The roles of p53, p21, and RB in regulation of proliferation and apoptosis in hepatocytes

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Declaration

This thesis and the work described herein is my own work. Where contributions have been made by others this is stated in the text.

Sharon Sheahan

July 2002
Acknowledgements

Well, “it’s a miracle at Knock”, as my grandmother would say. It’s finished at long last. Thanks first of all to David for giving me the opportunity to come and work here on a project I thoroughly enjoyed, and for his help throughout. Huge thanks to Sandrine who gave me so much of her time, patience, and enthusiasm throughout this project. Thanks to Helen for helping me out when I most needed it (fixing those 8 a.m. time-points so that I didn’t have to crawl back into the lab after the 3 a.m. time-points). Thanks to Dominic for teaching me the way of the adenovirus.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
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<tr>
<td>ΔN</td>
<td>transactivation-deficient</td>
</tr>
<tr>
<td>AIF</td>
<td>apoptosis-inducing factor</td>
</tr>
<tr>
<td>APC</td>
<td>anaphase-promoting complex</td>
</tr>
<tr>
<td>ARF</td>
<td>alternative reading frame</td>
</tr>
<tr>
<td>ASK1</td>
<td>apoptosis signal-regulating kinase</td>
</tr>
<tr>
<td>ATF2</td>
<td>activated transcription factor 2</td>
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<td>ATM</td>
<td>ataxia telangiectasia-mutated protein</td>
</tr>
<tr>
<td>ATR</td>
<td>ataxia telangiectasia-related</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL-associated X protein</td>
</tr>
<tr>
<td>BCL-2</td>
<td>B-cell lymphoma</td>
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<td>bHLH</td>
<td>basic helix-loop-helix</td>
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<td>BID</td>
<td>BH3-interacting domain</td>
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<tr>
<td>BRCA1</td>
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<td>BrdU</td>
<td>5-bromodeoxy-uridine</td>
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<td>Brm-related gene 1</td>
</tr>
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<td>Brm</td>
<td>Brahma</td>
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<td>CCAAT/enhancer binding protein α</td>
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<tr>
<td>C/EBPβ</td>
<td>CCAAT/enhancer binding protein β</td>
</tr>
<tr>
<td>c-Abl</td>
<td>Abelson murine leukemia oncogene 1</td>
</tr>
<tr>
<td>CAK</td>
<td>cdk-activating kinase</td>
</tr>
<tr>
<td>c-AMP</td>
<td>cyclic adenosine mono phosphate</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
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<td>Cdc25C</td>
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</tr>
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<td>Cdk</td>
<td>cyclin-dependent kinase</td>
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<tr>
<td>CHK1</td>
<td>checkpoint kinase 1</td>
</tr>
<tr>
<td>CHK2</td>
<td>checkpoint kinase 2</td>
</tr>
<tr>
<td>CIP/KIP</td>
<td>cdk-inhibitory protein/kinase-inhibitory protein</td>
</tr>
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<td>cyclin kinase 1</td>
</tr>
<tr>
<td>CK2</td>
<td>cyclin kinase 2</td>
</tr>
<tr>
<td>CKI</td>
<td>cdk-inhibitor</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CREB</td>
<td>c-AMP-response-element-binding protein</td>
</tr>
<tr>
<td>CRM1</td>
<td>chromosomai regional maintenance</td>
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<td>CtBP</td>
<td>C-terminal-binding protein</td>
</tr>
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<td>CTD</td>
<td>C-terminal domain</td>
</tr>
<tr>
<td>CtIP</td>
<td>CtBP-interacting protein</td>
</tr>
<tr>
<td>DAB</td>
<td>diaminobenzidine</td>
</tr>
<tr>
<td>dH₂O</td>
<td>distilled water</td>
</tr>
<tr>
<td>DHFR</td>
<td>dihydrofolate reductase</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNMT1</td>
<td>DNA methyltransferase 1</td>
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<tr>
<td>DP</td>
<td>dimerisation protein</td>
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<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>E2F</td>
<td>early region 2 factor</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<tr>
<td>FEN1</td>
<td>flap endonuclease 1</td>
</tr>
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<td>Floxed</td>
<td>flanked by LoxP sites</td>
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<td>growth arrest and DNA damage protein 45</td>
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<td>GNER</td>
<td>global nucleotide excision repair</td>
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<td>HAT</td>
<td>histone acetylase</td>
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<td>HBP</td>
<td>high density lipoprotein binding protein</td>
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<td>HCC</td>
<td>hepatocellular carcinoma</td>
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<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
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<td>human papillomavirus</td>
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<td>HRP</td>
<td>horse radish peroxidase</td>
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<td>Id2</td>
<td>inhibitor of DNA binding 2</td>
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<td>interferon γ</td>
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<td>IGF-1R</td>
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<td>interleukin</td>
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<td>inhibitor of kinase 4</td>
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<tr>
<td>IR</td>
<td>ionizing radiation</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>LoxP</td>
<td>locus of crossover of PI</td>
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<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>MCM</td>
<td>mini chromosome maintenance protein</td>
</tr>
<tr>
<td>MDM2</td>
<td>mouse double minute clone 2 oncoprotein</td>
</tr>
<tr>
<td>MDMX</td>
<td>MDM2-related protein X</td>
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<td>MEFs</td>
<td>mouse embryonic fibroblasts</td>
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<td>MMTV</td>
<td>mouse mammary tumour virus</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MTS</td>
<td>multiple tumour suppressor</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide</td>
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<td>NER</td>
<td>nucleotide excision repair</td>
</tr>
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<td>NES</td>
<td>nuclear export signal</td>
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<td>NF-kB</td>
<td>nuclear factor kappa-B</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localisation signal</td>
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<tr>
<td>OD</td>
<td>optical density</td>
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<tr>
<td>P/CAF</td>
<td>p300/CBP-associated factor</td>
</tr>
<tr>
<td>p300</td>
<td>transcription adaptor protein required to drive p53-dependent expression</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating-cell nuclear antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PIDD</td>
<td>p53 inducible protein with death domain</td>
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</tbody>
</table>
PIG  p53-inducible genes
PKC  protein kinase C
PNS  peripheral nervous system
Pol I - III  RNA polymerase I - III
PP1α  protein phosphatase 1α
PRC  pre-replicative complex
RAP74  general transcription factor iiif polypeptide 1 74 kilodalton
Rb  retinoblastoma gene
RB  retinoblastoma protein
RBBP48  RB-binding protein 48
RBP  retinoblastoma binding protein
RCE  retinoblastoma control element
ROS  reactive oxygen species
SAPK  stress-activated protein kinases
SCF  Skp1/cullin/F-box
SDI  senescent cell derived inhibitory protein
SH3  src homology 3
SSC  standard saline-citrate
SST  sequence-specific transactivation
STAT  signal transducer and activator of transcription
SUMO  small ubiquitin-related modifier-1
SV40 TAg  simian virus 40, T antigen
SWI/SNF  switching defective/sucrose nonfermenter
TA  transactivation
TAF  TBP-associated factor
TAFq50  TBP-associated factor 250 kilodalton
TBE  tris borate EDTA
TBP  TATA-binding protein
TBS  tris buffered saline
TCNER  transcription-coupled nucleotide excision repair
TE  tris EDTA solution
TFIID  transcription factor II D
TFIIF  transcription factor II F
TGF-β1, 2  transforming growth factor β 1, 2
TNF-α  tumour necrosis factor
TNFR  TNF-α receptor 1
TRAF  TNFR-associated factor
TSG  tumour suppressor gene
UV  ultra violet
Wnt1  wingless-type MMTV integration site 1
x-gal  5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XPB  xeroderma pigmentosum type B
XPD  xeroderma pigmentosum type D
Abstract
Precise control of proliferation and apoptosis is essential for the prevention of cancer. Pathways involving p53, p21, and RB are central to the regulation of these processes, and are dysregulated in almost all human cancers. This thesis describes an investigation into the effects of inactivating components of the p53-p21-RB pathway in order to better understand the mechanisms underlying its role in preventing cancer.

Hepatocytes isolated from transgenic mice bearing mutations in p53, p21, Rb, and combinations of all three, were employed to provide insight into the pathways controlling hepatocyte proliferation and apoptosis. In the case of the Rb mutation, Cre/LoxP conditional gene targeting was used to overcome the problem of embryonic lethality of Rb knock-out. In vitro Rb gene deletion mediated by adenovirus-Cre was successfully developed and characterised. This revealed that adenovirus-Cre-mediated gene deletion is extremely efficient in hepatocytes and that adenoviral infection had no affect on cell viability or growth. Expression of Cre, however, stimulated DNA synthesis while expression of LacZ adversely affected viability at higher MOI. These findings underline the need for careful characterisation of gene deletion systems employing both adenovirus and Cre recombinase.

Analysis of hepatocytes deficient in p53, Rb, and p21, revealed severe defects in proliferation accompanied by an increase in ploidy, and nuclear and mitotic abnormalities. Analysis of hepatocytes deficient in combinations of all three genes provided insight into the level of interdependency between these genes, suggesting that there are two major pathways regulating hepatocyte proliferation: a p21-RB pathway that operates independently of p53, and a p53-p21 pathway that operates independently of RB.

The p53-p21-RB pathway is critically important in regulating cellular responses to stress. The role of p53, p21, and RB in regulation of hepatocyte responses to DNA damage was investigated. It was found that each of these proteins is required for the maintenance, but not initiation, of UV-induced arrest. Both p53-dependent and -independent pathways of UV-induced apoptosis exist, and loss of Rb or p21 increased susceptibility to p53-independent, but not p53-dependent, apoptosis.
In conclusion, the work presented in this thesis demonstrates that adenovirus-Cre-mediated gene deletion is extremely efficient in hepatocytes. It also shows that p53, p21, and RB are critical in regulating proliferation and apoptosis both in the absence and presence of exogenous stress, underlining the importance of these proteins in regulation of cell growth and hence presumably in prevention of cancer.
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Chapter 1

Introduction

1.1 The *Retinoblastoma* Tumour Suppressor Gene

The *retinoblastoma* gene (*RB*) was the first human tumour suppressor gene identified and has been extensively studied as the prototype of this class of gene. It was initially identified as a genetic locus associated with the development of an inherited eye tumour, retinoblastoma (Knudson, 1971; Friend *et al*., 1986; reviewed in DiCiommo *et al*., 2000). Mutations in the *Rb* gene are found in approximately 30% of all human tumours, and the RB pathway (RB-p16-cyclin D-cdk [cyclin-dependent kinase] 4,6) is now known to be deregulated in most human cancers (for review see Weinberg *et al*., 1995; Sherr *et al*., 1996). Consistent with its role as a tumour suppressor, mice heterozygous for a *Rb* mutation are predisposed to tumorigenesis (Hu *et al*., 1994; Williams *et al*., 1994a; Harrison *et al*., 1995), while introduction of a wild-type *RB* gene into cells lacking functional RB suppressed cell growth and tumorigenicity (Bookstein *et al*., 1990; Goodrich *et al*., 1990; Takahashi *et al*., 1991; Qin *et al*., 1992).

1.1.1 Structure of the Retinoblastoma protein

The human *RB* gene contains 27 exons and encodes a nuclear phosphoprotein of 928 amino acids with a molecular mass of approximately 110kD (Lee *et al*., 1987; Hong *et al*., 1989). The corresponding murine gene has 91% homology to the human gene and encodes a protein of 921 amino acids (Bernards *et al*., 1989). The RB protein contains three distinctive domains: the N-terminal region, the central A and B domains, and the C-terminal region, each bearing various protein-binding and phosphorylation sites (Figure 1.1). Domains A and B interact with each other to form the A/B pocket (Chow & Dean, 1996; Lee *et al*., 1998), the defining characteristic of the pocket proteins, which, along with RB, include p107 and p130 (see section 1.1.2). The pocket is necessary for most of the biological properties of these proteins and it is this region of RB that suffers the most mutations detected in human tumours (Bookstein & Lee, 1991). It contains multiple protein-binding sites
including the LXCXE-motif-binding site and the E2F (early region 2 factor)-binding site (Lee et al., 1998).

**Figure 1.1: Structure of the Retinoblastoma protein**


The C-terminal region contains a NLS (nuclear localisation signal) (Zacksenhaus et al., 1993), cyclin-binding motifs required for RB phosphorylation (Adams et al., 1999), binding sites for the c-Abl (Abelson murine leukemia oncogene 1) tyrosine kinase and MDM2 (mouse double minute clone 2 oncoprotein) (Welch & Wang, 1993; Xiao et al., 1995), and is required for RB-mediated growth suppression (Qin et al., 1992; Qian et al., 1992). The c-Abl-RB interaction blocks c-Abl tyrosine kinase activity (Welch & Wang, 1993; Whitaker et al., 1998), while RB can form a trimeric complex with MDM2 and p53, preventing MDM2-mediated degradation of p53 and facilitating p53-dependent apoptosis (Hsieh et al., 1999). The C-terminal
region also contains a caspase cleavage site, and cleavage of RB is observed during apoptosis induced by a variety of death inducers (for review see Tan & Wang, 1998). Phosphorylation of the C-terminus induces an interaction with the A/B pocket, thereby disrupting interaction with LXCXE proteins (Harbour et al., 1999).

The role of the N-terminal region is unclear, but it is thought to contribute to the tumour supressor function of RB (Riley et al., 1997), perhaps through negative regulation of DNA replication via its association with MCM7 (mini chromosome maintenance 7), a component of replication licensing factor (Sterner et al., 1998).

