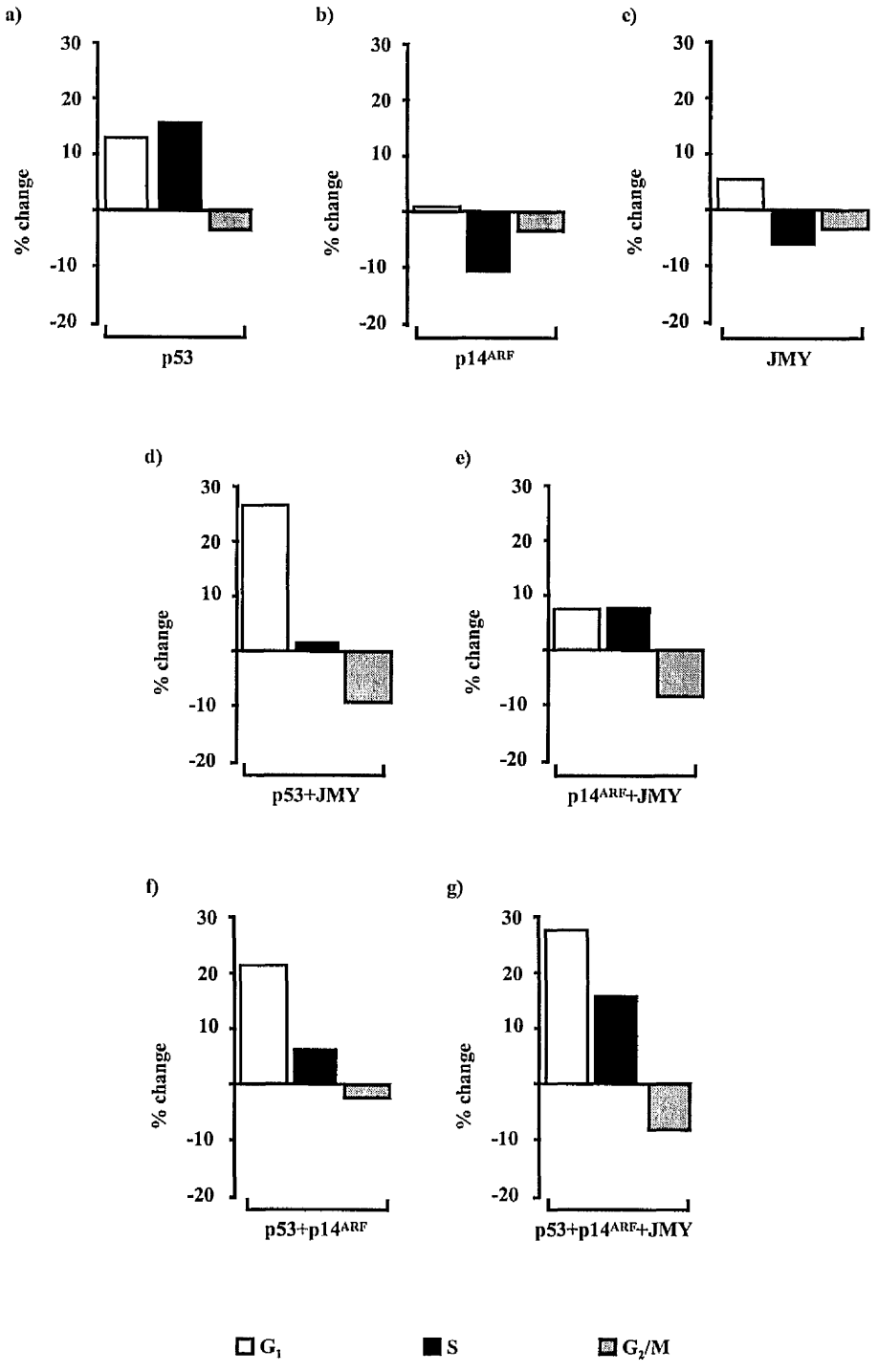
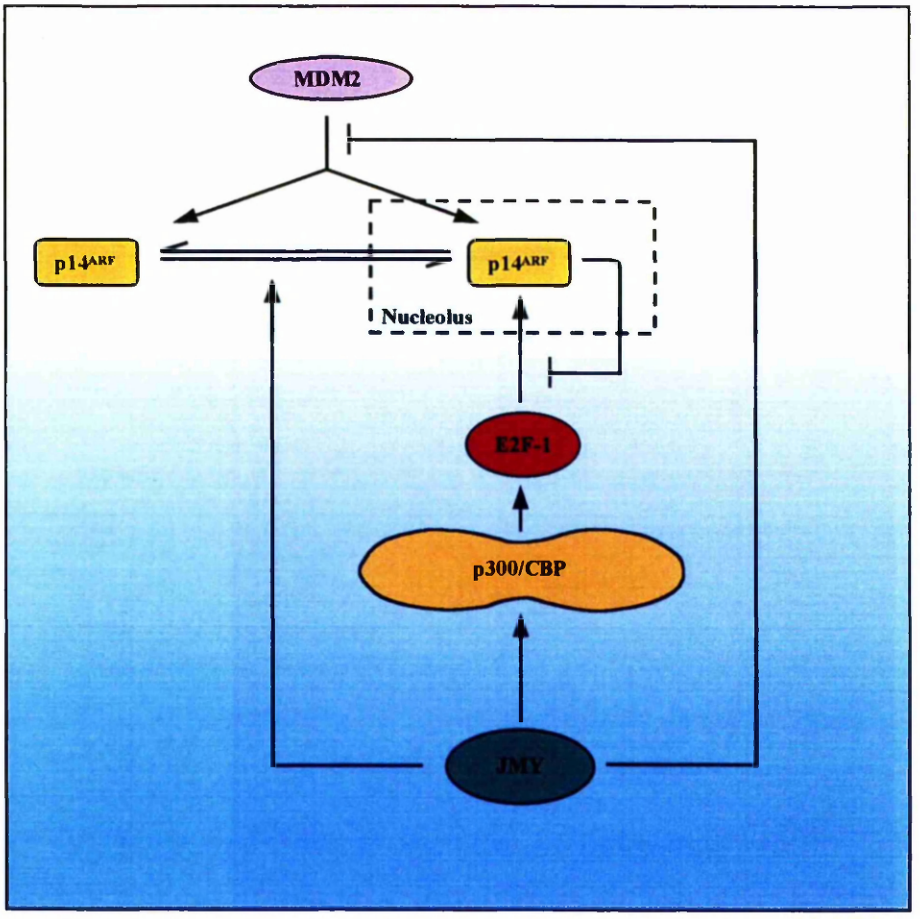

a)
b)
c)
d)
e)
f)
g)
30
20
10
-10
-20
% change
p53
p14ARF
JMY
p53+JMY
p14ARF+JMY
p53+p14ARF
p53+p14ARF+JMY
G1
S
G2/M

Figure 5.9. Summary diagram of JMY's regulation of $p14^{ARF}$.

JMY impacts on $p14^{ARF}$ in a number of ways. JMY induces the transcription of $p14^{ARF}$ via its association with p300 and their action on E2F-1 *trans*activation. JMY promotes the movement of $p14^{ARF}$ from the nucleolus to the nucleus and prevents $p14^{ARF}$ physiological association with the oncogene MDM2. In addition $p14^{ARF}$ downregulates the transcription driven by E2F-1 acting on the exon 1β promoter suggesting an auto-regulatory feedback loop for $p14^{ARF}$ expression.


MDM2
p14ARF
p14ARF
Nucleolus
E2F-1
p300/CBP
JMY

6. Transcriptional repression and activation by pRb.

6.1. Introduction.

The pRb tumour suppressor protein acts as a "master regulator" of cellular growth as a consequence of its ability to regulate the activities of transcription factors (Sellers and Kaelin, 1996). The key cell cycle controlling transcription factor family, E2F, is a physiological target of pRb's repressive function and it is the destruction of this regulatory pathway that viral oncoproteins use to exhibit their growth promoting activities (Vousden, 1995; Dyson, 1998).

The pRb pathway is frequently inactivated by mutations in tumour cells that have become immortal. Specifically the pRb protein is susceptible to mutational events that destroy its tumour suppressor function primarily due to a loss in its ability to regulate gene transcription by E2F.

Recently a number of studies have highlighted that functional cross-talk occurs between pRb and the tumour suppressor p53. It is becoming apparent that growth regulators, such as p53 and pRb, are not individually responsible for the phenotypes that they induce but rather it is the concerted effect of multiple cellular proteins that determines a cells fate. In particular it appears that the oncogene MDM2 and transcriptional co-activator family p300/CBP are key to pRb's role in the regulation of the cell cycle.

Given the conductor like role of pRb in cellular control it is of clear significance to elucidate the mechanisms of transcriptional repression that it employs. In addition pRb's ability to cross-talk with the transcriptional apparatus is undoubtedly an important mechanism by which pRb function is controlled.

6.2. pRb's repression and chromatin modulation.

A well characterised target of pRb repressive activity is the S-phase promoting transcription factor E2F (La Thangue, 1994; Dyson, 1998). The over-expression of a Gal4-E2F-$1^{380-437}$ fusion protein, that physiologically binds to pRb, effectively activates transcription from a promoter gene construct containing Gal4 DNA binding consensus sequences (Figure 6.1 and 6.2). The Gal4-E2F-$1^{380-437}$ fusion protein consists of a Gal4 DNA binding domain fused to the *trans*activation domain region of E2F-1 from amino acid 380 to 437 (Lee *et al.*, 1998). As expected pRb when overexpressed with Gal4-E2F-$1^{380-437}$ was able to dose-dependently repress Gal4-E2F-$1^{380-437}$ *trans*activation (Figure 6.1b).

Given that transcriptional repression correlates with DNA condensation and nucleosome formation it was of interest to determine the effect of nucleosome modifications on gene transcription. Acetylation is one such nucleosome modification that is believed to act by relaxing the histone/DNA interaction to facilitate the access of the transcriptional apparatus and *trans*activation (Brownell and Allis, 1996). The opposite of acetylation is the removal of acetly groups from histone tails and is termed deacetylation. Deacetylation is effectively inhibited by the naturally occurring *Streptomyces* compound trichostatin A (Figure 6.1a).

The treatment of cells with trichostatin A increases the level of basal gene transcription presumably as a result of a gross relaxation in chromatin structure (Figure 6.1 and 6.2). Interestingly the ability of pRb to repress E2F mediated transcription was completely abolished in the presence of trichostatin A (Figure 6.1c). Significantly it appears that pRb repression of E2F-1 transcriptional activation is at least in part mediated by a gross chromatin structural change that is potentially signalled through deacetylation.