1.1.2 The RB family of pocket proteins
Two other proteins, p107 and p130, are structurally and functionally related to RB, and together with RB they constitute a family of pocket proteins (Figure 1.2) (for review see Mulligan & Jacks, 1998; Classon & Dyson, 2001). Like RB, p107 and p130 induce growth arrest of cells when overexpressed (Zhu et al., 1993; Claudio et al., 1994). While all three proteins are closely related in the pocket domain, RB bears little similarity to the other family members outside of the pocket, and p107 and p130 are more closely related to each other than either is to RB. The spacer sequences, which separate A and B subdomains of the pocket, are conserved between p107 and p130, but have little similarity with RB. They contain a binding-site for cyclin A-cdk2 and cyclin E-cdk2, allowing p107 and p130 to form complexes with, and inhibit these cdks (Figure 1.2A) (Zhu et al., 1995a, 1995b; Adams et al., 1996; Lacy & Whyte, 1997; Woo et al., 1997; Castano et al., 1998; Coats et al., 1999).
Figure 1.2: The RB family of pocket proteins

A

RB

N A S B C

p130

N A S B C

p107

N A S B C

Cyclin A/E-cdk binding site

B

p107

RB

p130

G0 G1 S

Cyclin D kinase activity

Cyclin E/A kinase activity

C

Rb partners

E2F-1
E2F-2
E2F-3
E2F-4

p107/p130 partners

E2F-4
E2F-5

Model describing the RB family of pocket proteins.

A. p107 and p130 contain a cyclin-cdk binding site within the spacer region enabling them to inhibit cdk activity.  
B. Pocket proteins exhibit different expression patterns during the cell cycle.  
C. Pocket proteins interact with different E2F family members.
Pocket proteins differ in their expression patterns (Figure 1.2B) (for review see Nevins, 1998; Grana et al., 1998). p130 protein levels are high in quiescent and differentiated cells, and low in actively cycling cells (Mayol et al., 1995, 1996; Smith et al., 1996). In contrast, p107 protein levels are low in quiescent and differentiated cells, and increase as quiescent cells enter the cell cycle (Beijersbergen et al., 1995). RB protein levels are relatively constant in both quiescent and cycling cells, although they increase slightly when quiescent cells are stimulated to proliferate (Ikeda et al., 1996).

Pocket proteins associate differentially with E2F family members (Figure 1.2C). In general, RB interacts with E2F-1 to -4, whereas p107 and p130 bind to E2F-4 and E2F-5. p130-E2F4 complexes are the predominant E2F complexes found in quiescent and differentiated cells where it is thought they mediate transcriptional repression and the G0/G1 transition. In contrast, RB-E2F and p107-E2F complexes are found in proliferating cells where they regulate the G1/S transition (for review see Sardet et al., 1997; Mayol & Grana, 1998).

Pocket proteins are phosphorylated in a cell-cycle dependent manner (for review see Grana et al., 1998). Several phosphorylated forms of RB exist, and this varies depending on the cell type. In contrast, 2 forms of p107, hypo- and hyperphosphorylated p107, and 4 forms of p130, unphosphorylated and phosphorylated forms 1, 2 and 3 are found in most cell types (Mayol et al., 1995).

1.1.2.a RB family knockout mice

The development of gene targeting technology allowed the generation of mice heterozygous and homozygous for null Rb alleles. Rb+/− mice are viable, but develop pituitary and thyroid tumours upon loss of the wild-type Rb allele (Hu et al., 1994; Williams et al., 1994a; Harrison et al., 1995). Homozygous null mice die in utero between 13-15 days of gestation, indicating that RB is not required in early stages of murine development (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Rb−/− embryos showed abnormalities in erythropoiesis, neurogenesis and lens development, associated with ectopic S phase and apoptosis. At this stage of development, the affected tissues normally express high levels of RB (Jiang et al., 1997), and while capable of initiating differentiation, they failed to attain a fully differentiated state.
(Lee et al., 1994; Morgenbesser et al., 1994). Rescue of Rb<sup>−/−</sup> embryos to birth by expression of an RB transgene revealed a role for RB in muscle differentiation (Zacksenhaus et al., 1996). These findings suggested that RB plays a role in regulation of differentiation as well as proliferation and apoptosis (see sections 1.1.3.c, 1.1.3.a and 1.1.3b respectively).

In contrast to Rb, mice deficient in p107 or p130 are viable and develop normally (Cobrinik et al., 1996; Lee et al., 1996). Mice deficient in both p107 and p130 exhibit defective endochondral bone development and shortened limbs associated with deregulated chondrocyte cell cycle exit, and they fail to respire and die perinatally (Cobrinik et al., 1996). This indicates that these proteins can substitute for each other during development. Unlike Rb, neither heterozygous nor homozygous deletions of p107, p130, or both, lead to tumorigenesis in mice (Cobrinik et al., 1996; Lee et al., 1996), in agreement with the fact that these genes are rarely mutated in human cancer (Mulligan & Jacks, 1998).

Rb<sup>−/−</sup>p107<sup>−/−</sup> (Lee et al., 1996) and Rb<sup>−/−</sup>p130<sup>−/−</sup> (Lipinski & Jacks, 1999) embryos die two days earlier than Rb<sup>−/−</sup> embryos, supporting the notion that RB family members can substitute for one another functionally. In contrast to Rb<sup>−/−</sup> mice, Rb<sup>−/+</sup>p107<sup>−/−</sup> mice exhibit retinal dysplasia (Lee et al., 1996), which was not found in animals with any other combination of mutation of Rb family members. Rb<sup>−/−</sup>p107<sup>−/−</sup> chimeric mice developed bona fide retinoblastoma, indicating that in the mouse, loss of both Rb and p107 is required for retinoblastoma to develop (Robanus-Maandag et al., 1998). This is in contrast to the situation in humans where loss of RB alone is sufficient for the development of retinoblastoma. Taken together, these findings suggest a significant functional overlap between pocket proteins, which may involve both genetic redundancy and functional compensation mechanisms. This functional overlap makes it difficult to define precise biological roles for these proteins and is likely to result in underestimation of their unique functions in wild-type cells.

Analysis of MEFs (mouse embryonic fibroblasts) from the various knock-out mice provided additional insight into the degree of functional overlap between these proteins. Rb<sup>−/−</sup> MEFs exhibit a shortened G1 phase, reduction in growth factor requirement, and deregulation of E2F-responsive genes including cyclin E and p107
In contrast, p107\(^{-/-}\) and p130\(^{-/-}\) MEFs show normal length of G1 phase and growth factor dependency, and no deregulation of cell cycle genes. p107\(^{-/-}\)p130\(^{-/-}\) MEFs, however, showed deregulation of different genes than those found in Rb\(^{-/-}\) MEFs (Hurford \textit{et al.}, 1997). Triple knock-out MEFs exhibit a very different phenotype to either single or double knock-out MEFs. They do not arrest in G1 after contact inhibition, serum starvation, or DNA damage. Moreover, they do not undergo differentiation or senescence, and are highly prone to transformation by the Ras oncogene (Dannenberg \textit{et al.}, 2000; Sage \textit{et al.}, 2000). These new findings point to an intimate relationship between the pocket proteins and indicate that they are likely to mediate both unique and interdependent functions.

\subsection*{1.1.3 Functions of RB}

RB is a transcriptional cofactor involved in transcriptional activation and repression of genes that regulate proliferation, apoptosis, and differentiation (discussed below).

\subsubsection*{1.1.3.a Regulation of Proliferation}

\paragraph*{1.1.3.a.i Transcriptional Repression}

RB executes its biological functions by both positively and negatively regulating transcription. Transcriptional activation is associated with differentiation (see 1.1.3.c), while transcriptional repression is associated with inhibition of proliferation. RB can repress transcription by several different mechanisms (Figure 1.3). First, it binds to the transcriptional activation domain of E2F and blocks its transcriptional activity (Flemington \textit{et al.}, 1993; Helin \textit{et al.}, 1993) (see 1.1.3.a.ii). Second, the RB-E2F repressor complex thus formed actively represses transcription when bound to a promoter (Adnane \textit{et al.}, 1995; Bremner \textit{et al.}, 1995; Sellers \textit{et al.}, 1995; Weintraub \textit{et al.}, 1995) by interfering with the basal transcription machinery (Figure 1.3). While tethered to the promoter via E2F, RB interacts with adjacent transcription factors such as PU.1 and ELF1, preventing them, and E2F, from interacting with the TBP (TATA-binding protein) component of the TFIID (transcription factor II D) basal transcriptional complex (Weintraub \textit{et al.}, 1995; Pearson \textit{et al.}, 1997; Ross \textit{et al.}, 1999). RB binds to TAF\(_{50}\) (TBP-associated factor 250kD), and inhibits its ability to phosphorylate itself and the RAP74 (general transcription factor iif
polypeptide 174 kilodalton) subunit of TFIIF (transcription factor II F) (Siegert et al., 1999), and thus, may impair the assembly of the general transcriptional complex required for transcriptional initiation (Shao et al., 1995, 1997). RB represses transcription by all three eukaryotic RNA polymerases via direct binding to components of Pol I (RNA polymerase I) and Pol III, and by blocking interaction of Pol II-associated transcription factors with the basal transcription machinery (for reviews see Dynlacht, 1997; Grana et al., 1998; Ciarmatori et al., 2001; Scott et al., 2001).

**Figure 1.3: Transcriptional Repression by RB**

**Transcriptional Activation by E2F**

A. *Transcriptional activation by E2F.* E2F binds to its DP partner, which stimulates E2F DNA-binding activity at a consensus E2F site. E2F activates transcription via interaction with transcriptional coactivators such as p300/CBP, and with TBP, a component of the basal transcriptional complex.

**Transcriptional Repression by RB**

B-C. *Transcriptional repression by RB.* B. RB binds to the transactivation domain of E2F inhibiting its transcriptional activity and interaction with the transcriptional machinery. C. RB recruits the chromatin remodelling enzymes HDAC and SW1/SNF, forming repressor complexes that repress transcription.
Third, RB has been shown to repress transcription through recruitment of distinct classes of chromatin-remodelling enzymes (for review see Ferreira et al., 2001) (Figure 1.3). RB can interact with HDACs (histone deacetylases) through the pocket domain and an LXCXE-like motif. RB can bind simultaneously to HDAC and E2F, allowing the formation of a transcriptional repressor complex at promoters containing E2F-binding sequences (Brehm et al., 1998; Ferreira et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998). This complex actively represses transcription by deacetylating lysine residues in the N-terminal tails of core histones that protrude out of the nucleosome, leading to the formation of a repressive chromatin structure that denies transcription factors access to their binding sites (Hassig et al., 1997). In addition, recruitment of HDAC to E2F may act to offset E2F-associated HAT (histone acetylase) activity, which increases its DNA-binding and transcriptional activity (Martinez-Balbas et al., 2000). RBBP48 (RB-binding protein 48) has recently been shown to be a component of the histone deacetylase complex recruited by RB, where it is associated with E2F-1, suggesting that it too may be involved in transcriptional repression by RB (Nicolas et al., 2000).

RB interacts with Brm (Brahma) and BRG1 (Brm-related gene 1), mammalian homologs of the yeast SNF2/SW12 (sucrose nonfermenter 2/switching defective 12) ATPases that are part of the SW1/SNF nucleosome remodeling complex (Dunaief et al., 1994; Strober et al., 1996; Trouche et al., 1997) (Figure 1.3). These complexes use ATP hydrolysis to influence access to promoters by altering the formation and positioning of nucleosomes along a promoter (reviewed in Tyler & Kadonaga, 1999). SW1/SNF complexes appear to be involved in transcriptional repression (Holstege et al., 1998) as well as activation of genes (Tyler & Kadonaga, 1999). RB can bind simultaneously to Brm and E2F, perhaps forming a SW1/SNF-RB-E2F complex at promoters (Trouche et al., 1997) (Figure 1.3). Brm can enhance the ability of RB to inhibit E2F transcriptional activity (Trouche et al., 1997), and BRG1 can cooperate with RB to induce cell cycle arrest (Dunaief et al., 1994; Strobeck et al., 2000). These findings suggest a role for the SW1/SNF nucleosome remodeling complex in RB function.

It has recently been shown that RB can interact simultaneously with BRG1 and HDAC, allowing formation of an HDAC-RB-SW1/SNF complex (Figure 1.3)
This repressor complex inhibits transcription of cyclin E and cyclin A and arrests cells in G1 (see section 1.1.4.a and Figure 1.7). Phosphorylation of RB by cyclin D-cdk4 disrupts association with HDAC, allowing cyclin E expression and progression into S phase. However, the RB-SW1/SNF complex persists and maintains repression of cyclin A and cdc2 (cell division cycle 2), thereby inhibiting mitosis (Zhang et al., 2000). This is consistent with other studies showing that the RB-E2F repressor complex inhibits expression of cyclin A. The resultant decrease in cdk2 activity leads to disruption of the PCNA (proliferating-cell nuclear antigen)-chromatin interaction, thereby inhibiting S phase progression (Sever-Chroneos et al., 2001), and allows activation of the APC (anaphase-promoting complex), which degrades cyclin B, thereby inhibiting mitosis (Lukas et al., 1999). Accumulation of cyclin E-cdk2 activity blocks RB-SW1/SNF activity at least in part by phosphorylation of BRG1, relieving repression of cyclin A and cdc2 (Zhang et al., 2000). Inactivation of the repressor complex allows cyclin A expression and activation of cyclin A-cdk2, which phosphorylates the cdh1 subunit of APC (anaphase-promoting complex). This blocks its ubiquitin ligase activity and allows accumulation of cyclin B and progression of cells into mitosis (Lukas et al., 1999). Thus, the HDAC-RB-SW1/SNF complex allows the sequential expression of cyclins E, A and B, which regulate exit from G1 and from S phase (Figure 1.7).

In addition to blocking the activity of the basal transcription machinery, and recruiting chromatin-remodeling enzymes, RB is able to repress transcription via interaction with other transcriptional corepressors including CtIP(C-terminal-interacting protein)/CtBP (C-terminal-binding protein) (Meloni et al., 1999), RBP1 (RB-binding protein 1) (Lai et al., 1999), HBP1 (high density lipoprotein-binding protein) (Shih et al., 1998), and DNMT1 (DNA methyltransferase 1) (Robertson et al., 2000). The function and relative contribution of these corepressors to RB-mediated repression remains to be determined.