Given that transcriptional upregulation by co-activators is associated with a relaxed chromatin structure and that the co-activator p300 contains an intrinsic HAT activity it was of interest to examine p300's role in pRb's repression of E2F mediated transcription. As expected and in agreement with previously published work, p300 effectively activated Gal4-E2F-$1^{380-437}$ mediated transcription in a transient reporter assay (Trouche *et al.*, 1996; Lee *et al.*, 1998) (Figure 6.1d). The ability of pRb to repress co-activated Gal4-E2F-$1^{380-437}$ transcription was un-compromised in the presence of p300, suggesting that pRb's function in E2F-1 transcriptional repression is dominant over that of p300 co-activation (Figure 6.1d). The treatment of cells with trichostatin A did not affect the level of co-activated transcription seen with p300 and Gal4-E2F-$1^{380-437}$ but completely abolished pRb's repressive function (Figure 6.1e). Interestingly the level of Gal4-E2F-$1^{380-437}$ *trans*activation was unaffected in the presence of trichostatin A suggesting that activated transcription employs maximal chromatin relaxation in terms of inhibition of deacetylation. In addition p300's ability to co-activate transcription was unaffected by the induction of hyperacetylation, by trichostatin A treatment, implying that co-activation in the context of p300 acting on the artificial promoter is outside its HAT function or that the level of acetylation is

maximal upon p300 treatment of cells. An explanation for the inability of hyperacetylation to stimulate activated and co-activated transcription is that artificial promoters are not correctly condensed into nucleosomes.

These results suggest that pRb's ability to repress E2F-1 mediated transcription is in part dependent on the chromatin structural changes. More specifically pRb's repressive effect overrides the activating effects of the p300 co-activator implying that the loss of repression is absolutely vital in transcriptional co-activation.

6.3. HDAC-1 enhances pRb's repressive function.

The active repression of gene transcription is carried out by a number of co-repressor complexes. Co-repressor complexes such as SIN3 and NCo-R act as muti-subunit platforms that are specifically targeted to gene promoters where they silence transcription. A component of the co-repressor complexes are the histone deacetylase family of enzymes (HDAC's). Histone deacetylases are families of proteins that help repress transcription by promoting the association of histones and DNA into nucleosomes. Enzymatically HDAC's as a result of their intrinsic histone deacetylation activity are able to remove the acetyl groups from histone lysine tails to promote nucleosome assembly and chromatin condensation. The deacetylation activity of HDAC's links their cellular role to one as global transcriptional repressors. Some HDAC's also contain a putative LXCXE motif that suggests a pRb interface (Kaelin, 1999).

The co-repressor complex, SIN3, interestingly also contains the pRb binding proteins pRbAp46 and pRbAp48. Given that the stimulation of hyperacetylation, by

trichostatin A treatment, resulted in a loss in pRb repressive function it was conceivable that pRb's ability to recruit and use histone deacetylase enzymes was responsible at least in part for their ability to repress transcription. To this end, the transcriptional activity of Gal4-E2F-$1^{380-437}$ in combination with pRb was studied by the co-expression of HDAC-1. Interestingly in SAOS-2 cells, that lack pRb, HDAC-1 had no effect on the level of E2F-1 mediated transcription or basal transcription (Figure 6.2). However the co-expression of pRb and HDAC-1 reduced the level of Gal4-E2F-$1^{380-437}$ mediated transcription to a level below that seen with pRb (Figure 6.1b and 6.2a). It is evident that pRb's ability to repress transcription is therefore specifically enhanced by HDAC-1 co-expression.

Given HDAC-1's ability to heighten pRb's ability to repress E2F mediated transcription it was of interest to determine if this affect was due to HDAC-1 intrinsic histone deacetylase activity. The deacetylase inhibitor trichostatin A completely abolished pRb mediated repression of E2F in the presence of HDAC-1 (Figure 6.2b). It therefore appears that HDAC-1's ability to reduce the level of E2F mediated transcription in the context of pRb corresponds to its function as a deacetylase. Interestingly HDAC-1's enhanced repression of pRb/E2F-1 was never complete; suggesting that pRb's physiological repressive function is not only through its recruitment of HDAC-1 but also relies on its previously described functions (La Thangue, 1994; Weintraub *et al.*, 1995; Dyson *et al.*, 1998; Ross *et al.*, 1999).

p300's ability to activate E2F-1 mediated transcription was unaffected in the presence of HDAC-1 and absence of pRb (Figure 6.2c). pRb's repression of E2F-1 *trans*activation in the presence of p300 and HDAC-1 was equivalent to that seen in

the absence of exogenous HDAC-1 (Figure 6.1d and 6.2c). The treatment of HDAC-1, p300 and pRb overexpressing cells with trichostatin A resulted in a level of transcription by Gal4-E2F-$1^{380-437}$ equivalent to that seen by the treatment of cells with p300 (Figure 6.1e and 6.2d).

Together, these results show that pRb repressive function is enhanced by HDAC-1. In particular, other groups have demonstrated that pRb binds directly to HDAC's in order to transiently repress transcription (Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998; Brehm and Kouzarides, 1999). In addition it appears that p300's function in the co-activation of E2F-1 transcription is directly affected by pRb, as pRb's effect dominates p300 function. pRb's effect on p300 is however dependent on deacetylation and presumably occurs via HDAC. p300 directly promotes *trans*activation while HDAC-1 appears to promote *trans*repression. Competition between these two factors helps determine pRb's ability to repress E2F-1 mediated transcription.

6.4. pRb co-operates in transcriptional activation.

pRb's role as a transcriptional repressor is well characterised, however its ability to activate transcription is poorly understood (Sellers and Kaelin, 1996). The ability of pRb, in certain circumstances, to upregulate transcription has been associated with the integrity of the pocket region and so may also play a role in pRb's suppression of tumourigenesis. An interesting feature of JMY is its ability to bind directly to pRb (Shikama, personal communication). It was therefore of interest to study the role of pRb in the JMY/p300/p53 response.

JMY in combination with p53 showed its characteristic up-regulation of the p53 responsive promoter *bax* (Figure 6.3a). Interestingly, pRb co-operated with p53 in the induction of pBax promoter driven expression implying that pRb possesses the properties of a transcriptional activator for p53 in the context of Bax expression (Figure 6.3a). The induction of p53 driven expression by pRb was further enhanced by the addition of exogenous JMY suggesting that pRb co-operates in JMY's function as a p53 co-activator (Figure 6.3a). The level of co-activation was over three fold of that seen either with JMY/p53 or with p53/pRb implying a degree of co-operation between the three proteins. Furthermore, the pRb tumour derived mutant devoid of exon 22, RbΔ22, retained the able to drive p53 mediated transcription and equally co-operated with JMY (Figure 6.3a). Given that pRbΔ22 has lost its ability to act as a G_1/S phase repressor it is interesting to speculate that the activation of the Bax promoter by pRb is a mutational event that occurs in tumour cells that prevents the proliferation of cells with deregulated growth control.

As Bax is not the only p53 responsive promoter it was of significance to study the pRb effect on another p53 responsive promoter. The $p21^{Waf1/Cip1}$ promoter is effectively co-activated by p53 expression however JMY does not significantly co-activate p53 mediated *transa*ctivation in the context of $p21^{Waf1/Cip1}$ (Figure 6.3b). pRb alone or in combination with JMY was unable to *trans*activate p53 expression of the $p21^{Waf1/Cip1}$ promoter (Figure 6.3b). The pRbΔ22 mutant is also unable to co-operate in p53 mediated *trans*activation of the $p21^{Waf1/Cip1}$ promoter (Figure 6.3b). The ability of pRb to co-operate with both p53 and JMY in the co-activation of the p53 response is therefore specific to the Bax promoter.

The co-activation of the p53 response was further analysed using mutants in both pRb and JMY. As the pRbΔ22 mutant is devoid of a region of the pocket it was of interest to examine the affect if any of the N-terminus of pRb. To this end a pRb mutant, $pRb^{379-792}$ that is deleted in the N-terminal 378 amino acids was used and scored in the reporter assay. The $pRb^{379-792}$ mutant was significantly reduced compared to wild-type pRb and pRBΔ22 in the activation of p53 mediated transcription and similarity the co-operation between JMY and $pRb^{379-792}$ in activation was also reduced relative to wild-type pRb and pRbΔ22 (Figure 6.3a and 6.4a). Together these results imply that the N-terminus of pRb is essential for maximal co-operation in the upregulated p53 *trans*activation of Bax.

Furthermore, a JMY dominant negative mutant that represses p53 mediated transcription was examined for its ability to co-operate with pRb in p53 mediated transcription. As previously shown the JMY^{1-403} mutant effectively represses p53 mediated transcription (Shikama *et al.*, 1999) (Figure 6.4b). Significantly the repressive effect of JMY^{1-403} was dominant over pRb activating effect as the co-transfection of pRb and JMY^{1-430} reduced the level of pRb/p53 mediated transcription (Figure 6.4b). Given that JMY^{1-403} potentially interferes with the endogenous p300/p53 interaction it is conceivable that pRb's activating potential is signalled through p300. Significantly the repressive activity of JMY^{1-403} was further enhanced by the co-tranfection of the $pRb^{379-792}$ mutant to levels representing basal reporter activity (Figure 6.4b). Together these results suggest that both pRb and JMY can co-operate in p53 mediated *trans*activation and that a potential intermediary molecule in this pathway is p300.

6.5. pRb influences JMY's cellular localisation.

The ability of p53 to influence JMY's cellular localisation is potentially an intregal point in JMY's ability to co-activate p53 mediated transcription. As pRb co-operates with JMY in p53 mediated *trans*activation it was of interest to determine if JMY nuclear localisation was affected by pRb.

The over-expression of p53 and JMY resulted in their characteristic co-nuclear staining pattern (Figure 6.5a). pRb over-expression in SAOS-2 cells did not alter the characteristic whole cell staining pattern of JMY and pRb was as expected exclusively nuclear (Figure 6.5b). Interestingly the over-expression of p53, pRb and JMY did not affect p53 nuclear localisation but JMY's nuclear staining pattern in the presence of p53 was disrupted and a cytoplasmic staining population of JMY was clearly visible in cells overexpressing pRb (Figure 6.5c).

These results suggest that pRb interrupts p53's ability to influence the cellular localisation, at least in part, of JMY. Given that pRb and JMY co-operated in p53's *trans*activation it was expected that a nuclear complex between all three proteins would be visible. However the disruption of JMY's nuclear pattern by pRb suggests that the enhanced *trans*activation of p53 is mediated through a cytoplasmic intermediary path. An intriguing possibility is that pRb removes JMY to the cytoplasm where it receives the necessary signals that stimulate its function as a co-activator, a hypothesis supported by the reduced ability of JMY^{NLS} to activate p53 mediated transcription (Figure 4.3a). Although supportive many other possibilities can not be ruled out.