1.1.3.a.ii The E2F family of transcription factors
The term E2F encompasses a family of transcription factors, of which six members have been identified to date (E2F-1 to -6) (Figure 1.4) (for review see Slansky et al., 1996; Dyson et al., 1998). E2F DNA-binding activity requires the formation of a
heterodimer composed of an E2F component and a member of the DP (dimerisation proteins 1 and 2) family of transcription factors (Figure 1.4). E2F-1 to -5 have a pocket protein-binding domain located within a C-terminal transactivation domain (Slansky et al., 1996) that allows them to interact with specific members of the RB family (for review see Sardet et al., 1997) (Figure 1.2). E2F transcription factors do not contain the LXCXE motif; their binding to RB requires the A/B pocket and C-terminal amino acids. This E2F binding site is called the large A/B pocket (Qin et al., 1992; Kaelin et al., 1992; Helin et al., 1992). E2F-6 lacks a C-terminal transactivation and RB-binding domain and is thought to repress transcription (Morkel et al., 1997; Trimarchi et al., 1998). Most E2F family members are expressed in a wide variety of tissues but have distinct roles in cell cycle control and apoptosis. E2F-1 to -3 can efficiently induce S phase, whereas E2F-4 and E2F-5 do so only weakly. Although overexpression of E2F-1, E2F-2, or E2F-3 has been shown to induce apoptosis (Vigo et al., 1999), it is widely believed to be a specific property of E2F-1 (DeGregori et al., 1997; Lissy et al., 2000; Vogelstein et al., 2000). However, a recent study strongly supports a role for E2F-3 in the induction of apoptosis (Ziebold et al., 2001).
Figure 1.4: The E2F family of transcription factors

Model describing the major structural and functional domains of the E2F and DP families of transcription factors (adapted from Classon & Dyson, 2001).
**Regulation of E2F**

The activity of E2F is tightly regulated by a series of transcriptional and post-transcriptional events, including protein synthesis, subcellular localization, binding to DP proteins and pocket proteins, phosphorylation, acetylation and degradation (for review see Dyson et al., 1998; Black & Azizkhan, 1999). The expression of E2F-1 to -3 is low in quiescent cells but is strongly induced during G1. This accumulation is thought to be mediated, at least in part, by derepression of E2F promoters during the transition from G0 into the cell cycle (Johnson et al., 1994a, 1995; Sardet et al., 1995; Leone et al., 1998). This is in contrast with the relatively constant level of expression of E2F-4 and E2F-5 (Sardet et al., 1995). The E2F-1 promoter appears to be under autoregulatory control and is activated by the expression of E2F-2 and -3, as well as E2F-1 itself (Hsiao et al., 1994; Johnson et al., 1994a). Subcellular localization of E2Fs is regulated in a cell cycle-dependent manner. E2F-1 to -3 are located in the nucleus due to the presence of a N-terminal NLS. E2F-4 and -5 lack this NLS and are predominantly cytoplasmic, except in G0 and early G1 when they are also found in the nucleus through interaction with their pocket protein partners. This suggests that E2F-4 and -5 are likely to be involved in transcriptional repression during G0 and early G1, rather than activation in G1, and that this repression is relieved due to cytoplasmic sequestration of E2F-4 and -5 during late G1 and S phase, following their dissociation from pocket proteins (Muller et al., 1997; Verona et al., 1997).

Interaction of E2F with its DP partner stimulates E2F DNA-binding activity at a consensus E2F site (Figure 1.5). However, hypophosphorylated RB binds to the transactivation domain of E2F-1, blocking its transcriptional activity (Flemington et al., 1993; Helin et al., 1993), inducing G1 arrest. Phosphorylation of RB by cyclin-cdks during G1 disrupts the RB-E2F interaction, allowing E2F transcriptional activity and S phase entry (Figure 1.5). Phosphorylation of E2F-1 itself, perhaps also by cyclin-cdks, can enhance its activity by inhibiting its interaction with RB (Fagan et al., 1994). Phosphorylation of DP-1 by cyclin A-cdk2 during S phase abrogates E2F DNA-binding and transcriptional activity (Figure 1.5) (Dynlacht et al., 1994; Krek et al., 1994), allowing S phase exit. Loss of this regulation by cyclin A-cdk2 activates an S-phase checkpoint that results in growth arrest or apoptosis.
E2F binds to its DP partner, which stimulates E2F DNA-binding activity at a consensus E2F site. RB binds to the transactivation domain of E2F inhibiting its transcriptional activity, inducing G1 arrest. Phosphorylation of RB by cyclin D-cdk4,6 and cyclin E-cdk2 inactivates RB so that it can no longer bind E2F. E2F then activates transcription, allowing S phase entry. Phosphorylation of DP by cyclin A-cdk2 abrogates E2F DNA-binding and transcriptional activity, while ubiquitination of E2F targets it for proteasomal degradation. Inactivation of E2F allows S phase exit and progression into mitosis. In G1, unphosphorylated RB binds once again to E2F, inducing G1 arrest.
E2Fs are also regulated at the level of protein degradation. Free E2Fs are unstable proteins, targeted for ubiquitination through the C-terminal region containing the transactivation and pocket protein-binding domains (Figure 1.5). E2Fs in complexes with pocket proteins are protected from degradation, perhaps due to overlapping E2F sequences required for ubiquitination and interaction with pocket proteins (Hateboer et al., 1996; Campanero & Flemington, 1997). In the case of E2F-1, it has been shown that the N-terminus interacts with the SCF^Skp2 (Skp1/cullin/F-box) ubiquitin ligase, which transfers ubiquitin to E2F-1, thus marking it for degradation (Figure 1.5) (Marti et al., 1999). Acetylation has recently been shown to regulate E2F. P/CAF (p300/CBP-associated factor) and p300/CBP (CREB-binding protein) can acetylate E2F-1, thereby increasing its DNA-binding ability, transcriptional activity and protein half-life (Martinez-Balbas et al., 2000).

Another level of E2F-1 regulation involves the MDM2 protein. It can bind to the transactivation domain of E2F-1 and stimulate its activity (Martin et al., 1995), but it can also inhibit E2F-1-induced apoptosis and accumulation of p53 (Kowalik et al., 1998). Downregulation of MDM2 correlated with accumulation of E2F-1, and a requirement for the p53-binding domain of MDM2 for E2F-1 degradation was demonstrated (Blattner et al., 1999), suggesting that p53 and E2F-1 may be regulated in a similar manner by MDM2. Whether MDM2 acts as a ubiquitin ligase for E2F-1, as in the case of p53, is not yet known.

**Transcriptional Regulation by the Rb-E2F complex**

E2F can both positively and negatively regulate proliferation by activating or repressing the expression of cell cycle genes, respectively. The interaction between E2F and RB determines whether E2F activates or represses transcription (Figure 1.3). Precisely how E2F activates transcription is unknown, however, various mechanisms have been suggested. E2F-1 can bind to co-activators that have HAT activity, such as CBP (Trouche et al., 1996) and p300 (Lee et al., 1998), and to the general transcription factor TBP (Hagemeier et al., 1993) (Figure 1.3). Additionally, upon binding to DNA, E2F induces a structural bend, thought to be important for optimal spacing between proteins in the transcriptional complex (Cress & Nevins, 1996).
A large body of evidence supports the role of E2F as a transcriptional activator. 1) E2F-binding sites are present in a large number of growth-regulatory genes involved in cell cycle control, including the proto-oncogenes c-Myc, N-Myc and B-Myb, genes required for DNA synthesis such as DHFR (dihydrofolate reductase), thymidine kinase, thymidylate synthetase, DNA polymerase α and PCNA, genes encoding proteins that mediate the recognition and utilization of replication origins including Orc1, cdc6 and MCMs, and genes required for cdk activity such as cyclin D1, cyclin E, cyclin A, cdk2 and cdk4 (see Yamasaki et al., 1998; Leone et al., 1998, and references therein). Mutation of the E2F site in some of these promoters, including cyclin E (Ohtani et al., 1995) and DHFR (Means et al., 1992), results in a dramatic reduction of activity during cell cycle progression, suggesting that these E2F-binding sites mediate transcriptional activation during S-phase entry. 2) E2F-1 overexpression can induce quiescent cells to enter S phase and prevent cells from entering quiescence upon serum deprivation (Johnson et al., 1993); lead to transformation of an immortalized cell line (Singh et al., 1994) and transformation of primary rat embryo cells in cooperation with activated ras (Johnson et al., 1994b); overcome G1 arrest mediated by TGF-β (transforming growth factor β) (Schwarz et al., 1995) and p16 (Lukas et al., 1996); induce tumour formation in mice (Johnson et al., 1994b; Xu et al., 1995; Guy et al., 1996, Pierce et al., 1998a, 1998b). 3) Interaction of RB with E2F results in inhibition of E2F transcriptional activity and the ability of RB to interact with E2F correlates with its ability to induce growth arrest (Hiebert et al., 1992, 1993). Inactivation of E2F-1 reduced tumourigenesis in Rb+/− mice (Yamasaki et al., 1998), and ectopic S phase entry in Rb+/− embryos (Tsai et al., 1998). 4) The ability of viral oncoproteins to transform cells is dependent upon their ability to disrupt RB-E2F complexes and liberate “free” E2F (Chellapan et al., 1991, 1992).

These results led to the conclusion that E2Fs function primarily as positive regulators of cellular proliferation and can function as oncogenes when overexpressed, hence the widely accepted ‘activation model’ of E2F activity (Figure 1.3). According to this model, in the absence of RB, E2F activates transcription of genes required for S phase entry, while interaction of E2F with RB inhibits its transcriptional activity. Despite their oncogenic potential, mutations in E2F in human
cancer are rare and only sporadic cases of amplification have been observed (Saito et al., 1995).

Although the above studies clearly demonstrate a role for E2F as a transcriptional activator, mutational analysis of a large number of E2F-regulated promoters indicate that E2F sites can act as negative regulatory elements. Mutation of E2F sites in promoters such as cdc2 (Dalton et al., 1992), b-Myb (Lam et al., 1993), and E2F-1 (Hsiao et al., 1994; Johnson et al., 1994a, 1995), led to an increase in transcriptional activity rather than repression. A role for E2F as a transcriptional repressor was strongly supported by the unexpected finding that E2F-1 knockout mice develop normally with little hypoproliferation and actually develop a variety of tumours, indicating that E2F-1 can function as a tumour suppressor gene as well as an oncogene (Field et al., 1996; Yamasaki et al., 1996). These findings led to the proposal of a repression model of E2F activity, whereby E2F mediates transcriptional repression of promoter activity during G0 and early G1 in association with pocket proteins (Figure 1.3).

It is still unclear whether E2F acts primarily as a transcriptional activator, repressor, or both, and it may be that the role of the E2F site varies in different promoters and tissues, acting as a positive or negative element depending on the presence of a pocket protein (Weintraub et al., 1992, 1995) and a particular E2F-family member (Takahashi et al., 2000). This would help to explain the ability of E2F-1 to function as an oncogene or tumour suppressor in a tissue-specific manner. Alternatively, it may be that different E2Fs regulate the expression of distinct sets of target genes, resulting in different biological properties. For example, E2F-3 may be critical for the activation of genes required for DNA replication (Humbert et al., 2000), while the induction of apoptosis by E2F-1 (DeGregori et al., 1997) may underlie its tumour suppressor function. It is clear that transcriptional control by E2F is complex, and difficulties in interpreting results have arisen at least in part due to redundancy and functional compensation among the E2F family members.

1.1.3.b Regulation of Apoptosis

Apoptosis is a genetically controlled program of cell death characterised by its morphological characteristics, including cell shrinkage, membrane blebbing,
chromatin condensation, and nuclear fragmentation (Wyllie et al., 1980). It plays an important role in embryonic development and tissue homeostasis, and disruption of the apoptotic process can result in disease. For example, excessive apoptosis can lead to neurodegenerative diseases, while suppression can lead to cancer (for review see Evan, 1998). That RB plays a role in protection against apoptosis was first suggested upon examination of Rb<sup>-/-</sup> embryos, which exhibit high levels of apoptosis in the CNS (central nervous system), the PNS (peripheral nervous system), the hematopoietic system, lens, and skeletal muscle. Apoptosis was dependent on E2F-1 and p53 in the CNS and lens, and independent of E2F-1 and p53 in the PNS and skeletal muscle (Morgenbesser et al., 1994; Macleod et al., 1996; Jiang et al., 2000). It was recently shown that E2F-3 is responsible for both p53-dependent and p53-independent apoptosis in Rb<sup>-/-</sup> embryos (Ziebold et al., 2001).

The ability of E2F proteins to induce apoptosis helps to explain the anti-apoptotic activity of RB (Figure 1.6). The exact mechanism by which E2Fs induce apoptosis is not clear but both transactivation and repressor functions are likely to be involved (Figure 1.6). For example, E2F-1-induced p53-dependent apoptosis appears to be mediated at least in part through induction of p14<sup>ARF</sup> (Alternative Reading Frame; p19<sup>ARF</sup> in mouse) (Bates et al., 1998a), which in turn activates p53 through inhibition of MDM2 (see section 1.2.4.a and Figure 1.12) (Pomerantz et al., 1998; Zhang et al., 1998; Honda & Yasuda, 1999; Tao & Levine, 1999; Weber et al., 1999). In addition, E2F-1 can induce expression of APAF1 (apoptosis-associated factor 1) and p73, both of which can induce apoptosis (Irwin et al., 2000; Stiewe et al., 2000; Moroni et al., 2001). By contrast, a transactivation-defective but DNA-binding competent mutant of E2F-1 can induce p53-independent apoptosis in some cell types (Hsieh et al., 1997; Phillips et al., 1997). This suggested that, at least in these cases, transcriptional repression of apoptotic genes by the RB-E2F complex, rather than the transactivation function of E2F-1, is involved in its apoptotic function. Finally, E2F-1 can induce apoptosis by a death-receptor dependent mechanism, by downregulating TRAF2 (TNFR-associated factor) protein levels and thereby inhibiting activation of TNFR (tumour necrosis factor receptor)-mediated survival signals including NF-κB (nuclear factor kappa-B) (Phillips et al., 1999). Mechanisms of E2F-3-induced apoptosis are yet to be elucidated.
Inappropriate S phase entry (Lee et al., 1994; Tsai et al., 2002) may account for apoptosis caused by $Rb$-deficiency independently of E2Fs and p53. The ability of wild-type cells to rescue their $Rb$-deficient neighbours from apoptosis suggests that RB may also regulate the expression of survival factors which protects cells from apoptosis (Figure 1.6) (Robanus-Maandag et al., 1994; Williams et al., 1994b; Lipinski et al., 2001).

Findings that RB is proteolytically cleaved by caspases during apoptosis induced by UV (ultra violet) irradiation, anti-Fas antibody, and TNFα (tumour necrosis factor α) (Janicke et al., 1996; Tan et al., 1997; Diederich et al., 1998), and that overexpression of RB can protect cells from IR (ionizing radiation)-, p53-, and TGF-β-induced apoptosis (Haas-Kogan et al., 1995; Haupt et al., 1995; Fan et al., 1996), provided further evidence of a role for RB in protection against apoptosis (see Tan & Wang, 1998 for review). Cleavage of RB results in its degradation (Janicke et al., 1996; Tan et al., 1997) and release of E2F-1, which can induce apoptosis. Thus, inactivation of $Rb$ should sensitise tumour cells to apoptosis, unless they also acquire mutations that inactivate apoptotic pathways, such as p53. Indeed, p53 is usually mutated in sporadic cancers that involve mutations of the $Rb$ gene. Similarly, transformation induced by viral oncoproteins (eg. E1A and E1B, E6 and E7) requires the simultaneous inactivation of $Rb$ and p53.
RB inhibits transactivation of pro-apoptotic genes, and may positively regulate the production of survival factors. Inactivation of RB by phosphorylation, cleavage, or mutation, allows E2F transactivation of pro-apoptotic genes and downregulation of survival factors.
1.1.3.c Regulation of Differentiation

In addition to facilitating differentiation by allowing cell cycle withdrawal, RB activates tissue-specific gene expression, which generally involves augmentation of the activity of tissue-specific transcription factors associated with differentiation. These include C/EBPβ (CCAAT/enhancer binding protein β) (Chen et al., 1996a), MyoD (Gu et al., 1993; Novitch et al., 1996; Sellers et al., 1998), c-JUN (Nead et al., 1998; Nishitani et al., 1999), and NF-IL6 (Chen et al., 1996b), involved in adipocyte, myogenic, keratinocyte, and monocyte/macrophage differentiation, respectively. RB also activates transcription of TGF-β2 via ATF-2 (Kim et al., 1992), TGF-β1 via the RCE (retinoblastoma control element) (Kim et al., 1991), the ubiquitous transcription factor SP1 by relieving MDM2-mediated repression (Johnson-Pais, 2001), and p21 via SP1 sites in the p21 promoter (Decesse et al., 2001).

One of the mechanisms by which RB may positively regulate transcription and differentiation is via its interaction with Id2 (inhibitor of DNA-binding 2), an inhibitor of bHLH (basic helix-loop-helix) transcription factors involved in differentiation. Indeed, loss of Id2 has recently been shown to suppress many of the defects observed in Rb−/− embryos (Lasorella et al., 2000). In some situations the SW1/SNF-RB complex is associated with transcriptional activation, and can enhance BRG1-dependent transcriptional activation by the glucocorticoid receptor, which is associated with HAT activity (Fryer & Archer, 1998).

1.1.3.d Regulation of Genomic Stability

In addition to its well known roles in regulation of proliferation, apoptosis and differentiation, a role for RB in regulation of genomic stability is beginning to emerge. Recent studies have implicated a role for RB in regulation of chromosome segregation, M phase progression, and chromatin remodeling (for review see Zheng & Lee, 2001), although the mechanisms by which it exerts such regulation are as yet unclear. They are likely to involve the interaction of RB with PP1α (protein phosphatase) (Durfee et al., 1993), H-NUC (Chen et al., 1995), mitosin (Zhu et al., 1995c), topoisomerase II (Bhat et al., 1999), and HEC1 (Zheng et al., 2000). Mitosin is thought to be involved in kinetochore assembly (Zhu et al., 1995c), while PP1α and HEC1 regulate multiple mitotic events, including kinetochore functions, mitotic
chromatin configuration, and mitotic cyclin degradation (Zheng & Lee, 2001). H-NUC is a subunit of the APC complex that controls the onset of sister chromatid separation and metaphase-anaphase transition, in addition to mitotic cyclin degradation (Koepf et al., 1999). Topoisomerase II orchestrates the higher order compaction of chromatin to form highly condensed mitotic chromosomes (reviewed in Withoff et al., 1996; Wang et al., 1997a). Consistent with a role for RB in regulation of chromatin structure, chromatin was found to be more relaxed in Rb−/− cells (Herrera et al., 1996).

RB is also involved in regulation of centrosome duplication (Meraldi et al., 1999; Mussman et al., 2000), precise control of which is required for accurate chromosome segregation. Phosphorylation of RB regulates coordination of both G1/S and G2/M transitions (see section 1.1.4.a), via the timely induction and degradation of cyclins. That RB is required for proper coordination of S phase and mitosis, accurate chromosome segregation, both of which are necessary to avoid hyperploidy and aneuploidy, supports a role for RB in the maintenance of genome stability, and may help to explain why inactivation of RB leads to multiple genetic alterations required for tumorigenesis (Zheng & Lee 2001). Consistent with a role for RB in regulation of genome stability, it has been suggested that RB plays a role in GNER (global nucleotide excision repair) (Therrien et al., 1999), although other studies have argued against a role for RB in DNA repair (Smith et al., 2000; Zhu et al., 2000).

1.1.4 Regulation of RB

RB is constitutively expressed in all normal mammalian cell types examined (Lee et al., 1987; Bernards et al., 1989), suggesting that the activity of RB is not regulated at the transcriptional level, although it has been shown that RB and E2F can regulate the RB promoter (Shan & Lee et al., 1994). Instead, RB activity is regulated post-translationally by serine/threonine phosphorylation mediated by cdks in a cell-cycle dependent manner (Buchkovich et al., 1989; Chen et al., 1989; DiCaprio et al., 1989; Mihara et al., 1989) (see below). Phosphorylation of RB by cdks is inhibited by the activity of CKIs (cyclin-dependent kinase inhibitors) (see section 1.1.4.b), while acetylation of RB by p300/CBP has been shown to inhibit phosphorylation of RB by
cyclin E-cdk2, and may regulate protein-protein interactions (Chan et al., 2001). In addition to its phosphorylation by cdk's, RB can be phosphorylated by other kinases. For example, p38MAPK (mitogen-activated protein kinase) is thought to phosphorylate and inactivate RB in response to Fas (Wang et al., 1999a), while JNK (c-Jun N-terminal kinase) phosphorylates RB in response to IR (Chauhan et al., 1999).

1.1.4.a Phosphorylation of RB by Cdk's

Hypophosphorylated RB predominates in G0 and early to mid-G1 phases of the cell cycle. It undergoes hyperphosphorylation in late G1 and remains in this hyperphosphorylated state until the end of mitosis when it becomes dephosphorylated. At least 16 cdk consensus sequences have been identified to date, and several cyclin-cdk complexes have been implicated in phosphorylation of RB. The cyclin-cdk complexes most prominently implicated in phosphorylation of RB are cyclin D-cdk4/6, cyclin E-cdk2, and cyclin A/cdk2. It is thought that cyclin D-cdk4/6 phosphorylates RB in early G1, cyclin E-cdk2 phosphorylates it near the end of G1, and cyclin A-cdk2 is required to maintain phosphorylation during S phase (Sherr et al., 1996). However, the cdk's that actually phosphorylate RB in vivo, and the consensus sites they act upon is still unclear, as is the precise mechanism by which RB phosphorylation leads to its inactivation, although recent studies have shed considerable light on the mechanism of sequential phosphorylation of RB (Harbour et al., 1999; Zhang et al., 2000). The most recently proposed model for RB phosphorylation is summarised below (Figure 1.7).
RB forms a repressor complex with E2F, HDAC, and SW1/SNF, that represses transcription and induces G1 arrest. Upon induction of cyclin D by growth factors, cyclin D-cdk4,6 phosphorylates RB, disrupting the interaction with HDAC, relieving repression of cyclin E and allowing S phase entry. Cyclin E-cdk2 then phosphorylates RB, disrupting the interaction with SW1/SNF and E2F, allowing expression of cyclin A and S phase progression. Cyclin A-cdk2 further phosphorylates RB and activates cyclin cdc2. Cyclin B-cdc2 induces mitosis, during which RB is dephosphorylated by I-phosphatase, allowing RB to interact once more with E2F and the chromatin-remodelling complexes, inducing G1 arrest.
Figure 1.7: Regulation of RB by phosphorylation

Growth Factors $\rightarrow$ Cyclin D
Cdk4,6

Sw1/SNF

Cyclin A
Cyclin E
G1 Arrest

Cyclin E
Cdk2

Sw1/SNF

Cyclin A
Cyclin E
S phase entry

Cyclin A
S phase progression

Cyclin A
Cdk2

Cyclin B
Cdc2
Mitosis

PP1α
D-type cyclins are induced in response to growth factor stimulation (Matsushime et al., 1991), and form complexes with cdk4 and cdk6, resulting in their activation and subsequent phosphorylation of RB (Ewen et al., 1993; Kato et al., 1993). It was recently shown that phosphorylation of Ser795 at the C-terminal region of RB by cyclin D-cdk4/6 is the first site to be phosphorylated (Grafstrom et al., 1999), causing the C-terminus of RB to interact with the positively charged lysine patch surrounding the LXCXE-binding site in the B domain of the pocket (Harbour et al., 1999). This blocks HDAC binding to the pocket and thus active transcriptional repression mediated by the pocket and HDAC (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998; Luo et al., 1998). Phosphorylation by cyclin D-cdk4,6 also facilitates interaction of cyclin E-cdk2 with the pocket (Harbour et al., 1999). Cyclin E protein levels increase due to loss of transcriptional repression (Zhang et al., 2000) and form complexes with cdk2, which phosphorylate Ser 567 located in pocket domain A, at the A-B interface. This causes disruption of the pocket structure, preventing RB from binding and inactivating E2F (Harbour et al., 1999), and cells enter S phase. It has been suggested that phosphorylation of RB by cyclin D-cdk4,6 does not disrupt the RB-SW1/SNF repressor complex, and that this complex maintains repression of cyclin A, thus inhibiting exit from S phase. Cyclin E-cdk2 phosphorylation of RB and/or BRG1 disrupts the RB-SW1/SNF complex, allowing expression of cyclin A, accumulation of cyclin B, and entry into mitosis (Zhang et al., 2000). RB becomes dephosphorylated in M phase by PPlα (Durfee et al., 1993; Ludlow et al., 1993; Yan & Mumby, 1999). Thus the sequential phosphorylation of RB coordinates progression into both S phase and mitosis.

1.1.4.b Regulation of RB phosphorylation by CKIs

Cdk protein levels are invariant throughout the cell cycle, indicating that their activity is regulated posttranslationally. There are four major mechanisms involved in regulation of cdk activity: binding of cyclin subunit, inhibitory phosphorylation, activating phosphorylation, and association with cyclin kinase inhibitors (CKIs) (for review see Morgan, 1997). Two families of CKIs have been identified based on their structures and cdk targets (for review see Sherr & Roberts, 1995, 1999). The INK4A (Inhibitor of cdK4) family comprises four members: p16INK4A (Serrano et al., 1993),
(Hannon & Beach, 1994), \(p_18^{\text{INK4C}}\) (Guan et al., 1994; Hirai et al., 1995), and \(p_19^{\text{INK4D}}\) (Chan et al., 1995; Hirai et al., 1995), characterised by the presence of ankyrin-like repeats, which are involved in protein-protein interactions. They inhibit cdk activity by acting as competitive inhibitors of D-type cyclins by forming specific complexes with their catalytic partners cdk4 and cdk6 (Serrano et al., 1993; Hannon & Beach, 1994; Hirai et al., 1995), thereby blocking RB phosphorylation and inducing cell cycle arrest. The inhibitory action of this family of proteins is therefore largely dependent on the presence of RB (Guan et al., 1994; Koh et al., 1995; Lukas et al., 1995).

Among this family, \(p_16^{\text{INK4A}}\) (also known as MTS1, for multiple tumour suppressor) is considered to be the most significant due to its role as a tumour suppressor. The \(p_16\) locus maps to chromosome 9p21, a region frequently mutated in human tumours (Kamb et al., 1994; Nobori et al., 1994), and the \(p_16\) gene is mutated or repressed by methylation in many types of cancer cell lines and primary tumours at a frequency second only to p53 inactivation (for review see Hirama & Koeffler, 1995; Ruas & Peters, 1998). \(p_16\) is part of the RB pathway (RB/\(p_16/cyclin\ D/cdk4\) proposed to act as a single oncogenic target that is disrupted in most, if not all, human tumours, either through inactivation of RB or \(p_16\), or through amplification of cyclin D or cdk4 (Serrano, 1997).