6.6. Conclusions.

The ability of pRb to repress transcription is clearly associated with its role and ability to bind E2F-1. Interestingly it appears that pRb in addition to its ability to directly repress E2F-1 *trans*activation is also able to utilise chromatin modulators such as HDAC-1 to bring about its repressive function. Given that co-activators such as p300 are believed to functionally upregulate the activity of transcription factors it is of significance to note that the repressive function of pRb is dominant over that of the co-activators. In addition p300's HAT activity was maximal in the activation of E2F-1 and was unaffected by the induction of hyperacetylation.

In addition to its role in transcriptional repression pRb possesses a transcriptional activating potential. Specifically pRb co-operated in JMY's ability to co-activate p53 *trans*activation of the bax promoter and interestingly this activity was independent of a region of the pocket in pRb. Given that a naturally found mutant form of pRb behaved as wild-type in the co-operation of the p53 response it is interesting to speculate that tumour cells that have lost pRb function as a gene repressor retain its ability to functionally activate transcription and so is important in its role as a tumour suppressor.

In particular the co-operation in *trans*activation of the p53 response between JMY and pRb alters the cellular localisation of JMY. The JMY/p53 nuclear population is disrupted and in particular JMY is shifted to the nucleus. The exact functional significance of JMY localisation is however unknown.

Figure 6.1. pRb mediated repression.

a). Trichostatin A.

The structure of the deacetylase inhibitor trichostatin A is shown.

b). pRb represses E2F-1.

SAOS-2 cells were transiently tansfected with the pG5-E1b luciferase vector (1μg) together with 100ng of Gal4-E2F-$1^{380-437}$. Repression was achieved by the over-expression of the pCMV-Rb expression vector in the quantities 1μg (+) and 2μg (++). The values shown represent the average of two readings of luciferase expression relative to the expression of an internal β-galastosidase control vector.

c). pRb uses deacetylases to repress E2F-1 *trans*activation.

SAOS-2 cells were transfected as in figure 6.1b. Trichostatin A was added to transfected cells at a final concentration of 150nM 10 hours prior to harvesting. Reading represents the expression level of luciferase relative to that of an internal β-galactosidase control gene.

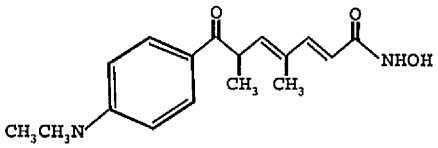
d). p300 and pRb repression.

SAOS-2 cells transfected with pG5-E1b luciferase (1μg), 100ng Gal4-E2F-$1^{380-437}$ and pRb at 1μg (+) and 2μg (++). In addition the expression vector for pCMV-p300 (5μg) was included. The relative level of luciferase and internal β-galastosidase expression was measured and plotted as relative activity.

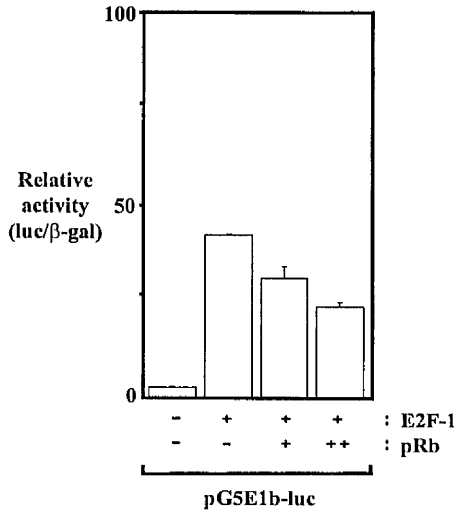
e). Trichostatin A treatment.

SAOS-2 cells were transfected as in figure 6.1d. Trichostatin A was added to transfected cells at a final concentration of 150nM 10 hours prior to harvesting. Reading represents the expression level of luciferase relative to that of an internal β-galactosidase control gene.

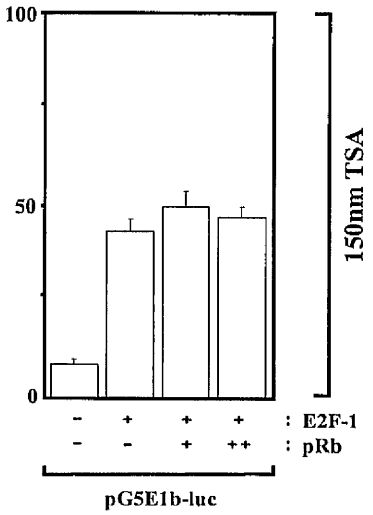
a)


O
O
NHOH
CH3 CH3
CH3CH2N

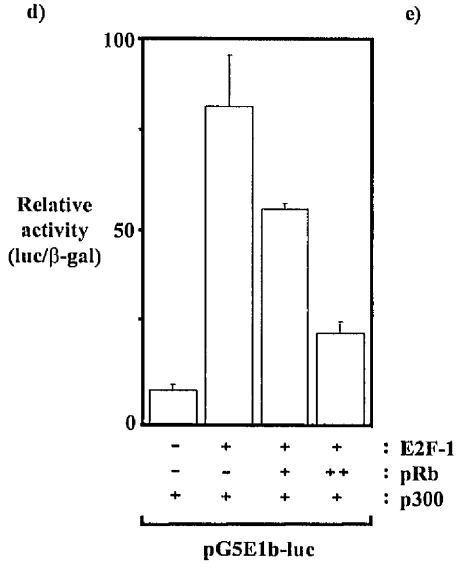
b)


100
50
0
Relative activity (luc/β-gal)
- + + + : E2F-1
- - + ++ : pRb
pG5E1b-luc

c)


100
50
0
150nm TSA
- + + + : E2F-1
- - + ++ : pRb
pG5E1b-luc

d)


100
50
0
Relative activity (luc/β-gal)
- + + + : E2F-1
- - + ++ : pRb
+ + + + : p300
pG5E1b-luc

e)

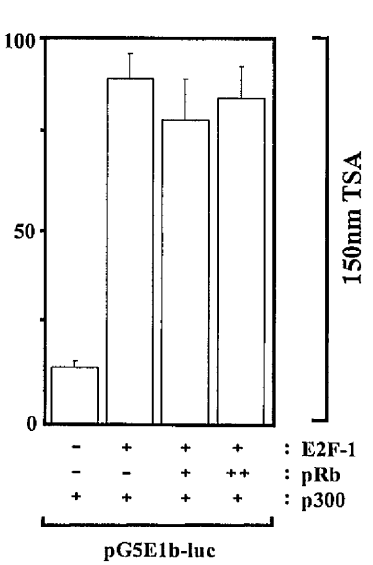

100
50
0
150nm TSA
- + + + : E2F-1
- - + ++ : pRb
+ + + + : p300
pG5E1b-luc

Figure 6.2. HDAC-1 enhances pRb mediated transcriptional repression.

a). pRb repression of E2F-1 is enhanced by HDAC-1.

SAOS-2 cells were transiently tansfected with the pG5-E1b luciferase vector (1μg) together with 100ng of Gal4-E2F-$1^{380-437}$. Repression was achieved by the over-expression of pCMV-Rb expression vector in the quantities 1μg (+) and 2μg (++) respectfully. In addition 2μg of the expression vector for HDAC-1 was included. The values shown represent the average of two readings of luciferase expression relative to the expression of an internal β-galastosidase control vector.

b). pRb/HDAC-1 repressive activity is overcome by trichostatin A.

SAOS-2 cells were transfected as in figure 6.2a except trichostatin A was added to transfected cells at a final concentration of 150nM 10 hours prior to harvesting. Reading represents the expression level of luciferase relative to that of an internal β-galactosidase control gene.

c). p300 and pRb/HDAC-1 repression.

SAOS-2 cells were transiently tansfected with pG5-E1b luciferase vector (1μg) together with 100ng of Gal4-E2F-$1^{380-437}$. Repression was achieved by the over-expression of pCMV-Rb expression vector in the quantities 1μg (+) and 2μg (++) respectfully. In addition 2μg of the expression vector for HDAC-1 and 5μg of pCMV-p300 encoding vectors were included. The values shown represent the average of two readings of luciferase expression relative to the expression of an internal β-galastosidase control vector.

d). Trichostatin A treatment.

SAOS-2 cells were transfected as in figure 6.2c except trichostatin A was added to transfected cells at a final concentration of 150nM 10 hours prior to harvesting. Reading represents the expression level of luciferase relative to that of an internal β-galactosidase control gene.

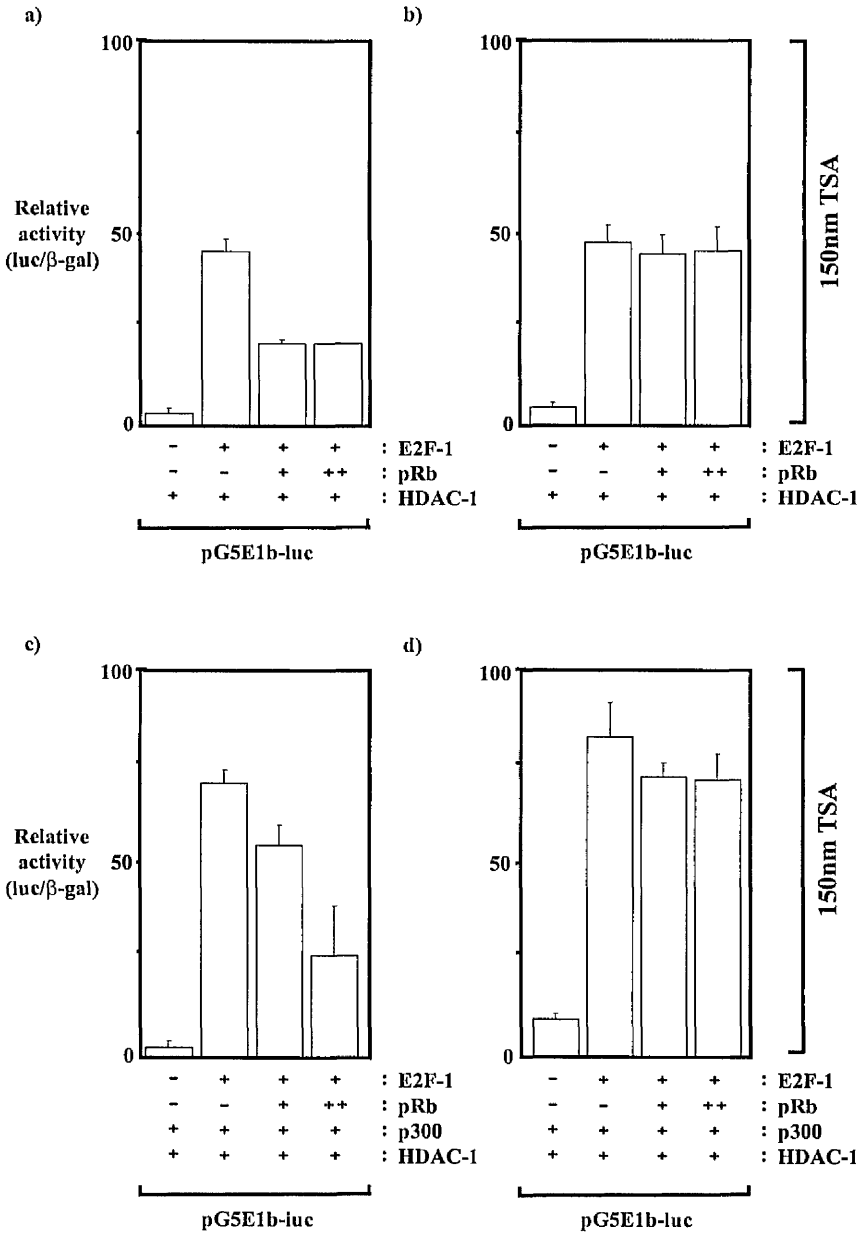

a)
b)
100
50
0
Relative activity (luc/β-gal)
150nm TSA
- + + + : E2F-1
- - + ++ : pRb
+ + + + : HDAC-1
pG5E1b-luc
c)
d)
+ + + + : p300

Figure 6.3. Co-operation between p53 and pRb in JMY co-activation.