The second family of CKIs, referred to as the CIP/KIP (Cdk-Inhibitory Protein/Kinase-Inhibitory Protein) family, consists of \(p_21\)CIP1, \(p_27\)KIP1, and \(p_57\)KIP2 (see section 1.3.2). Unlike the INK4 family of inhibitors, CIP/KIP proteins make specific interactions with both cyclin and cdk and act as stoichiometric inhibitors (Russo 1996). The CIP/KIP proteins exhibit inhibitory activity towards a broader range of cdks, albeit with different efficiencies (Harper et al., 1995) to the INK4 family. They can also promote the assembly of active cyclinD-cdk4,6 complexes and target them to the nucleus (Zhang et al., 1994; LaBaer et al., 1997) (see section 1.3.3.a and Figure 1.15D).
1.2 The p53 Tumour Suppressor Gene

p53 protein was originally identified by virtue of its interaction with SV40 TAg (simian virus large T antigen) (Linzer & Levine et al., 1979; Lane & Crawford, 1979), and, as a result, it was first classified as a tumour antigen and then as an oncoprotein (Jenkins et al., 1985). The discovery that inactivation of p53, either by mutation or interaction with viral or cellular proteins, is the most frequent alteration observed in cancer cells, led to its reclassification as a tumour suppressor (for review see Levine et al., 1997). Mutations in p53 have been linked to Li-Fraumeni syndrome in humans, an inherited susceptibility disorder in which affected individuals are at risk of developing a variety of cancers (Malkin et al., 1990). p53-deficient mice also have a high incidence of tumours (Donehower et al., 1992; Purdie et al., 1994).

1.2.1 Structure of the p53 protein

The human p53 gene contains 11 exons and encodes a protein of 393 amino acids with a molecular mass of 53kD (Benchimol et al., 1985; Oren, 1985) (Figure 1.8). p53 has been highly conserved during evolution (Soussi et al., 1990) and murine p53 contains 387 amino acids. The p53 protein is composed of four major functional domains. The acidic N-terminal region contains the transcriptional activation domain (Unger et al., 1992), which interacts with components of the basal transcription machinery such as TBP (Seto et al., 1992) and TAFs (TBP-associated factors) (Thut et al., 1995), and p300/CBP (Gu & Roeder, 1997; Lill et al., 1997). It contains several phosphorylation sites and is critically involved in regulating p53 stability and activity via a NES (nuclear export signal) (Zhang & Xiong, 2001) and interaction with MDM2 (Haupt et al., 1997; Kubbutat et al., 1997) (see section 1.2.4). The N-terminal region also contains a proline rich region, which has been implicated in both p53-mediated growth arrest (Walker & Levine, 1996) and apoptosis (Venot et al., 1998). It contains five repeats of the SH3 (src homology 3) binding motif PXXP, suggesting that it may mediate interactions with SH3 domain-containing proteins. The central region contains the sequence-specific DNA-binding domain (Pavletich et al., 1993), exhibits exonuclease activity (Mummenbrauer et al., 1996; Bakhanshvilii, 2001), and sustains the vast majority of p53 mutations (Hollstein et al., 1994). The
Model describing the structural domains of p53 and some of the key modification sites important for p53 stabilisation and activation (see section 1.2.4). NLS: the major nuclear localisation signal. NES: nuclear export signal. CTD: C-terminal domain. P: phosphorylation site, Ac: acetylation site, Su: sumoylation site. Ser: serine, Lys: lysine.

C-terminal region contains a tetramerisation domain (Jeffrey et al., 1995), a non-sequence-specific DNA-binding domain (Wang et al., 1993), a NES (Stommel et al., 1999), three NLSs (nuclear localisation signals) (Shaulsky et al., 1990; Liang & Clarke, 1999a, b) and multiple phosphorylation and acetylation sites (see section 1.2.4). p53 monomers homodimerise to form the p53 tetramer which contains a pair of dimers and is the most efficient DNA binding form of the protein (Jeffrey et al., 1995). The non-specific DNA binding domain binds damaged DNA (Nelson & Kastan, 1994) and is involved in DNA and RNA strand annealing (Wu et al., 1995). This region is thought to have a negative regulatory function, due to allosteric inhibition of DNA-binding by the central region (Hupp & Lane, 1994; Hupp et al., 1995). A recent study proposed that the C-terminal region binds directly to DNA, thereby inhibiting DNA binding by the central region (Yakovleva et al., 2001).
Another recent study, however, reported that the C-terminal region does not negatively regulate p53 DNA binding activity (Espinosa & Emerson, 2001).

1.2.2 The p53 family of proteins

p53 is a member of a family that includes the two structurally related proteins p63 and p73 (for reviews see Lohram & Vousden, 2000; Moll et al., 2001) (Figure 1.9). The DNA-binding region bears the highest degree of homology between these proteins, with p63 and p73 being more similar to each other than to p53. Unlike p53, which has a single promoter that encodes a single protein, p63 and p73 use alternate promoters and exon splicing to generate several different isoforms grouped into two different classes of proteins, those that contain the transactivation domain (TAp73α-ζ, and TAp63α-γ) and those that don’t (ΔNp73α and β, and ΔNp63α-γ). The TA forms can transactivate p53 target genes and induce cell cycle arrest and apoptosis; ΔN forms act as dominant-negative inhibitors of p53 family members (Chen, 1999; Kaelin 1999). The α forms of p63 and p73 contain SAM (sterile α motif) domains, protein interaction motifs found in proteins implicated in development. Like p53, p73 is stabilised by oncogenes (Zaika et al., 2001), and some forms of DNA damage via the c-Abl tyrosine kinase (Agami et al., 1999; Yuan et al., 1999), resulting in apoptosis. Unlike p53, p73 is degraded through the proteasome independently of MDM2, although MDM2 does inhibit p73 transcriptional activity (Zeng et al., 1999).

Unlike p53, p63 and p73 are not frequently mutated in human cancer (Kaelin, 1999), although a potential role in tumour suppression cannot be ruled out, as the signals and pathways regulating the stability and activity of p63 and p73, and the influence that different family members have on each other, are only beginning to emerge.
Figure 1.9: The p53 protein family

<table>
<thead>
<tr>
<th>p53</th>
<th>Transactivation</th>
<th>Proline-rich</th>
<th>DNA binding</th>
<th>C-terminus</th>
</tr>
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<tbody>
<tr>
<td>TAp73α</td>
<td>Transactivation</td>
<td>Proline-rich</td>
<td>DNA binding</td>
<td>C-terminus</td>
</tr>
<tr>
<td>TAp63α</td>
<td>Transactivation</td>
<td>Proline-rich</td>
<td>DNA binding</td>
<td>C-terminus</td>
</tr>
<tr>
<td>ΔNp73</td>
<td>N</td>
<td>Proline-rich</td>
<td>DNA binding</td>
<td>C-terminus</td>
</tr>
<tr>
<td>ΔNp63</td>
<td>N</td>
<td>Proline-rich</td>
<td>DNA binding</td>
<td>C-terminus</td>
</tr>
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</table>

Model describing the structural domains of the p53 family proteins. Unlike p53, which has a single promoter, p73 and p63 genes generate both transactivation-proficient (TA) and transactivation-deficient (ΔN) proteins by using alternate promoters and exon splicing. There are 6 TAp73 (α-ζ) and 3 TAp63 (α-γ) isoforms, and 3 ΔNp73 (α-γ) and 2 ΔNp73 (α and β) isoforms. α forms of p63 and p73 contain SAM domains involved in protein-protein interactions. ΔN forms can act as dominant negative inhibitors of p53 family members.

1.2.2.a p53 family knock-out mice

p53−/− mice were initially described as being developmentally normal, suggesting that p53 is dispensable for growth, differentiation, and embryonic development (Donehower et al., 1992; Jacks et al., 1994; Purdie et al., 1994). Subsequent studies, however, revealed that a small number of female embryos developed exencephaly due to failure of the neural tube to close properly, and died before birth (Armstrong et al., 1995; Sah et al., 1995). p53−/− mice are highly predisposed to malignancy and develop predominantly lymphomas, and sarcomas to a lesser extent. p53 heterozygotes are also predisposed to malignancy but with increased latency, and in
contrast to \( p53^{+/−} \) mice, they develop predominantly sarcomas, lymphomas to a lesser extent, and some develop adenocarcinomas (Donehower et al., 1992; Jacks et al., 1994; Purdie et al., 1994). The differences in the tumour spectrum between \( p53^{+/−} \) mice and \( p53 \) heterozygotes may reflect the difference in timing during development when the mutation occurs. MEFs derived from \( p53^{+/−} \) mice have a significantly shorter doubling time, increased ability to grow under conditions of low density, and a lack of senescence even at high passage (Harvey et al., 1993).

In contrast to \( p53^{+/−} \) mice, mice deficient in \( p63 \) or \( p73 \) are not predisposed to malignancy, but exhibit severe developmental abnormalities. \( p63^{−/−} \) mice show defects in limb and skin development as well as craniofacial malformations (Mills et al., 1999; Yang et al., 1999). Consistent with this, heterozygous germline mutations of \( p63 \) in humans cause EEC syndrome, characterised by limb defects and facial clefts (Celli et al., 1999). \( p73^{−/−} \) mice develop neurological, pheromonal, and inflammatory defects (Yang et al., 2000).

### 1.2.3 Functions of \( p53 \)

\( p53 \) functions as a transcription factor by binding to consensus target DNA sequences and regulating the transcription of target genes, thereby integrating a wide range of cellular processes including growth arrest, apoptosis, differentiation, senescence, DNA repair and angiogenesis, in response to a variety of genotoxic stress (for reviews see Almog & Rotter, 1997; May & May, 1999; Albrechtsen et al., 1999; Sionov & Haupt, 1999). Although \( p53 \) is not required for normal cell growth or differentiation, it plays an essential role in preventing the accumulation of genetic lesions and maintaining genomic integrity, hence its role as “guardian of the genome”. It achieves this through the induction of growth arrest to prevent the replication of damaged DNA, or apoptosis to eliminate the aberrant cells. Hence, regulation of proliferation and apoptosis are crucial to the role of \( p53 \) as a tumour suppressor and are discussed below. Whether \( p53 \) induces either a growth arrest or apoptosis in response to a particular stress is determined by a variety of factors. These include the cell type, the availability of survival factors, the severity of the stress, the duration and level of \( p53 \) expression, the ability to repair DNA, the presence of activated oncogenes, the RB status, and the BAX (BCL-2-associated X
protein)/BCL-2 (B-cell lymphoma) protein ratio (for review see Sionov & Haupt, 1999).

1.2.3.a Regulation of Proliferation

p53 is an essential regulator of the G1 (Kastan et al., 1992; Kuerbitz et al., 1992) and G2 (Agarwal et al., 1995; Stewart et al., 1995) checkpoints in response to DNA damage, which ensure that damaged DNA is repaired before it is replicated or segregated. In response to DNA damage, p53 accumulates in a transcriptionally active form (see section 1.2.4) and activates expression of its target genes (Figure 1.10). Primary amongst these is the cyclin kinase inhibitor p21 (El-Deiry et al., 1993), an important component of both p53-dependent G1 (Deng et al., 1995; Brugarolas et al., 1995; Waldman et al., 1995; Polyak et al., 1996) and G2 arrest (Polyak et al., 1996; Waldman et al., 1996; Dulic et al., 1998; Medema et al., 1998). MEFs from p21<sup>−/−</sup> mice are only partially defective in G1 arrest, however, following DNA damage, compared with p53<sup>−/−</sup> MEFs, indicating that a p53-dependent, p21-independent pathway of growth arrest also exists (Brugarolas et al., 1995; Deng et al., 1995). p21 inhibits the activity of cyclin-cdk2 and maintains RB in a hypophosphorylated state which prevents S phase entry (Dulic et al., 1994; Slebos et al., 1994; Brugarolas et al., 1999). p21 also promotes growth arrest by binding to PCNA, the DNA polymerase δ processivity factor, inhibiting the elongation step in DNA replication, while permitting DNA repair (Li et al., 1994). p21 inhibition of cdk2 activity prevents activation of cyclin B-cdc2, and therefore entry into mitosis.

p53-induction of GADD45 (growth arrest and DNA damage gene 45) in response to DNA damage leads to G1 arrest in some cell types (Kastan et al., 1992; Zhan et al., 1998). Although the mechanism of this arrest is unknown, it is thought to involve its interaction with PCNA (Smith et al., 1994). GADD45 is thought to play a role in the p53-dependent G2 arrest following UV but not IR, through disruption of the cyclin B-cdc2 complex via its interaction with cdc2 (Wang et al., 1999b; Zhan et al., 1999).
Transcriptional activation of 14-3-3δ by p53 (Hermeking et al., 1997) is an important part of the G2 DNA damage checkpoint. It sequesters Cdc25C (cell division cycle 25C) in the cytoplasm so that it cannot activate cdc2 (Peng et al., 1997), and also sequesters cyclin B-cdc2 in the cytoplasm preventing this complex from entering the nucleus and initiating mitosis (Hermeking et al., 1999). Additional mechanisms of p53-induced G2 arrest include transcriptional repression of cdc2, cyclin B1 (Taylor et al., 1999; Flatt et al., 2000; Krause et al., 2000), and Topoisomerase II (Withoff et al., 1996; Wang et al., 1997b).

p53 can repress transcription by squelching, whereby high levels of p53 bind to TBP and TAFs, sequestering them away from the promoters of genes that do not contain p53-binding sites (Seto et al., 1992; Farmer et al., 1996; Mack et al., 1993, Ragimov et al., 1993). Repression can also be due to binding of p53 to the p300 histone actetyl transferase, blocking the binding of this protein to genes that depend on actetylation for activity (Avantaggiati et al., 1997). Finally, p53 can inhibit cell cycle progression by binding to cyclin H and MAT1, components of the CAK (cdk-activating kinase) enzyme, which activates cyclin-cdk's required for cell cycle progression (Schneider et al., 1998).

1.2.3.b Regulation of Apoptosis

p53 can induce apoptosis in response to DNA damage (Kastan et al., 1991; Kuerbitz et al., 1992; Clarke et al., 1993; Lowe et al., 1993), expression of viral or cellular oncogenes (Hermeking & Eick, 1994; Lowe et al., 1994), withdrawal of growth factors (Canman et al., 1995), or the absence of RB (Morgenbesser et al., 1994; Macleod et al., 1996), via transcription-dependent and -independent apoptotic pathways (Haupt & Oren, 1996) (for review see Bates & Vousden, 1999). There are two major apoptotic pathways in mammalian cells: the death-receptor pathway and the mitochondrial pathway (for review see Hengartner, 2000) (Figure 1.11 A). p53 induces expression of proteins involved in activation of both apoptotic pathways (Figure 1.11 A and B). The death-receptor pathway involves trimerisation of death receptors, such as Fas/APO1 by binding of Fas ligand, which leads to recruitment of pro-caspase 8 via the adaptor molecule FADD (Fas-associated death domain), and autoactivation of caspase 8. Activated caspase 8 then cleaves and activates the effector caspase 3, and can also activate the mitochondrial pathway through cleavage
of BID (BH3-interacting domain). Activation of the mitochondrial pathway can occur independently of the death-receptor pathway, and involves release of cytochrome c from the mitochondria, often through activation of a pro-apoptotic member of the BCL-2 family such as BAX or BID. Cytochrome c associates with APAF-1 and caspase 9 to form the apoptosome, which cleaves and activates caspase 3, resulting in cleavage of specific substrates required for execution of the apoptotic process.

p53-induced apoptotic target proteins include Fas/APO-1 (Owen-Schaub et al., 1995), PIDD (p53-inducible protein with death domain) (Lin et al., 2000), BAX (Miyashita & Reed, 1995), PUMA (Nakano et al., 2001; Yu et al., 2001), Noxa (Oda et al., 2000), APAF-1 (Moroni et al., 2001), AIP1 (Oda et al., 2000), IGF-BP3 (insulin-like growth factor-binding protein) (Buckbinder et al., 1995), PIGs (p53-inducible genes) (Polyak et al., 1997), NF-κB (Ryan et al., 2000), and p21B (Nozell & Chen, 2002) (for review see May & May, 1999; Hickman et al., 2002) (Figure 1.11 B). Fas and PIDD induce apoptosis via the death receptor pathway (Owen-Schaub et al., 1995; Lin et al., 2000) (Figure 1.11 A). BAX promotes apoptosis by facilitating the release of AIF (apoptosis-inducing factor) and cytochrome c from the mitochondria (Narita et al., 1998; Susin et al., 1999), and by antagonizing the anti-apoptotic activity of BCL-2 (Oltvai et al., 1993) (Figure 1.11 A). PUMA (Nakano et al., 2001; Yu et al., 2001) and Noxa (Oda et al., 2000) also antagonize the anti-apoptotic activity of BCL-2 and promote the release of cytochrome c. APAF-1, together with cytochrome c, activates caspase 9, forming the apoptosome (Rodriguez & Lazebnik, 1999). AIP1 (apoptosis-inducing protein 1) induces apoptosis by disrupting mitochondrial membrane potential (Oda et al., 2000), and p21B is also thought to induce apoptosis via the mitochondrial pathway (Nozell & Chen, 2002). IGF-BP3 sequesters the survival factor IGF-1 (Buckbinder et al., 1995), and PIGs mediate apoptosis through the generation of ROS (reactive oxygen species) (Johnson et al., 1996). Transcription-independent p53 apoptosis involves transrepression of genes including IGF-1R, which blocks IGF-1 survival signaling (Prisco et al., 1997), and BCL-2, which prevents the formation of reactive oxygen intermediates (Hockenberry et al., 1993), and release of AIF and cytochrome c from the mitochondria (Susin et al., 1999).
Model describing p53 activation of apoptosis by the death-receptor pathway and the mitochondrial pathway. p53 can activate apoptosis in response to DNA damage by inducing Fas which activates the death-receptor pathway, and by inducing BAX and BID, which activate the mitochondrial pathway. p53 repression of BCL-2 also activates the mitochondrial pathway. Each pathway culminates in caspase 3 activation and cleavage of specific substrates required for the execution of apoptosis.
The critical role of p53-induced apoptosis in limiting tumor progression was demonstrated in several transgenic mouse models. Mice expressing HPV E7 (inactivates RB) in the retina (Howes et al., 1994) or lens (Pan & Griep, 1994) exhibit extensive apoptosis. When crossed to either p53 null mice (Howes et al., 1994) or mice expressing HPV E6 (inactivates p53) (Pan & Griep, 1994), apoptosis was reduced and the mice developed lens and retinal tumors. Mice expressing a SV40 TAg in the choroid plexus, which can bind RB but not p53, developed slow growing tumors. When crossed onto a p53 null background, the mice developed aggressive tumors with attenuated apoptosis (Symonds et al., 1994).

1.2.3.c Regulation of Genomic Stability

p53 is involved in maintenance of genomic stability, a function that is critical to its role as a tumor suppressor. Loss of p53 leads to gene amplification (Livingstone et al., 1992; Yin et al., 1992), fibroblasts from p53 null mice exhibit chromosome abnormalities (Harvey et al., 1993), and p53 mutations in both human and mouse tumors have been associated with aneuploidy and genomic instability (Blount et al., 1994; Donehower et al., 1995). p53 is thought to maintain genomic stability by preventing homologous recombination, and participating in DNA repair, in addition to inducing cell cycle arrest or apoptosis of cells containing DNA damage, thereby preventing propagation of potentially deleterious mutations (for review see Albrechtsen et al., 1999).

The role of p53 in DNA repair is not yet clear and remains controversial (for review see McKay et al., 1999). For example, several studies implicated a role for p53 in GNER but not TCNER (transcription-coupled nucleotide excision repair), resulting in reduced efficiency of repair and increased genomic instability in p53-deficient cells (Ford & Hanawalt, 1995, 1997; Ford et al., 1998; Prost et al., 1998a, 1998b; Smith et al., 2000; Wani et al., 2000), whereas other studies reported that p53 participates in both GNER and TCNER (Therrien et al., 1999; Zhu et al., 2000). p53 induces expression of GADD45 (Kastan et al., 1992) and p48 (Hwang et al., 1999), which have been linked to DNA repair, and associates with and inhibits the helicase activity of XPB (xeroderma pigmentosum type B) and XPD (xeroderma pigmentosum type D), components of the TFIIH (transcription factor II H) repair
complex (Leveillard et al., 1996; Wang et al., 1995). p53 can bind to damaged DNA (Reed et al., 1995), and possesses intrinsic 3' to 5' exonuclease activity (Mummenbrauer et al., 1996; Bakhanashvili, 2001), a mechanism employed to ensure sequence fidelity during DNA replication and DNA repair.

Consistent with its role in protecting genome integrity, p53 has been implicated in regulation of a post-mitotic checkpoint, whereby cells lacking p53 do not arrest in response to mitotic spindle inhibitors and reinitiate DNA synthesis, becoming polyploid (Cross et al., 1995). The finding that p53−/− MEFs contain multiple centrosomes, resulting in aberrant chromosomal segregation, indicated a role for p53 in regulation of centrosome duplication (Fukasawa et al., 1996). p53 regulates both centrosome duplication, and coordination of centrosome and DNA duplication, via p21-dependent (Tarapore et al., 2001) and -independent pathways, perhaps involving GADD45 (Hollander et al., 1999). Chromosome instability and aneuploidy associated with inactivation of p53 in human cancers strongly correlates with centrosome hyperamplification (Weber et al., 1998; Carroll et al., 1999).

1.2.4 Regulation of p53
p53 is normally present at very low levels in cells due to its extremely short half-life (Rogel et al., 1985), and exists mainly in a latent, inactive state. Although p53 is regulated at both the level of transcription (Schwartz et al., 1993) and translation (Fu & Benchimol, 1997), the primary mechanism of p53 regulation is post-translational, allowing a rapid response to a range of stress conditions including DNA damage, oncogene activation, heat shock, hypoxia, mitotic spindle damage, changes in redox potential, and reduction in the ribonucleoside triphosphate pool (reviewed by Giaccia & Kastan, 1998). Post-translational modifications, including phosphorylation, dephosphorylation, acetylation, sumoylation and glycosylation, alter both the level of protein stability (reviewed by Ashcroft & Vousden, 1999), and biochemical activation (reviewed by Oren, 1999; Vousden et al., 2002), allowing accumulation of active p53 protein.

1.2.4.a p53 Protein Stability
The very short half-life of p53 is due to the constant ubiquitin-dependent degradation of newly synthesised p53 by the 26S proteasome. MDM2 is one of the principal
regulators of p53, demonstrated by the early embryonic lethality of MDM2-deficient mice, which is entirely prevented by simultaneous deletion of p53 (Jones et al., 1995; Montes de Oca Luna, et al., 1995) (for review on MDM2 see Momand et al., 2000). MDM2 employs several mechanisms to maintain p53 under tight control (Figure 1.12). Primary among these is its ability to act as an E3 ubiquitin ligase, transferring ubiquitin to p53 (Honda et al., 1997; Fang et al., 2000), thereby targeting it for degradation by the proteasome, as well as mediating p53 export from the nucleus to the cytoplasm where degradation occurs (Roth et al., 1998; Boyd et al., 2000; Geyer et al., 2000). In addition to MDM2, the MAPK JNK1 is thought to regulate p53 stability in normal cells by acting as a ubiquitin ligase (Fuchs et al., 1998a), while in cells infected with HPV (human papilloma virus)-16/18, the papillomavirus E6 oncoprotein interacts with the cellular E6AP protein forming an E3 ubiquitin ligase, which targets p53 for degradation (Scheffner et al., 1993).

One way to stabilise p53, therefore, is to inhibit MDM2. p14/p19ARF (hereafter referred to as ARF) does this in a variety of ways (Figure 1.12). It inhibits MDM2-mediated ubiquitination of p53 (Honda & Yasuda, 1999; Midgeley et al., 2000), blocks nucleocytoplasmic shuttling of MDM2 required for p53 degradation (Tao & Levine, 1999), binds directly to MDM2 and promotes its degradation (Pomerantz et al., 1998; Zhang et al., 1998), and sequesters MDM2 into the nucleolus (Weber et al., 1999; Lohrum et al., 2000). ARF is required for p53 stabilisation in response to a variety of cellular stresses, including DNA damage (Khan et al., 2000), and hyperproliferative oncogenic stimuli (Sherr, 1998).

RB inhibits degradation of p53 by binding to MDM2, and as a result, protects p53 apoptotic activity, but not transcriptional activity from MDM2 inhibition (Hsieh et al., 1999). The c-Abl tyrosine kinase (Sionov et al., 2001) and the MDM2-related protein, MDMX (MDM2-related protein X) (Jackson & Berbich, 2000), also inhibit the ability of MDM2 to mediate p53 degradation.
Figure 1.12: Regulation of p53 by the MDM2-ARF pathway

Model describing the regulation of p53 by the MDM2-ARF pathway. p53 binds consensus p53 site and activates transcription of several genes including MDM2, interaction with transcriptional coactivators such as p300/CBP, and with TBP component of the basal transcriptional complex. MDM2 binds to the transactiva domain of p53 inhibiting its transcriptional activity and interaction with transcriptional machinery. MDM2 also mediates ubiquitination of p53 and facilit p53 nuclear export and proteasomal degradation. ARF inhibits degradation of p53 facilitating MDM2 degradation and sequestering MDM2 in the nucleolus, as well directly inhibiting MDM2-mediated ubiquitination and nuclear export of p53.
Figure 1.12: Regulation of p53 by the MDM2-ARF pathway

- p53 binds to the MDM2-ARF pathway
- Ubiquitination (Ub) marks p53 for proteasomal degradation
- p53 is degraded in the cytoplasm
- ARF is induced and localizes to the nucleolus
- CBP and TBP are involved in the regulation of p53

Proteasomal degradation

nucleus
cytoplasm

nucleolus
Model describing the regulation of p53 by phosphorylation. DNA damage activates the ATM/ATR kinases, which in turn phosphorylate and activate Chk2 and Chk1, respectively. ATM and ATR, together with Chk1 and Chk2, phosphorylate p53, while ATM phosphorylates MDM2, which blocks MDM2 binding to and mediating degradation of p53. Phosphorylation also blocks the nuclear export signal (NES), allowing accumulation of p53. p53 then binds to a consensus p53 site and activates transcription of several genes resulting in growth arrest or apoptosis.
Phosphorylation of p53 is another mechanism by which to inhibit the p53-MDM2 interaction and therefore stabilise p53. Following DNA damage and other types of stress, p53 is phosphorylated by various kinases at multiple sites within the N- and C-termini (Figure 1.8). The PI-3 kinases ATM (ataxia telangectesia-mutated) and ATR (ATM-related), and their downstream targets Chk1 (checkpoint 1) and Chk2 (checkpoint 2), phosphorylate p53 on Ser20 and Ser15 in response to IR (ATM/Chk2) and UV (ATR/Chk1) radiation, respectively (Banin et al., 1998; Canman et al., 1998; Tibbets et al., 1999; Hirao et al., 2000; Shieh et al., 2000) (Figure 1.13). This phosphorylation interferes with the ability of MDM2 to interact with p53 and mediate its degradation, leading to accumulation of p53. Phosphorylation of MDM2 by ATM (Khosravi et al., 1999; Maya et al., 2001), and phosphorylation of p53 by stress-activated JNK1, also stabilise p53 by inhibiting the interaction with MDM2 (Fuchs et al., 1998b). Decreased MDM2 sumoylation resulting in its enhanced ubiquitination and degradation (Buschmann et al., 2000, 2001), as well as downregulation of MDM2 expression (Arriola et al., 1999; Ashcroft et al., 2000), are also involved in p53 stabilisation after DNA damage.

An alternative way by which to achieve p53 accumulation, independently of MDM2, is to inhibit p53 nuclear export mediated by its NESs via the CRM1 (chromosomal regional maintenance)-dependent nuclear export pathway. DNA-damage-induced phosphorylation of p53 on ser15, which resides within the N-terminal NES (Figure 1.8), has been shown to block p53 nuclear export (Zhang & Xiong, 2001), while tetramerisation of p53 is thought to occlude the C-terminal NES (Stommel et al., 1999).