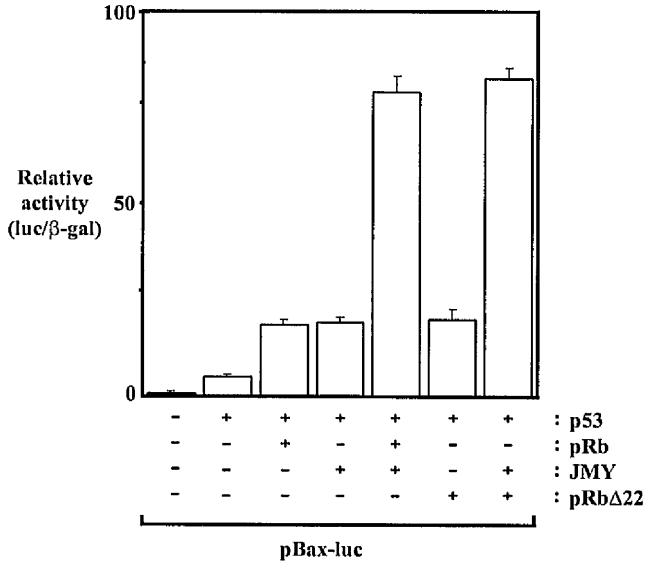
a). p53 and pRb co-operate in transcription.

SAOS-2 cells were transfected with 500ng of the p53 responsive promoter pBax-luciferase together with 50ng of p53 and 5μg of pRb or RbΔ22 expression vectors. In addition 5μg of JMY expression vector was added to the indicated treatments. Relative expression is depicted normalised relative to the level of β-galastosidase internal control.

b). p53, pRb and JMY do not co-operate in $p21^{Waf1/Cip1}$ co-activation.

SAOS-2 cells were transfected with the WWP-luciferase (1μg) reporter vector construct together with a p53 vector (50ng). In addition either CMV-pRb (5μg) or pCMV-pRbΔ22 (5μg) were added. The expression vector for JMY (5μg) was added to the indicated treatments. Relative expression is depicted normalised relative to the level of β-galastosidase internal control.

a)


100
50
0
Relative activity (luc/β-gal)
- + + + + + + : p53
- - + - + - - : pRb
- - - + + - + : JMY
- - - - - + + : pRbΔ22
pBax-luc

b)

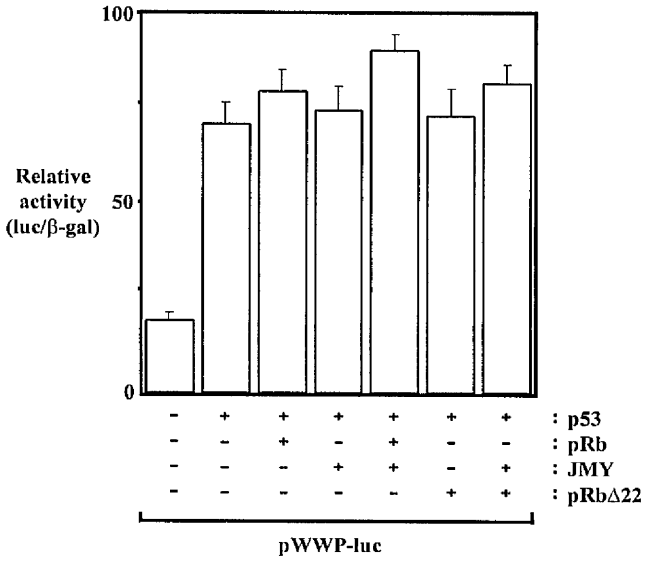

100
50
0
Relative activity (luc/β-gal)
- + + + + + + : p53
- - + - + - - : pRb
- - - + + - + : JMY
- - - - - + + : pRbΔ22
pWWP-luc

Figure 6.4. Mutational analysis of the p53, pRb and JMY co-activation affect.

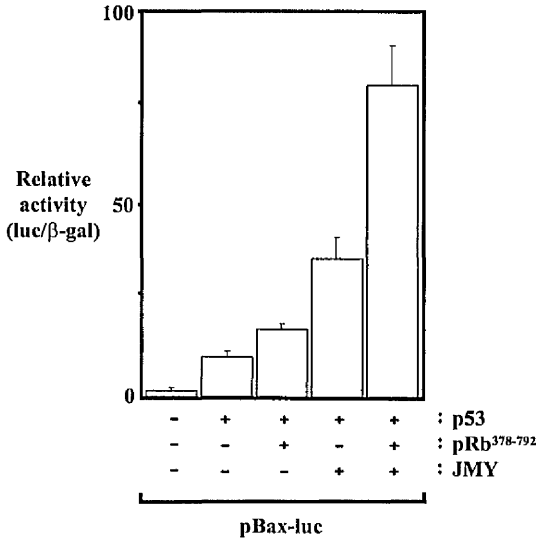
a). Requirement for pRb N-terminus in *trans*activation by p53.

The pBax luciferase (500ng) reporter vector together with a p53 (50ng) expression vector were transfected into SAOS-2 cells. In addition the expression vector for $pRb^{378-792}$ (5μg) was added either alone or in combination with a JMY (5μg) expression vector. Relative expression is depicted normalised relative to the level of β-galastosidase internal control.

b). Behaviour of a dominant negative JMY in transcriptional activation

The pBax luciferase (500ng) reporter vector together with a p53 (50ng) expression vector were transfected into SAOS-2 cells. In addition the expression vector for $pRb^{378-792}$ (5μg) was added either alone or in combination with a JMY^{1-403} (5μg) expression vector. Relative expression is depicted normalised relative to the level of β-galastosidase internal control.

a)


100
50
0
Relative activity (luc/β-gal)
- + + + + : p53
- - + - + : pRb378-792
- - - + + : JMY
pBax-luc

b)

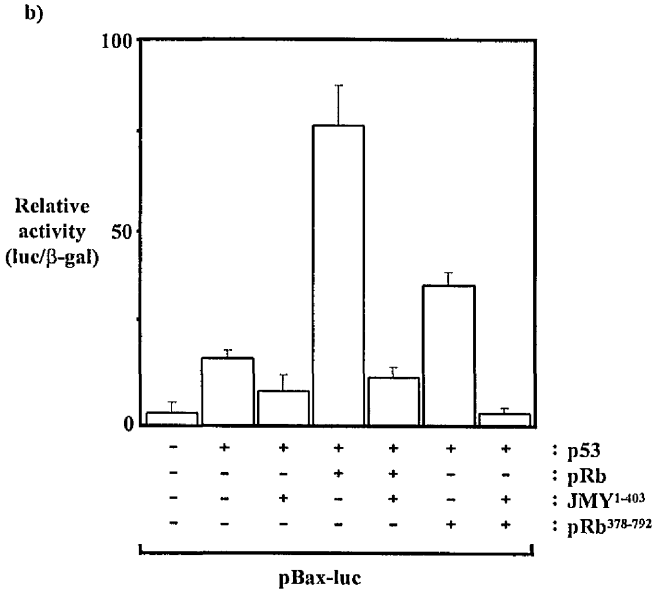

100
50
0
Relative activity (luc/β-gal)
- + + + + + + : p53
- - - + + - - : pRb
- - + - + - + : JMY1-403
- - - - - + + : pRb378-792
pBax-luc

Figure 6.5. Cellular localisation effects of pRb and p53 on JMY.

a). p53's affects JMY's cellular localisation.

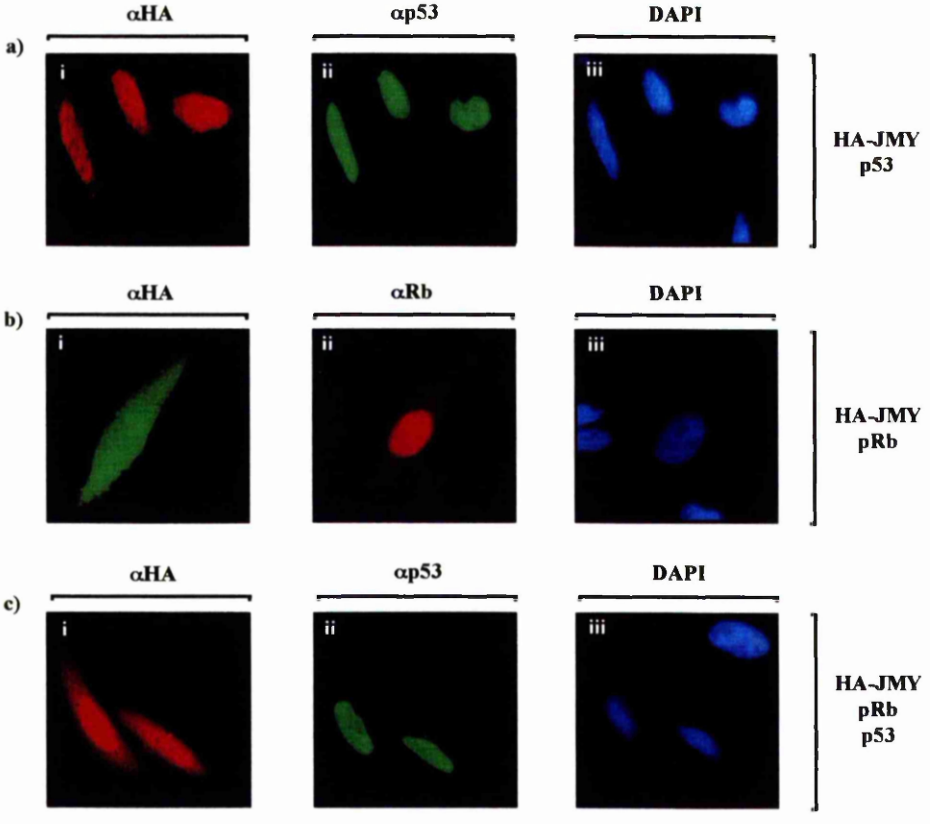
The intracellular distribution of exogenous JMY and p53 were examined by direct immunoflorescence. 5μg of HA-JMY and p53 expression vectors were transiently transfected into SAOS-2 cells and their localisation visualised by staining with an anti-mouse HA antibody for JMY and an anti-rabbit antibody for p53. Anti-HA binding was detected using a rhodamine conjugated anti-mouse immunogloblin (i) and p53 using an anti-rabbit fluorescein conjugated antibody (ii). DAPI was included to stain nuclei (iii).

b). pRb's and JMY localisation.

The intracellular distribution of exogenous JMY and pRb were examined by direct immunoflorescence. 5μg of HA-JMY and pRb expression vectors were transiently transfected into SAOS-2 cells and their localisation visualised by staining with an anti-rabbit HA antibody for JMY and an anti-mouse antibody for pRb. Anti-HA binding was detected using a fluorescein conjugated anti-rabbit immunogloblin (i) and pRb using an anti-mouse rhodamine conjugated antibody (ii). DAPI was included to stain nuclei (iii).

c). JMY, p53 and pRb.

The intracellular distribution of exogenous JMY and p53 were examined by direct immunofluorescence in cells that were also overexpressing exogenous pRb (5μg). 5μg of HA-JMY and p53 expression vectors were transiently transfected into SAOS-2 cells and their localisation visualised by staining with an anti-mouse HA antibody for JMY and an anti-rabbit antibody for p53. Anti-HA binding was detected using a rhodamine conjugated anti-mouse immunogloblin (i) and p53 using a fluorescein anti-rabbit conjugated antibody (ii). DAPI was included to stain nuclei (iii).


αHA
αp53
DAPI
a)
i
ii
iii
HA-JMY
p53
αHA
αRb
DAPI
b)
i
ii
iii
HA-JMY
pRb
αHA
αp53
DAPI
c)
i
ii
iii
HA-JMY
pRb
p53

7. Discussion.

7.1. Transcriptional co-activation by p300.

The biological activity of the p300/CBP family is at least in part dependent on their ability to augment the function of a variety of transcription factors such as, c-Jun, MyoD, CREB and E2F-1/DP-1 (Chrivia *et al.*, 1993; Arias *et al.*, 1994; Eckner *et al.*, 1996b; Perkins *et al.*, 1997; Shikama *et al.*, 1997; Lee *et al.*, 1998; Torchia *et al.*, 1998). Indeed E1A's ability to perturb the cell cycle by blocking differentiation and transcriptional control has been associated with its ability to directly interfere with p300/CBP's function as a co-activator of p53 (Avantaggiati *et al.*, 1997; Gu *et al.*, 1997; Lill *et al.*, 1997a; Lee *et al.*, 1998; Shikama *et al.*, 1997).

Specifically, here I demonstrated, in a fashion similar to others, that p300 acts as a transcriptional co-activator for both p53 and E2F-1 (Trouche *et al.*, 1996; Avantaggiati *et al.*, 1997; Lee *et al.*, 1998) (Figure 7.1). The phosphorylation of p53 in response to cellular stress is arguably a mechanism that controls its stability and activity (Siliciano *et al.*, 1997). p300 functionally acetylates p53 and increases the sequence specific binding activity of p53 (Gu and Roeder, 1997; Sakaguchi *et al.*, 1998). In addition *trans*activation domain phosphorylation of p53 stimulates its binding to p300 and consequently an increase in the level of p53 acetylation (Lambert *et al.*, 1998). Recent evidence has also demonstrated that the acetylation of E2F-1 by p300 directly influences E2F-1 DNA binding activity and stability (Martínez-Balbás *et al.*, 2000). An intriguing possibility is that the p300 co-activator complex co-ordinates and regulates the p53 and E2F-1 cellular responses.

7.2. JMY enhances p53-dependent transcription.

Evidence from mammalian cells has implicated the p300 co-activator complex as having a significant role in mediating transcription. p300's co-activator function is known to be regulated by its association with a number of proteins such as PCIP, P/CAF, and SRC-1 (Yang *et al.*, 1996; Chen *et al.*, 1997; Spencer *et al.*, 1997). Given that JMY physically associates with p300 and that p300 functionally co-activates p53 it was considered that JMY would modulate the activity of the p300/p53 response.

Analysis of a series of p53 responsive promoters demonstrated that JMY significantly co-operated with p300 in the co-activation of p53 (Shikama *et al.*, 1999). Significantly, JMY exhibited the properties of a gene specific co-activator as the *bax* and *PIG3* promoters were efficiently co-activated upon JMY over-expression. In contrast the *mdm2* and *waf1* p53 responsive promoters were largely unaffected by increased levels of JMY. It appears that JMY may show promoter specific effects *in vivo* as the co-activation of the endogenous *mdm2*, *bax* and *waf1* genes mirrored that seen with artificially reporters. Thus this study suggests that JMY performs a physiological role in co-activating the p53 response and importantly demonstrates that JMY's co-activation is more pronounced on a selective sub-group of p53 target genes (Figure 7.1).

The differential regulation of the p53 response by JMY is identical to results that demonstrate that p300 itself regulates the specificity of the p53 response for certain p53 responsive promoters (Lee *et al.*, 1998). Indeed the viral E1A oncogene has been shown to specifically target p300's co-activation of MDM2 expression (Thomas and White, 1998). It therefore appears that JMY behaves in a similar manner to its

partner, p300, in order to differentially regulate the p53 response. In fact it is conceivable that JMY's mechanism of action may account for the differential regulation of p53 inducible genes by p300.

7.3. JMY arguments p53-dependent apoptosis.

p53's ability to induce apoptosis correlates with both transcriptional dependent and independent mechanisms (Caelles *et al.*, 1994; Haupt *et al.*, 1995). The over-expression of Bax effectively induces apoptosis and the inactivation of p300/CBP can, in certain situations, prevent p53-dependent apoptosis (Oltavi *et al.*, 1993; Miyashita and Reed, 1995; Lill *et al.*, 1997a). p53's induction of the *PIG* subset of genes also correlates with the induction of apoptosis (Polyak *et al.*, 1996). In particular it appears that the induction of apoptosis following the *trans*activation of specific target genes by p53 is cell type specific, which may correlate with the presence of specific amounts of additional effector molecules, such as JMY.

Fibroblasts derived from *p300*$^{-/-}$ embryos display a profile of transcriptional defects including a defective proliferation phenotype, implying that p300 is essential for cell proliferation and development (Yao *et al.*, 1998). It therefore appears that JMY's ability to transcriptionally activate p53 gene expression may be dependent on its association with p300.

JMY efficiently co-activated *bax* and *PIG3* expression, and significantly co-operated with p53 in the induction of apoptosis. The induction of p53-dependent apoptosis by JMY is independent of p300's over-expression and interestingly p300 over expression itself is insufficient to induce p53 apoptosis (Lee *et al.*, 1998; Shikama *et*

al., 1999). Rather it appears that JMY acts together with p300 to promote p53-dependent apoptosis and implies that JMY is the physiological effector in the induction of apoptosis. Furthermore, since JMY could argument apoptosis without the addition of p300 it suggests that JMY rather than p300 is the limiting partner in p53's induction of apoptosis. These results directly imply that effector molecules such as JMY regulate the p53 response through their association with p300 (Figure 7.1).

In agreement with JMY being important in p53's induction of apoptosis it was demonstrated that the JMY *in vivo* complex contains a very high level of p53. It is interesting to speculate that through its promoter specific co-activator effects JMY acts downstream of p53's activation in the induction of apoptosis. Clearly p300 and JMY co-operate in the promoter specific activation of p53 responsive genes but their ability to bias gene expression is not yet fully understood.

p53's cellular level helps determining p53's role in cell cycle arrest and apoptosis (Macleod *et al.*, 1996; Polyak *et al.*, 1996). However JMY's ability to up-regulate p53-dependent apoptosis is independent of p53 post-translational stabilisation as JMY itself does not post-translationally activate p53. It therefore appears that a controlling factor in p53's role in apoptosis and cell cycle arrest is the presence and activity of JMY, and factors that mimic JMY's function.

7.4. The importance of 3' isoforms of JMY.

As many of the vitally important cell cycle regulators, such as the p53 family, are extensively spliced it was of significance to observe that the JMY mRNA was

alternatively spliced. Specifically the 3' region of the JMY mRNA showed diverse splicing and, in particular, the proline rich domain was a common region lost as a result of splicing. Given JMY's ability to co-activate p53's apoptotic response it was of considerable interest to observe that the splice isoform, JMYΔP, displayed properties that were functionally distinguishable from wild-type JMY. JMYΔP was unable to co-activate the *bax* promoter but, significantly, showed a preference over JMY in the co-activation of the *waf1* promoter. In agreement, the JMYΔP splice variant showed a dramatic increase in the endogenous induction of $p21^{Waf1/Cip1}$ protein levels in a p53-dependent manner.

p53's induction of cell cycle arrest is closely associated with the induction of $p21^{Waf1/Cip1}$ and block of E2F transcription (El-Diery *et al.*, 1993). It was therefore notable that JMYΔP was able to significantly co-operate with p53 in the induction of a G_1 cell cycle arrest. Conversely, JMYΔP did not affect the level of p53-dependent apoptosis. Given that p300 is required for $p21^{Waf1/Cip1}$ expression but is insufficient to induce p53-dependent apoptosis (Lee *et al.*, 1998) it has been proposed that apoptosis occurs when the level of p300 is not rate limiting. Here I propose that a second controlling event in the mediation of a p53-dependent G_1 arrest is the presence of specific JMY isoforms (Figure 7.1).

7.5. JMY controls the p53 response.

Transcriptionally inactive p53 can induce apoptosis (Caelles *et al.*, 1994; Haupt *et al.*, 1995). Here I demonstrated that the co-activation of p53 by JMY correlates well with *trans*activation and apoptosis. It is believed that the controlling switch between p53 function in apoptosis and cell cycle arrest relates to the cellular level of p53 (Gottlieb

and Oren, 1996; Burns and El-Deiry, 1999), here I clarify such an issue by demonstrating that a second controlling switch is the p300/JMY co-activator complex. Specifically p53 bound to p300/JMY will co-activate apoptotic genes, whilst p53 bound to p300/JMYΔP can induce the expression of cell cycle arrest associated genes (Figure 7.1). Even through JMY acts downstream of p53 to co-activate gene expression, it is conceivable that the signals that simulate cell cycle arrest and apoptosis also impact directly on JMY, controlling the relative intensities and activities of the JMY population.