### 1.2.4.b p53 Transcriptional Activity

Activation of p53 refers to its conversion from a protein that binds poorly to DNA to one that interacts efficiently with its target sequences, and activates transcription. DNA damage is a potent activator of p53, and the presence of a single double strand break is sufficient to induce a p53 response (Huang et al., 1996).

In addition to regulating p53 protein stability, MDM2 regulates p53 transcriptional activity (Figure 1.12). It does this by binding to and inhibiting the N-terminal transactivation domain of p53 (Momand et al., 1992; Oliner et al., 1993; Wu
et al., 1993), blocking the interaction of p53 with transcriptional coactivators such as p300/CBP (Wadgaonkar & Collins, 1999), as well as directly repressing transcription (Thut et al., 1997). MDM2 also inhibits p53 acetylation (Kobet et al., 2000; Ito et al., 2001), required for p53 activation. Inhibition of the p53-MDM2 interaction is therefore one way to activate p53, and this is achieved via phosphorylation of p53 by the ATM/ATR pathway (see section 1.2.4.a). Importantly, MDM2 is itself a transcriptional target of p53 (Barak et al., 1993; Wu et al., 1993), and this autoregulatory feedback loop is critical to the regulation of p53, perhaps contributing to termination of the p53 response once appropriate action has been taken.

Apart from ATM, ATR, Chkl, Chk2, and JNK1, other kinases implicated in phosphorylation of the p53 N-terminus include DNA-PK (DNA-protein kinase), p38MAPK, CK1 (cyclin kinase 1), and CAK (for review see Appella & Anderson, 2001). Different kinases are thought to phosphorylate specific sites in response to specific stimuli, thereby mediating a particular pattern of gene expression and cellular response. For example, p38MAPK phosphorylates ser46 in response to UV (Bulavin et al., 1999) (Figure 1.8), activating expression of p53AIP1 and apoptosis (Oda et al., 2000). Kinases implicated in phosphorylation of the p53 C-terminus include cdc2, cdk2, PKC (protein kinase C), and CK2 (cyclin kinase 2), although their roles in p53 activation are not well understood (for review see Apella & Anderson, 2001 and references therein) (Figure 1.8). Phosphorylation of p53 on Ser392 by CK2 is thought to stimulate p53 activity via conformational changes in the tetramer (Hupp & Lane, 1994; Hupp et al., 1995). In addition to p53 activation by phosphorylation, dephosphorylation of p53 on ser376 by ATM in response to IR has been shown to promote its interaction with the 14-3-3 protein, which can enhance p53 SST (sequence-specific transactivation) (Waterman et al., 1998).

In addition to phosphorylation, the C-terminus of p53 is acetylated following DNA damage, and phosphorylation of p53 at the N-terminus augments this acetylation (Sakaguchi et al., 1998; Liu et al., 1999). The role of acetylation, however, in p53 activation remains controversial (for review see Prives & Manley, 2001). The histone acetylases p300/CBP and P/CAF acylate p53 at Lys373, 382 (p300/CBP) and 320 (P/CAF) (Sakaguchi et al., 1998; Liu et al., 1999) (Figure 1.8), increasing p53 sequence-specific DNA binding and SST of target genes.
(Avantaggiati et al., 1997; Gu et al., 1997). In contrast, a recent study suggested that acetylation of p53 by p300 does not facilitate p53 transcriptional activity via increased DNA-binding, but that by recruiting p300 to its target promoters, p53 facilitates acetylation of nucleosomes, which in turn enhances transcription (Epsinosa & Emerson, 2001).

Sumoylation of p53 at Lys386 has been shown to activate p53 (Rodriguez et al., 1999; Muller et al., 2000), although a recent study contradicted this finding (Kwek et al., 2001). Finally, inhibition of p53 nuclear export leads to its activation (see section 1.2.4.a) (Freedman et al., 1998; Roth et al., 1998).

Thus, p53 undergoes a variety of post-translational modifications in response to cellular stress such as DNA damage, allowing accumulation and activation of p53. It appears that the particular pattern of modifications induced by a particular stress depends on the cell type, and determines the p53 response by allowing differential activation of target genes, culminating in cell cycle arrest or apoptosis (for review see Jayaraman & Prives, 1999).

1.3 The p21 Tumour Modifier Gene

p21\textsuperscript{CIP1/WAF1/SDI1} was the first CKI (cyclin kinase inhibitor) identified and was discovered almost simultaneously as a cdk2-interacting protein, CIP1 (Harper et al., 1993), as a p53-activated protein, WAF1 (wild-type p53-activated protein) (El-Deiry et al., 1993), and as a growth inhibitor from senescent cells, SDI1 (senescent cell-derived inhibitory protein) (Noda et al., 1994). Expression of p21 suppresses growth of normal (Harper et al., 1993) and tumour cells in vitro and in vivo (El-Deiry et al., 1993), suggesting that it functions as a tumour suppressor. However, the role of p21 in tumour suppression is controversial as p21 is rarely mutated in human cancers (Shiohara et al., 1994) and p21 null mice are not predisposed to malignancy (Deng et al., 1995), although they are more susceptible to chemical- and oncogene-induced carcinogenesis than wild-type mice (Topley et al., 1999; Weinberg et al., 1999; Adnane et al., 2000; Bearss et al., 2002). Consequently, p21 has been proposed to act as a tumour modifier gene rather than as a tumour suppressor gene (Jones et al., 1999; Hupp et al., 2000). The fact that p21 functions not only as a negative regulator of proliferation, but can also positively regulate proliferation and survival (see sections
1.3.3a and 1.3.3b), helps to explain why loss of \textit{p2l} is not selected for during tumourigenesis.

### 1.3.1 Structure of the p21 protein

The human \textit{p2l} gene contains 3 exons encoding a protein of 164 amino acids with a molecular mass of 21kD, and has been conserved during evolution (El-Deiry et al., 1993; Huppi et al., 1994) (Figure 1.14). The N-terminus of p21 contains cyclin- and cdk-binding domains, responsible for cyclin-cdk inhibition (Chen et al., 1995; Lin et al., 1996). The C-terminus contains a PCNA-binding region, a NLS, a cyclin-binding site, and two consensus caspase cleavage sites (Adams et al., 1996; Chen et al., 1996; Ball et al., 1997; Park et al., 1998; Zhang et al., 1999)

![Figure 1.14: Structure of the p21](image)

**N-terminus-binding proteins**

- E2F
- Procaspsase 3
- SAPK
- ASK1

**C-terminus-binding proteins**

- c-Myc
- C/EBPα


### 1.3.2 The CIP/KIP family of CKIs

\textit{p21} belongs to a family of proteins that includes \textit{p27KIPl} and \textit{p57KIP2}, characterized by a conserved N-terminal cyclin-cdk inhibitory domain. Like \textit{p21}, \textit{p27}
(Polyak et al., 1994; Toyoshima & Hunter, 1994) and p57 inhibit growth in vitro (Lee et al., 1995; Matsuoka et al., 1995). Unlike p21 and p57, p27 does not contain a PCNA-binding domain. Determination of the crystal structure of p27 bound to cyclin A-cdk2 provided insight into the mechanism of cyclin-cdk2 inhibition by CIP/KIP proteins. p27 binds to the cyclin subunit via the cyclin box, and to the N-terminal region of cdk2, inserting into the catalytic cleft, causing extensive conformational changes, and inhibiting ATP-binding (Russo et al., 1996). Association of p27 with the cyclin-cdk complex also sterically hinders activating phosphorylation of cdk2 Thr160 by CAK (Kato et al., 1994; Aprelikova et al., 1995). p21 and p57 are thought to inhibit cyclin-cdk activity by a similar mechanism.

1.3.2.a CIP/KIP family knock-out mice

p21$^{-/-}$ mice develop normally, exhibit a normal apoptotic response, and do not have an increased frequency of spontaneous tumorigenesis (Deng et al., 1995). However, p21$^{-/-}$ MEFs are partially defective in the p53-dependent G1 arrest response to DNA damage and nucleotide depletion (Brugarolas et al., 1995; Deng et al., 1995). These data indicate that factors other than p21 are required for p53-mediated tumour suppression, while p21 may play a particularly important role in the p53 response to DNA damage. p27$^{-/-}$ mice exhibit a phenotype of gigantism and organomegaly, female infertility, deafness, and retinal defects, associated with an inability of cells to withdraw from the cell cycle. However, p27$^{-/-}$ fibroblasts are not impaired in their growth arrest response to a variety of antimitogenic signals, and p27$^{-/-}$ mice are not predisposed to a general increase in tumour frequency (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996; Chen & Segil, 1999; Lowenheim et al., 1999), consistent with the absence of p27 mutations in human tumours (Pietenpol et al., 1995; Ponce-Castaneda, 1995). However, low expression of p27 protein occurs frequently in many types of human tumours and correlates strongly with tumour aggression and poor prognosis (Tsihlias et al., 1999), and p27$^{-/-}$ mice are susceptible to tumorigenesis after carcinogenic treatment (Fero et al., 1998). p57$^{-/-}$ mice die neonatally, with altered proliferation in the lens and cartilage, as well as developmental defects in several tissues (Yan et al., 1997; Zhang et al., 1997). Loss
of p57 in humans results in the complex overgrowth and cancer predisposition disease Beckwith-Wiedemann syndrome (Zhang et al., 1997).

### 1.3.3 Functions of p21

#### 1.3.3.a Regulation of Proliferation

p21 can both negatively and positively regulate proliferation (Figure 1.15). p21 inhibits proliferation by a variety of different mechanisms (Figure 1.15 A-C) inducing both G1 and G2 arrest (Niculescu et al., 1998). It is an important component of the p53-dependent G1 and G2 DNA damage checkpoints (see 1.2.3.a) but can also induce G1 arrest independently of p53, for example, in response to TGF-β (Datto et al., 1995; Li et al., 1995).

By inhibiting cyclin-cdk activity, p21 maintains RB in a hypophosphorylated state, which prevents S phase entry (Dulic et al., 1994; Slebos et al., 1994; Brugarolas et al., 1999), and p21 can also inhibit E2F activity independently of the RB-E2F pathway (Dimri et al., 1996; Rousseau et al., 1999; Delavaine & La Thangue, 1999) (Figure 1.15 B). Inactivation of E2F by p21, and activation of the p21 gene by E2F (Hiyama et al., 1998), sets up a feedback loop between these two proteins that is important in regulating cell cycle progression. p21 has recently been shown to regulate the levels and intracellular localisation of Cdc25A (cell division cycle 25A), an activity which may contribute to its negative regulation of cyclin E-cdk2 activity and proliferation (Jaime et al., 2002).

Similar to its regulation of G1, p21-dependent G2 arrest involves inactivation of cdk2 activity (Bates et al., 1998b; Dulic et al., 1998; Stewart et al., 1999) and inhibition of RB phosphorylation (Niculescu et al., 1998). Inhibition of cdk2 activity prevents activation of cyclin B-cdc2 and subsequent entry into mitosis (Guadagno & Newport, 1996). p21 expression appears to be important in coupling S phase with mitosis, where decreased levels of p21 may be insufficient to inactivate cdk5 required for DNA synthesis, but sufficient to inhibit cdk5 required for mitosis (Niculescu et al., 1998). Consistent with this, tumour cells lacking p21 fail to maintain G2 arrest following DNA damage, and undergo endoreduplication culminating in apoptosis (Waldman et al., 1996).
Figure 1.15: Regulation of Proliferation by p21

Model describing regulation of proliferation by p21. p21 positively regulates proliferation by promoting the assembly of cyclin-cdk complexes, and negatively regulates proliferation by inhibiting cyclin-cdk activity. p21 also inhibits proliferation via inhibition of E2F, PCNA, and c-Myc activities, and regulation of Cdc25A subcellular localisation.
Figure 1.15: Regulation of Proliferation by p21

Figure 1.15: Regulation of Proliferation by p21

C

Model describing regulation of proliferation by p21 via inhibition of PCNA. PCNA regulates the activity of DNA polymerases δ and ε in replication and repair. After DNA damage, increased levels of p21 inhibit PCNA-dependent replication but not PCNA-dependent repair. c-Myc abrogates the p21-PCNA interaction while p21 inhibits c-Myc transcriptional activity.
Model describing regulation of proliferation by p21 via interaction with cyclin-cdks. p21 facilitates the assembly of cyclinD-ckd complexes and targets them to the nucleus where they promote proliferation. p21 inhibits proliferation by inhibiting cyclin-ckd2 activity.
As an auxiliary factor for DNA polymerases δ and ε, PCNA facilitates loading of the polymerases onto DNA templates, and increases their activity in both replication and repair (Stillman, 1994) (Figure 1.15C). PCNA also stimulates the activity of FEN1 (flap endonuclease 1), an exonuclease required for DNA replication (Warbrick et al., 1997). Through its interaction with PCNA, p21 inhibits PCNA-dependent replication (long-range DNA synthesis), but not PCNA-dependent repair (short-patch DNA synthesis) (Li et al., 1994; Shivji et al., 1994; Rousseau et al., 1999), while competition between p21 and FEN1 for binding to PCNA might also contribute to PCNA inhibition by p21 (Warbrick et al., 1997). c-Myc inhibits the ability of p21 to inhibit DNA synthesis by abrogating the p21-PCNA interaction, while p21 inhibits c-Myc transcriptional activity (Kitaura et al., 2000) (Figure 1.15C). This reciprocal inactivation between p21 and c-Myc plays an important role in cell cycle regulation.