Results presented in this study are consistent with p53's role in apoptosis and cell cycle arrest and underline the dependence of p300 in these processes. In addition it is evident that p300/CBP co-activator complex components, such as JMY, are key molecular switches that control the p53 response. The p53 family member, p73, is known to possess promoter specific *trans*activation properties and to directly interact with p300 in order to carry out its cellular functions (Lee *et al.*, 1999). Whether JMY is part of this complex is an interesting but as yet unanswered question.

Interestingly, the association of p300 with MDM2 has been linked with the regulation of MDM2 cellular function. Indeed, MDM2 forms a ternary complex with p53 and p300 and it appears that the inclusion of p300 is essential for MDM2's ability to mediate degradation of p53 (Grossman *et al.*, 1998). Thomas and White (1998) proposed that p300's co-activation and regulation of p53 mediated transcription of the *mdm2* gene is in fact an important determinant in the physiological response of p53 (Thomas and White, 1998). Hypothetically, JMY may be a mediator in this

pathway; in such a way that competition for p300 by JMY and MDM2 may augment and define the p53 response.

7.6. Translocation of JMY.

The nuclear localisation of transcription factors and co-activator molecules is essential for their effective *trans*activation. Since transcriptional co-activators exert their effects on gene transcription in the nucleus, the control of their intra-cellular localisation must be an important regulatory mechanism. JMY is predominantly a whole cell protein but in the presence of p53 the JMY signal is predominantly nuclear (Figure 7.1). However, the nuclear localisation of JMY appears not to be important for its function as a co-activator, as a constitutive nuclear JMY^{NLS} protein is less active than wild-type JMY in transcriptional co-activation. It therefore appears that it is signals that trigger JMY's translocation into the nucleus from the cytoplasm, or those which effect nuclear import or export, that are vital in regulating its role as a co-activator. Indeed it is interesting to speculate that the cellular signals that govern cell cycle arrest and apoptosis are individually responsible for the translocation of specific JMY isoforms from the cytoplasm to the nucleus. Whether JMY's localisation is signalled through the mitogenic or oncogenic pathways is unknown.

7.7. Proline rich domains and transcription.

The p53 tumour suppressor has been implicated in the regulation of multiple cellular anti-proliferative pathways including those that lead to transient cell cycle arrest as well as those that lead to permanent cell death. p53's accumulation leads to G_1 arrest, however the co-expression of cells with E2F-1 or c-Myc forces G_1 arrested cells to undergo apoptosis (Martinez *et al.*, 1991; Wu and Levine, 1994). Therefore p53

mediated events in a cell are able to detect and respond appropriately to signals generated from a variety of nuclear signal transduction pathways to mediate the appropriate cellular outcome.

Several studies have demonstrated that there are discrete classes of p53 responsive genes that determine the functional outcome of p53 activation (Friedlander *et al.*, 1996; Ludwig *et al.*, 1996). It appears that the proline rich domain, located between the *trans*activation and DNA binding domain in p53, helps discriminate the *trans*activation function of p53 (Walker and Levine, 1996; Venot *et al.*, 1998; Zhu *et al.*, 1999). The importance of the proline rich domain of p53 is highlighted by the inability of Li-Fraumeni syndrome suffers, that display mutational events within the proline domain, to undergo a normal p53 response (Sun *et al.*, 1996).

Strikingly, several studies have demonstrated that the proline rich domain of p53 imparts promoter specific functions in an identical manner to those observed for JMY. Here I have confirmed that the proline rich domain of p53 is essential for the transcriptional activation of *PIG3* but is dispensable for MDM2 and $p21^{Waf1/Cip1}$ expression. Taken together the results presented in this thesis suggest a hypothesis in which proline rich domains in transcriptional activating proteins play a vital role in promoter specificity and co-ordination of the cellular response.

Studies relating to the proline rich domain of p53 have suggest a number of potential roles for such a domain, all of which have a relevance in the understanding of JMY's function. The proline rich domain in p53 mediates an activity that is critical for its tumour suppressor function *in vivo* and demonstrates that transcriptional activation by

p53 can be uncoupled from its apoptotic growth suppression function (Walker and Levine, 1996; Venot *et al.*, 1998; Zhu *et al.*, 1999). Given that a p53ΔP mutant responds to DNA damage it is conceivable that in the case of p53 that this domain is responsible for signalling a subset of p53's downstream anti-proliferative signals that ultimately promote apoptosis (Walker and Levine, 1996). Furthermore, Zhu *et al.* (1999) conclude that the proline rich domain of p53 as a consequence of its ability to regulate p53's activation of PIG3, p85, MDM2, and PIG11, is required for activating genes that participate directly in the signalling pathways which control apoptosis. In fact p85 is involved in the apoptotic response to oxidative stress and PIG3 produces reactive oxygen species that degrade the mitochondria and subsequently stimulate apoptosis (Polyak *et al.*, 1997; Yin *et al.*, 1998). Similarly, the proline rich domain in JMY is also responsible for the co-activation of p53 responsive genes that are specifically involved in apoptosis.

The promoter specificity of the proline rich domain of p53 has been linked with the binding of p53 to low affinity DNA binding sites (Walker and Levine, 1996). Venot *et al.* (1998) demonstrated that the production of reactive oxygen species is dependent on the proline rich domain in p53 and that the binding of p53 to low affinity DNA binding sites is mediated by the proline rich domain. It is therefore conceivable that the proline rich domain of p53 functions structurally to regulate the activity of p53. In an identical manner the C-terminus of p53 is known to regulate the sequence specific binding of p53 (Hupp *et al.*, 1992). However, in the case of JMY the binding of JMY to its known partner, p300, is unaffected by the deletion of the proline rich domain. Given that prolines are hydrophobic residues that induce bends in protein structures it is conceivably that the proline rich domain in JMY may

influence the tertiary structure of JMY and the association of other p300/JMY co-activator complex components.

Walker and Levine (1996) proposed that the proline rich domain in p53 mediates the ability of p53 to interact with SH_3 domain containing proteins and that this allows the integration of signals that specifically induce transcriptional activation of a subset of genes. In fact the proline rich domain of p53 that contains five repeats of the P-X-X-P motif theoretically forms a left handed polyproline type II helix that creates a binding site for SH_3 domains (Yu *et al.*, 1994). In agreement, the murine polyproline rich domain in p53 is a known docking site for the transmission of Gas1's dependent anti-proliferative signals and mutations within the proline rich domain abolish p53 mediated *trans*activation independent growth arrest induced by Gas 1 (Ruaro *et al.*, 1997). Additionally, the SH_3 domain containing c-Abl protein stimulates a p53-dependent cell cycle arrest (Goga *et al.*, 1995). Interestingly, antibodies directed against epitopes close to the proline rich domain in p53 have been shown to modulate the DNA binding activity of p53 which lends support to the idea that this domain is a docking site for activating factors that structurally influence p53's activity (Wolkowicz *et al.*, 1995; Friedlander *et al.*, 1996).

In some cell types several reports have demonstrated that cytokines are influential in the life/death decision by p53 (Gottlieb and Oren, 1996; Ko and Prives, 1996). It is therefore speculative to associate proline rich domains with docking to SH_3 containing proteins and the separation of the life and death signals by p53 and JMY. The identification of such hypothetical SH_3 bearing proteins that impact on p53 and

JMY, via their proline rich domains, will greatly help decipher and separate the signals that control cellular life and death.

The repressive function of p53 may accelerate the induction and progression of apoptosis. In agreement the WT1 and E1B proteins that prevent p53 mediated apoptosis have been shown to inhibit p53's ability to repress transcription (Maheswaran *et al.*, 1995; Sabbatini *et al.*, 1995a; Murphy *et al.*, 1996). The absence of the proline rich domain in p53 also severely compromises p53's ability to repress transcription (Venot *et al.*, 1998), which strengthens the idea that repression is important for p53's apoptotic function. In the case of JMY, however, mutants devoid of the proline rich domain do not affect p53's ability to stimulate transcription or induce apoptosis. It therefore appears that the proline rich domain in JMY discriminates its co-activator function and plays no role in the direct down-regulation of transcription.

An intriguing possibility is that proline rich domains are regulated by post-translational phosphorylation. p53's regulation by phosphorylation is well documented and is known to influence the differential binding of p53 to various consensus sites (Wang and Prives, 1995; Lohrum and Scheidtmann, 1996). The possibility that the proline rich domains in JMY and p53 are regulated by phosphorylation is an intriguing if unconfirmed possibility.

Zhu *et al.* (1999) highlighted that p53's activation of transient reporter genes differs from that of endogenous genes, given that transiently transfected promoters are not correctly packaged in to chromatin. Zhu *et al.* (1999) also imply that the proline

promoter specific functions of p53 are reminiscent to those of MyoD, that acts as a chromatin modulator and shows differential activation of transfected promoters in transient reporter assays. Consequently they propose that the proline rich domain of p53 may be necessary for chromatin remodelling events that counteracts the chromatin mediated repression of some p53 cellular target genes. However in the case of co-activation by JMY, endogenous gene regulation was identical to that observed in transient reporter assays implying that although not excludable that chromatin modulation is not the primary function of JMY's proline rich domain.

7.8. JMY co-activates expression of the $p14^{ARF}$ gene.

The expression of the $p14^{ARF}$ tumour suppressor protein is initiated from a specific exon (1β) within the *INK4a* locus (Sharpless and DePinho, 1998). E2F-1 over-expression effectively induces expression from the exon 1β promoter (Bates *et al.*, 1998; Roberston and Jones, 1998; Inoue *et al.*, 1999) and I demonstrated that this *trans*activation is effectively co-activated by p300. Results presented here also demonstrated that the transcriptional co-activator JMY (Shikama *et al.*, 1999), potentially as a consequence of its association with p300 up-regulates E2F-1 mediated expression of the $p14^{ARF}$ promoter (Figure 7.1). It therefore appears that JMY behaves in an identical synergistic manner as p300 in that it is able to co-activate both p53 and E2F-1 mediated transcription. Consequently, the results presented in this thesis are consistent with the p300 co-activator complex being a vital component in the communication between the E2F and p53 growth controlling pathways.

$p14^{ARF}$'s ability to elicit a p53 response is manifested by an increase in expression of a number of p53 responsive genes as a result in the loss of MDM2 function (Kamijo *et al.*, 1998; Pomerantz *et al.*, 1998; Stott *et al.*, 1998; Zhang *et al.*, 1998). JMY's ability to up-regulate $p14^{ARF}$ expression will hypothetically induce p53's cellular function and therefore potentially supports a role for JMY acting not only as a p53 co-activator but also as an up-stream activator. The E2F-1 and p53 proteins are key cell cycle regulators and the balance between their activities is likely to be critical in cell survival. JMY clearly impacts on both pathways and is a potential molecule that mediates the cross-talk between the two growth controlling pathways.

E2F-1 and p53 are known to functionally co-operate in the induction of apoptosis (Qin *et al.*, 1994; Wu and Levine, 1994; Kowalik *et al.*, 1995; Lee *et al.*, 1998). Indeed E2F-1 can induce apoptosis in the absence of p53 although this apoptotic function is to some extent transcriptionaly independent and may reflect an alleviation of transcriptional repression by E2F-1 (DeGregori *et al.*, 1997; Hsieh *et al.*, 1997; Phillips *et al.*, 1997; Lee *et al.*, 1998). Furthermore, $p14^{ARF}$ is a potentially an essential intermediate in E2F-1 induced apoptosis as $arf^{-/-}$ cells are resistant to E2F-1 induced apoptosis (Zindy *et al.*, 1998). However, p300 has been shown to enhance E2F-1 mediated apoptosis and E2F-1 is a known *trans*activate the *Drosphila* cell death regulator *reaper* (Asano *et al.*, 1996). In addition E1A requires the capacity to bind both pRb and p300 in order to induce apoptosis and so it is possible that $p14/19^{ARF}$ activation is essential in apoptosis (de Stanchina *et al.*, 1998). Here I demonstrated that a potential mediator of co-operation between E2F-1's up-regulation of p53 mediated apoptosis is JMY, through its ability to up-regulate $p14^{ARF}$ expression.

Interestingly the cellular level of p300 is believed to be rate limiting (Yao *et al.*, 1998), and results presented here potentially associate the rate limiting effects of p300 with a reduced level of p300's recruitment of JMY to promoters and consequently a reduction in transcription. Whether JMY is involved in the co-activation of other E2F-1 target genes that are associated with apoptosis and cell cycle arrest events is at present unknown

The regulation of $p14^{ARF}$ expression is poorly understood but here I demonstrated that $p14^{ARF}$ itself is able to regulate its own expression in a manner reminiscent to that of p53's regulation of $p14^{ARF}$ (Robertson and Jones, 1998; Stott *et al.*, 1998). The over-expression of $p14^{ARF}$ reduces E2F-1's *trans*activation of the $p14^{ARF}$ promoter and the auto-regulatory loop is seemingly specific for the exon 1β reporter as the p53 responsive promoter, *bax*, expression was largely unaffected by $p14^{ARF}$ over-expression. Given the ability to $p14^{ARF}$ to impact on the cells fate it is not surprising that the level of $p14^{ARF}$ is tightly regulated and that $p14^{ARF}$ itself regulates its own expression. Data presented here demonstrated that $p14^{ARF}$ itself regulates its own expression in a mechanism reminiscent for that of p53/MDM2, in which $p14^{ARF}$ expression is repressed by over-expression of $p14^{ARF}$ (Figure 7.1).

The repressive effect of $p14^{ARF}$ does not require pRb as the affect was observed in SAOS-2 cells that contain no functional pRb. It is therefore conceivable that the repression of the $p14^{ARF}$ promoter by $p14^{ARF}$ occurs through a component of the E2F-1 co-activator complex and such a potential component is JMY. The ability of $p14^{ARF}$ to negatively regulate the proliferation of murine embryonic fibroblasts

independently of p53 also agrees with the data presented in this thesis (Carnero *et al.*, 2000).

E2F-1 is responsible for the transition between G_1 and S and although $p14^{ARF}$ represses E2F-1 transcription of the exon 1β promoter no change in the G_1 population of cell was visible in cells overexpresssing $p14^{ARF}$. It therefore appears that $p14^{ARF}$'s inhibitory effect is specific for E2F-1 in the context of the $p14^{ARF}$ promoter. In agreement Stott *et al.* (1998) presented identical findings, in that $p14^{ARF}$'s over-expression in SAOS-2 cells does not alter the population of cells in the different stages of the cell cycle.

7.9. Functional interaction between JMY and $p14^{ARF}$.

A potential target for $p14^{ARF}$'s inhibitory function is JMY as the two proteins physically associate *in vitro* and *in vivo*. $p14^{ARF}$'s primary function in cells appears to be the sequestration of MDM2 from the p53 regulatory pathway (Stott *et al.*, 1998) and JMY's function is in the up-regulation of p53 *trans*activation (Shikama *et al.*, 1999). Both JMY and $p14^{ARF}$ are therefore regulators of potentially the same functional outcome. However JMY appears to prevent the association of MDM2 with $p14^{ARF}$ *in vivo*.

Given that JMY physically impacts on the MDM2/$p14^{ARF}$ interaction it was interesting to observe that JMY actually relocalised $p14^{ARF}$ from the nucleolus to the nucleus. The nucleolar localisation of $p19^{ARF}$ corresponds to its ability to bind MDM2 and release p53 while $p14^{ARF}$ actually sequesters MDM2 into nuclear bodies (Tao and Levine, 1999b; Weber *et al.*, 1999; Zhang and Xiong, 1999). It is therefore

conceivable that JMY interferes with the MDM2/p14^{ARF} interaction by directly de-localising p14^{ARF} to the nucleus. In agreement with others it therefore appears that p14^{ARF}'s cellular localisation determines its function and it appears that JMY directly affects such a function via its ability to sequester p14^{ARF} into the nucleus and prevent the formation of nuclear bodies. An interesting possibility is that JMY actually enhances the p14^{ARF} effect by localising it to the nucleus from where it can directly associate with MDM2 upon the appropriate signals, and thereby release p53. Conceivably the movement of p14^{ARF} out of the nucleolus is a prerequisite for its formation of nuclear bodies, and a candidate protein that posses such a function is JMY.

The nuclear localisation of p53 is essential for its function as a transcription factor (Gannon and Lane, 1991; Shaulsky *et al.*, 1991; Kamijo *et al.*, 1998; Roth *et al.*, 1998). In cells that express high levels of p19^{ARF} high molecular weight forms of p53 are detected that likely correspond to poly-ubiquitinated forms (Pomerantz *et al.*, 1998). So it has been suggested that p19^{ARF} may not inhibit the ubiquitation of p53 by MDM2 but rather may prevent the degradation of ubiquitated p53 (Kamijo *et al.*, 1998).

Here I demonstrated that the over-expression of p14^{ARF} and MDM2 in SAOS-2 cells results in the binding of a high molecular weight form of p14^{ARF} to MDM2. The functional significance of such a species of p14^{ARF} is unknown however given that MDM2 modifies p53 by ubiquation it is interesting to speculate that MDM2 may also be regulating p14^{ARF} cellular levels in an identical manner. Similarly, Kurokawa *et al.* (1999) observed similar high mobility forms of p19^{ARF} in fibroblasts prior to G_1 arrest

and they postulate that such species correspond to either degradation intermediates or post-translational intermediates. The post-translation modification of $p14^{ARF}$ by other mechanisms can not be ruled out and a known regulatory mechanism of the p53/MDM2 interaction is phosphorylation. Whether the specific species of $p14^{ARF}$ in the MDM2 complex corresponds to a similar regulated interaction remains to be elucidated. It is also interesting to note that MDM2's stability when complexed with $p19^{ARF}$ is reduced (Zhang *et al.*, 1998; Kurokawa *et al.*, 1999; Zhang and Xiong, 1999).

Interestingly, p53's regulation of the level of $p14^{ARF}$ (Roberston and Jones, 1998; Stott *et al.*, 1998) is also evident as p53 deficient cells display an elevated level of $p14^{ARF}$ (Quelle *et al.*, 1995; Kamijo *et al.*, 1997). The repressive effect of p53 is not however specific for the *ARF* locus as p53 represses a variety of other cellular promoters such as c-fos, c-Jun, PCNA and interleukin-6. Whilst the $p14^{ARF}$ promoter is regulated by p53 and JMY it is interesting to speculate that JMY's functional association with $p14^{ARF}$ may impact on these mechanisms of regulation. The auto-regulatory feedback loop observed for $p14^{ARF}$ is potentially the mechanism that regulates the steady state level of $p14^{ARF}$ and the reason why $p14^{ARF}$ is a short-lived cellular protein. Whether JMY's association with $p14^{ARF}$ is responsible for the down-regulation of $p14^{ARF}$ expression is unknown although it is possible that such an association is one mechanism by which $p14^{ARF}$ achieves its auto-regulation.

The up-regulation of $p21^{Waf1/Cip1}$ can induce a premature senescence phenotype in human fibroblasts (McConnell *et al.*, 1998) and E2F-1 is also able to induce a sensecence like phenotype in a p53 and $p14^{ARF}$ dependent manner (Kamijo *et al.*,

1999a; Dimri *et al.*, 2000). Clearly JMYΔP co-operates with p300 in the induction of $p21^{Waf1/Cip1}$ and $p14/19^{ARF}$'s release of p53's activity will presumably also induce $p21^{Waf1/Cip1}$ expression. These observations suggest that JMY may be involved in the fail-safe program that curtails tumourigenesis and age related pathologies, although no experimental evidence exists to date.

7.10. pRb transcriptional repression.

pRb's functional repression of E2F-1 mediated transcription has been directly associated with its ability to bind and prevent the function of the *trans*activation domain of E2F-1 (Weintraub *et al.*, 1995). Here I demonstrated results in that the pRb repressive function is also dependent on its ability to utilise the histone deacetylase family of enzymes in the transient repression of E2F-1 *trans*activation (Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998; Brehm and Kouzarides, 1999). In fact I demonstrated that HDAC-1 over-expression directly enhances pRb's repressive function (Figure 7.1).

Since p300/CBP co-activates E2F-1 transcription it is possible that pRb overrides this transcription by blocking the interaction of p300/CBP with E2F-1 (Trouche *et al.*, 1996; Luo *et al.*, 1998). Previous studies have demonstrated that pRb and p300 compete for binding to the *trans*activation domain of E2F-1 (Helin *et al.*, 1993; Lee *et al.*, 1998). However the ability of HDAC-1 to facilitate pRb repression is not explained by the overriding of p300 co-activation potential. It therefore appears that pRb ability to bind HDAC-1 allows the negation of the histone acetylase activity of p300/CBP associated with E2F and results in an active transcriptional repression complex.

Several studies have demonstrated that pRb can associate with E2F-1 and HDAC-1 simultaneously but the role of other associated proteins such as pRbAp46 and pRbAp48 can not be excluded in these assays. Luo *et al.* (1998) demonstrated that pRb efficient repression of E2F transcription is dependent on its association with HDAC but it also is evident that the repressive function of pRb is also as a result of its direct interaction with E2F. Interestingly, pRb's ability to bind and recruit histone deacetylases is independent of pRb's role in cell cycle arrest although further studies are required to clarify this point (Chan *et al.*, personal communication). Given that pRb associates with the chromatin modulators, BRG1, $TAF_{II}250$ and Caf-1 via RbAp48 its ability to influence transcription by the modulation of chromatin structure is almost unquestionable (Hassig *et al.*, 1997; Hassig and Schrieber, 1997). Consistently I demonstrated that the block of chromatin condensation leads to an up-regulation of transcription and a loss in pRb's repressive function presumably as a result in the loss of its function, or those of its associated partners, in the modulation of chromatin.

7.11. Cross talk between p53 and pRb through JMY.

Numerous studies have demonstrated that pRb has the properties of a transcriptional activator and that this function is pocket dependent (Kim *et al.*, 1992; Dunaief *et al.*, 1994; Singh *et al.*, 1995; Chen *et al.*, 1996b; Need *et al.*, 1998). The activator potential of pRb although poorly understood is believed to be vital for its tumour suppressor function (Sellers and Kaelin, 1996). In agreement pRb's ability to augment MyoD dependent gene expression has been associated with its role in

tumour suppression and ability to induce differentiation (Gu *et al.*, 1993; Sellers *et al.*, 1998).

Here I demonstrated that pRb activates p53-dependent transcription of the *bax* promoter and furthermore that pRb co-operated with JMY in this co-activation (Figure 7.1). The activating potential of pRb is specific for Bax in the context of p53 *trans*activation and relies, at least in part, on the N-terminus of pRb. Given that the N-terminus of pRb has few prescribed functions it appears that in the case of *bax* activation to stabilise the co-activator complex as loss of this region drastically reduces pRb activating potential. Whether this reduction in the effectiveness of pRb corresponds to a structurally related event or loss of a functional domain is unknown. Interestingly, E1A stimulates the binding of pRb to p300 (Wang *et al.*, 1995a) but as yet the influence of such an interaction on JMY's function is unknown.

Whereas the pocket region of pRb is vital in its ability to function as a transcriptional repressor it is apparent that the pocket region encoded by exon 22 is dispensable for its function as a p53 activator (Weinberg, 1995). The tumour derived mutant pRbΔ22 behaved like wild-type pRb in the activation and co-operation of p53 *trans*activation. Results presented here imply that the ability of pRb to augment JMY activity is exerted independently of pRb dependent growth arrest, which is known to require the integrity of the pocket (Weinberg, 1995).

Given that JMY's cellular localisation appears to be vitally important for its function as a co-activator it is intriguing to note that pRb actually displaces a population of JMY to the cytoplasm in the presence of p53. Given that JMY is potentially a

regulated protein it is interesting to assume that the induction of cytoplasmic JMY by pRb corresponds to an activation of JMY in a cytoplasmic dependent fashion in response to pRb's anti-proliferative signals. Whether the cytoplasmic population of JMY corresponds to a dormant, dead or active complex remains unclear.

Interestingly, $rb^{-/-}$ cells undergo E2F-1 mediated apoptosis and pRb is a direct target for TNF induced apoptosis as a functional consequence of inhibition of MDM2 function (Qin *et al.*, 1994; Reinder *et al.*, 1996; Hsieh *et al.*, 1997; Phillips *et al.*, 1997; Hsieh *et al.*, 1999). E2F-1 induced apoptosis is overcome by pRb expression in an E2F-1 dependent manner (Hsieh *et al.*, 1997). A physiological rationale for the actions of pRb on JMY function may be relevant as a process that allows the induction of apoptosis upon the inactivation of pRb's pocket caused, for example, by mutational events or the action of viral oncoproteins during tumourigenesis. Cells mutated in pRb that are defective in cell cycle arrest would presumably undergo E2F-1 apoptosis and additionally would be able to increase p53's transcriptional activation of Bax which would functionally lead to the elimination of potentially neoplastic cells.

pRb is also known to promote p53 apoptosis function as a consequence of inhibition of MDM2's function (Hsieh *et al.*, 1999). Hypothetically therefore JMY may be a mediator of the apoptotic functions of pRb as a consequence of MDM2's loss of function.

7.12. Overall conclusions.

Results presented in this thesis provide information that relates to the mechanistic and physiological role of the p300 complex in the regulation of the p53 response. In particular the data presented here demonstrate that JMY physically and functionally co-operates with p300 and moreover implies that splicing of JMY is a vitally important event in the discrimination of p53's role in cell cycle arrest and apoptosis. In particular I have demonstrated that the proline rich domain in JMY is a region that can functionally control JMY's cellular function.

This study has also identified JMY as a potential regulator of the $p14^{ARF}$ tumour suppressor pathway. Moreover that analysis of pRb's role as a transcriptional activator has suggested a potentially novel role for pRb in the suppression of tumourigenesis, and have also implicated JMY in such a pathway (Figure 7.1).

In order to fully understand the individual roles of the JMY isoforms in gene expression and cell cycle control, it will be necessary to document the cellular factors that associate with specific isoforms under specific growth conditions. Here I have suggested a number of potential mechanisms whereby JMY is able to elicit a specific p53 response however to date the actual mechanistic pathway is unknown (Figure 7.1). The understanding and separation of the roles of the individual JMY isoforms may well prove productive in the design of anti-tumour reagents and the fight against cancer.

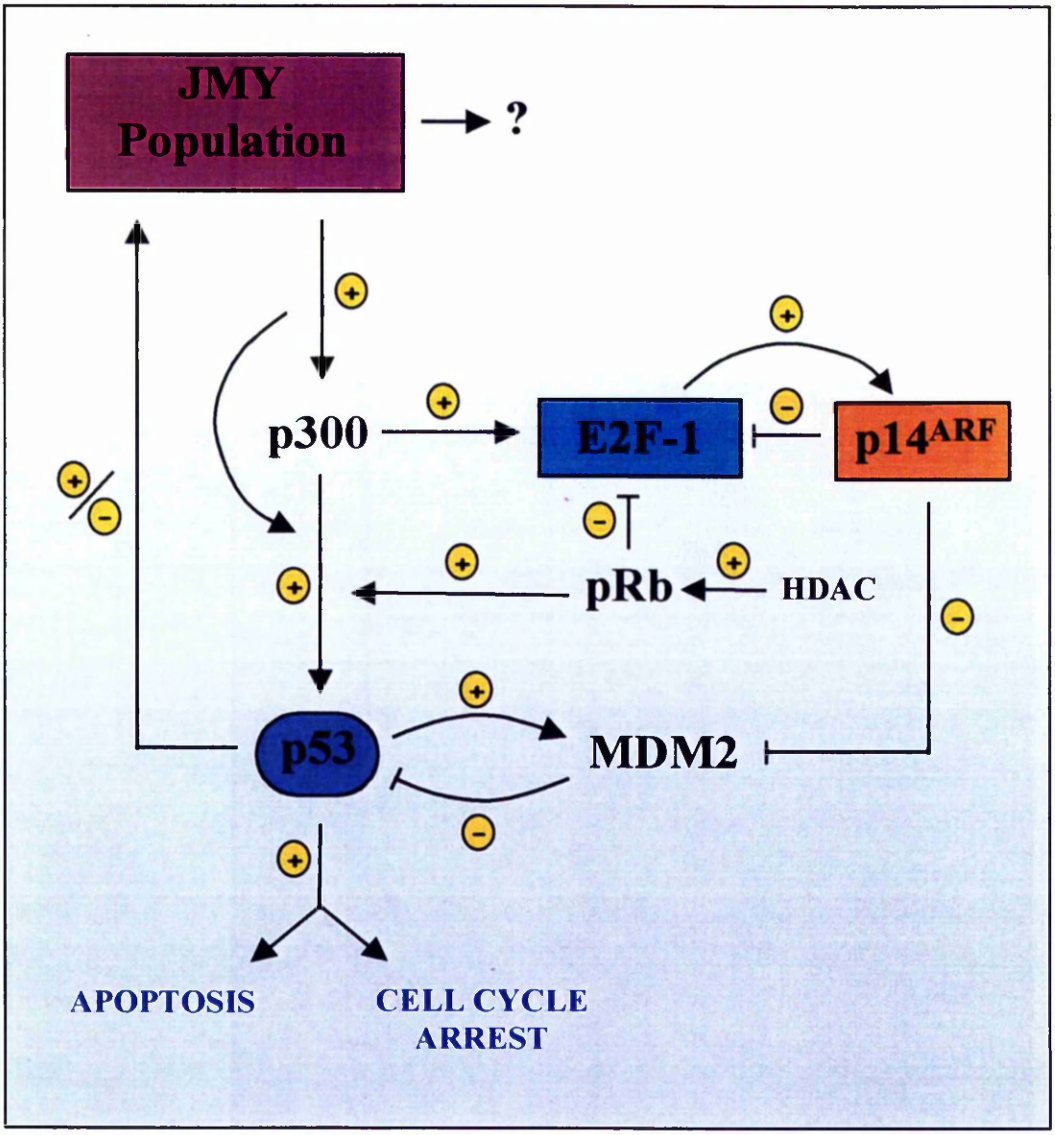
Figure 7.1. Summary of JMY's cellular role.

JMY lies at the nexus of the p53 and E2F growth control pathways, by nature of its ability to bind p300. The JMY cellular population acts positively via p300 to promote either p53 mediated cell cycle arrest or apoptosis. p53 itself plays a regulatory role in mediating the actions of the JMY population.

The overexpression of the tumour suppressor $p14^{ARF}$, that stimulates p53's cellular activity by releasing MDM2's control, is also governed by JMY. JMY stimulates $p14^{ARF}$ expression, that also regulates, in an auto-regulatory loop, its own expression. Surprisingly JMY appears to determine the specificity for the $p14^{ARF}$/MDM2 interaction.

In this study, it was also demonstrated that the histone deacetylase family of enzymes, HDAC's, help mediate pRb's repressive function. pRb, in the context of tumour derived mutations is able to stimulate p53 *trans*activation, and intriguingly co-operate with JMY in the stimulation of p53 mediated BAX protein expression. These findings imply a mechanism whereby tumour cells, that have lost pRb's growth control, have acquired a death mediated pathway, and that JMY is an important mediator in this pathway.

Clearly JMY's ability to influence two of the major growth controlling pathways is an indicator as to its cellular importance.


JMY Population
?
p300
E2F-1
p14ARF
pRb
HDAC
p53
MDM2
APOPTOSIS
CELL CYCLE ARREST

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