The ability of p21 to positively regulate proliferation is due to its ability to act not only as an inhibitor of cdk activity, but also to facilitate the association between cyclin D and its partner cdks and target them to the nucleus (Zhang et al., 1994; LaBaer et al., 1997; Cheng et al., 1999; Weiss et al., 2000) (Figure 1.15D). p21 is found in quaternary complexes in normal cells with cyclin-cdks and PCNA (Zhang et al., 1993), and it was thought that these cyclin-cdks could transition between active and inactive states through changes in p21 stoichiometry, whereby low concentrations of p21 promotes cyclin-cdk assembly, while high concentrations are inhibitory (Zhang et al., 1994) (Figure 1.15D). A subsequent study, however, showed that a single molecule of p21 was sufficient to inhibit cdk2 activity (Hengst et al., 1998). Thus, it appears that p21 negatively regulates cdk2 activity and positively regulates cdk4,6 activity. This cell cycle-promoting function of p21 may help to explain the high levels of p21 and cyclins observed in numerous human tumours (Barboule et al., 1998; Hibberts et al., 1999; Lonardo et al., 1999), and the lack of p21 mutations (Shiohara et al., 1994), as it may be beneficial for a tumour with increased levels of cyclins to preserve p21 function in order to stabilise cyclin-cdk activity required for cell cycle progression. Indeed, p21 dosage was shown to influence tumour progression in mammary tumour-susceptible Wnt-1 (wingless-type MMTV [mouse mammary tumour virus] integration site 1) transgenic mice (Jones et al., 1999).
$p21^{+/+}$ mammary tumours exhibited accelerated growth rates compared with either $p21^{+/+}$ or $p21^{-/-}$ tumours, consistent with the role of $p21$ as an adaptor protein for cyclin-cdk complex formation at low concentrations and as an inhibitor at high concentrations (Jones et al., 1999). A recent study has shown that $p21$ can function as a negative or positive regulator of proliferation depending on the specific cellular context (Bearss et al., 2002). Mammary tumour-susceptible MMTV-ras mice exhibited accelerated tumour incidence and growth rates in the absence of $p21$, whereas MMTV-c-Myc mice exhibited reduced tumour development (Bearrs et al., 2002). Thus, the dual role of $p21$ as both a positive and negative regulator of proliferation, in addition to its anti-apoptotic properties (see section 1.3.3.b), helps to explain the infrequency of $p21$ mutations in human tumours.

### 1.3.3.b Regulation of Apoptosis

$p21$ is generally thought to protect cells from apoptosis. $p21^{-/-}$ cells show increased susceptibility to UV-induced apoptosis compared with their wild-type counterparts (Stivala et al., 2001) and antisense oligonucleotides to $p21$ promote apoptosis during neurogenic differentiation (Poluha et al., 1996). In contrast, overexpression of $p21$ protects against apoptosis during myogenic differentiation (Wang & Walsh, 1996), and apoptosis induced by prostaglandin A2 (Gorospe et al., 1996), DNA damage (Bissonnette et al., 1998; Zhang et al., 1999a), and p53 (Polyak et al., 1996; Gorospe et al., 1997). Loss of $p21$ in ATM mice resulted in increased sensitivity to IR and delayed the onset of tumourigenesis (Wang et al., 1997).

$p21$ inhibits apoptosis by a variety of different mechanisms (Figure 1.16A). In human hepatoma HepG2 cells, $p21$ inhibits Fas-induced apoptosis by binding to procaspase 3 via its N-terminus, thereby inhibiting its cleavage and activation (Suzuki et al., 1999). In PBMs (peripheral blood monocytes) and U937 cells undergoing differentiation, cytoplasmic $p21$ inhibited apoptosis induced by a variety of stimuli, by binding to ASK1 (apoptosis signal-regulating kinase 1), and inhibiting MAPK cascade activation (Asada et al., 1999). $p21$ can inhibit other MAPK family members involved in induction of apoptosis including p38 and JNK (Shim et al., 1996). $p21$ might also inhibit apoptosis through its ability to bind PCNA and facilitate DNA repair whilst inhibiting replication (Li et al., 1994).
Figure 1.16: Regulation of Apoptosis by p21

Model describing regulation of apoptosis by p21. A. p21 inhibits activation of apoptosis by MAPK family members, caspase 3, and cyclin A-cdk2. p21 may also inhibit apoptosis by enhancing PCNA-dependent DNA repair. B. p21 inhibits DNA replication via inhibition of cyclin A-cdk2 and PCNA. Cleavage of the p21 C-terminus by caspase 3 removes the NLS and PCNA-binding site resulting in apoptosis due to increased cyclin A-cdk2 activity and perhaps less efficient PCNA-dependent repair.
Figure 1.16: Regulation of Apoptosis by p21

A

p21

Caspase 3 \ ASK-1 \ p38MAPK \ JNK \ Cyclin A-\cdk2 \ PCNA

\rightarrow \text{Apoptosis}

B

\text{Replication} \quad \text{Repair}

\text{CyclinACdk2} \quad \text{p21} \quad \text{PCNA} \quad \text{Pol6} \quad \text{Pol8}

\text{Caspase 3} \quad \text{p21} \quad \text{nucleus} \quad \text{cytoplasm}

\text{Replication} \quad \text{Repair}

\text{Apoptosis}
Recent studies have thrown light on the mechanism by which p21 antagonizes apoptosis. During apoptosis of human endothelial and hepatoma cells, the C-terminal region containing the NLS and PCNA-binding region of p21 is cleaved by caspase 3, and p21 exits the nucleus, resulting in increased cyclin A-cdk2 activity (Figure 1.16B). Although the mechanism of cyclin A-cdk2-induced apoptosis is not clear, upregulation of cdk2 activity is associated with the induction of apoptosis in a variety of cell types (Shi et al., 1996; Levkau et al., 1998; Zhou et al., 1998; Hakem et al., 1999), and a cleavage-resistant p21 was able to suppress apoptosis by inhibiting cyclin A-cdk2 activity (Levkau et al., 1998; Jin et al., 2000). Reciprocal inactivation between p21 and caspase 3 is therefore likely to play an important role in regulation of apoptosis.

While the majority of evidence supports a role for p21 in protection against apoptosis, overexpression of p21 has been associated with the induction of apoptosis in some cases. Overexpression of p21 induced apoptosis in human retinoblastoma cell lines (Kondo et al., 1997), and increased the level of p53-dependent apoptosis induced by DNA damage in thymocytes (Fotedar et al., 1999). Apoptosis induced by serum deprivation in mouse 3T3 fibroblasts was associated with upregulation of p21, and was inhibited by p21 antisense oligonucleotides (Duttaroy et al., 1997). A novel protein, p21B, expressed from an alternate promoter in the p21 locus that contains two unique exons and is not homologous to p21 (Nozell & Chen, 2002), can be induced by p53 and DNA damage and is associated with the induction of apoptosis, but not cell cycle arrest (Nozell & Chen, 2002). It appears that p21B would still be expressed in p21−/− mice (Deng et al., 1995), and differential regulation of p21 and p21B may be an important determinant of whether a cell undergoes cell cycle arrest or apoptosis.

1.3.3.c Regulation of Genomic Stability
Like RB and p53, p21 regulates a post-mitotic checkpoint that prevents reinitiation of DNA synthesis and polyploidisation in response to microtubule inhibitors (Stewart et al., 1999; Lanni & Jacks, 1998), and regulates both centrosome duplication, and coordination of centrosome and DNA duplication (Mussman et al., 2000; Tarapore et al., 2001). As discussed above, centrosome hyperamplification and polyploidisation
are associated with genomic instability and tumourigenesis. It is thought that p21 regulates centrosome duplication by suppressing premature activation of cyclin E-cdk2, which is required for the initiation of centrosome duplication (Lacey et al., 1999; Hinchcliffe et al., 1999; Mussman et al., 2000). p21 may also protect against genomic instability by facilitating DNA repair, although conflicting evidence has meant that its exact role in repair is somewhat controversial (Li et al., 1994; Shivji et al., 1994; McDonald et al., 1996; Wani et al., 2000; Smith et al., 2000; Stivala et al., 2001; Wani et al., 2002).

1.3.3.d Regulation of Differentiation

A role for p21 in differentiation was suggested by observations that p21 is highly expressed in differentiating tissues in vivo (Huppi et al., 1994; Macleod et al., 1995; Parker et al., 1995), and in vitro (Guo et al., 1995; Halevy et al., 1995; Parker et al., 1995; Jiang et al., 1994; Liu et al., 1996), although the lack of a phenotype in p21-deficient mice suggested otherwise. This was subsequently explained, at least in some tissues, by functional redundancy between family members. p21 and p57 redundantly control differentiation of muscle and lung alveoli (Zhang et al., 1999b), with the skeletal muscle phenotype bearing similarity to that observed in Rb' mice rescued to term by an RB transgene (Zacksenhaus et al., 1996). This suggests that the role of p21 and p57 in muscle differentiation is to inhibit cyclin-cdk activity and thereby maintain RB in a hypophosphorylated active state. p21 has also been shown to inhibit late stages of differentiation in some cell types (Di Cunto et al., 1998), suggesting that p21 can either negatively or positively regulate differentiation depending on cell type and the specific stage of differentiation.

1.3.4 Regulation of p21

p21 protein levels are relatively constant during the cell cycle (Li et al., 1994b), and p21 gene expression is regulated primarily at the level of transcription, by p53-dependent and -independent mechanisms. p53 is not required for p21 expression during development or in most tissues of the adult mouse (el-Deiry et al., 1995; Macleod et al., 1995; Parker et al., 1995). p21 is induced by a wide range of physiological and pathological factors including tumour suppressors such as p53, RB, and BRCA1 (breast cancer-associated gene 1), differentiation factors such as vitamin
D3, phorbol esters, and NGF (nerve growth factor), growth factors such as serum and PDGF (platelet-derived growth factor), cytokines such as TGF-\(\beta\), TNF-\(\alpha\), and IFN-\(\gamma\) (interferon-\(\gamma\)), and DNA damage (Di Leonardo et al., 1994; Li et al., 1999; Decesse et al., 2001; Kardassis et al., 1999 and references therein). Some factors such as p53 (El-Deiry et al., 1995) and IFN-\(\gamma\) (Chin et al., 1996) induce p21 via the distal region of its promoter, while others such as RB (Decesse et al., 2001) and TGF-\(\beta\) (Pardali et al., 2000), induce p21 via SP1 transcription factor binding to SP1 sites in the proximal region. It was recently shown that binding of p53 and SP1 to their cognate sequences on the p21 promoter resulted in synergistic transactivation of p21 (Koutsodontis et al., 2001). p300/CBP can cooperate with SP1 proteins to induce p21 expression (Billon et al., 1996; Owen et al., 1998). STAT (signal transducer and activating transcription factor) proteins are also involved in regulation of p21 transcription. EGF (epidermal growth factor) and IFN-\(\gamma\) induce p21 via STAT1 binding to STAT binding sites in the p21 promoter (Chin et al., 1996), while IL-6 (interleukin-6) induces p21 via STAT3 (Bellido et al., 1996).

p21 can also be regulated at the posttranscriptional level. p21 is an unstable protein with a half-life of less than 30 minutes and is a target for proteasome-mediated degradation by ubiquitin-dependent (Maki & Howley, 1997; Rousseau et al., 1999) and -independent pathways (Sheaff et al., 2000). UV-C enhances the stability of p21 mRNA (Gorospe et al., 1998) while C/EBP\(\alpha\) (Timchenko et al., 1996) and TSG101 (tumour suppressor gene 101) (Oh et al., 2002) enhance stability of p21 protein. In hepatocytes, C/EBP\(\alpha\) has been shown to protect p21 from degradation (Timchenko et al., 1997). p21 protein stability is also regulated by phosphorylation. The MAPK family members p38 and JNK1 stabilise p21 by phosphorylation at Ser130 (Kim et al., 2002), while the survival kinase AKT/PKB stabilizes p21 by phosphorylation at Ser146 (Li et al., 2002). Phosphorylation of p21 at Ser146 and Thr145 disrupts PCNA-binding (Scott et al., 2000; Li et al., 2002), which has been shown to protect p21 from degradation, in contrast to cyclin-cdks, which enhance degradation (Cayrol & Ducommun, 1998). An additional mechanism of p21 regulation involves sequestration by cyclin D-cdk4,6 complexes (reviewed in Sherr & Roberts, 1999).
1.4 Transgenic mouse models of cancer

The generation of mice carrying targeted mutations in tumour suppressor genes has greatly improved our understanding of the process of carcinogenesis, allowing detailed analysis of the function of such genes in processes such as proliferation and apoptosis, the dysregulation of which lead to cancer. Rb, p53 and p21 have all been successfully knocked-out in the mouse (see sections 1.1.2.a, 1.2.2.a, and 1.3.2.a), however, the Rb null mutation is embryonic lethal, thereby precluding study of the consequences of Rb inactivation later in development or in specific tissues. Conditional gene targeting, however, allows inactivation of genes in a tissue-specific and temporally defined manner, thus overcoming the problem of embryonic lethality. Two methods of conditional gene targeting, the Cre (cyclization recombination) /LoxP (locus of X-over of P1) and FLP/FRT systems, have been widely used for manipulating the genomes of mammalian cells and of mice in vivo. The Cre/LoxP system has been used with the most frequent success and is discussed below.

1.4.1 The Cre/LoxP system of conditional gene targeting

The Cre/LoxP system of E. coli bacteriophage P1 functions during the replicative cycle of the phage to circularise the viral genome (Segev & Cohen, 1981), and to maintain correct unit copy segregation of the prophage within host cells (Austin et al., 1981). The bacteriophage P1 38kD Cre protein is a member of the integrase family of site-specific DNA recombinases (Sternberg & Hamilton, 1981) that recognizes a palindromic 34-bp site on the P1 genome called LoxP. The 34-bp LoxP site consists of two 13-bp inverted repeats flanking an 8-bp non-palindromic core region (Hoess et al., 1982; Hoess et al., 1986) (Figure 1.17A). Each of the 13-bp inverted repeats of the LoxP site binds a single Cre monomer such that four Cre molecules are thought to be required per recombination event (Mack et al., 1992). The tetrameric structure then introduces sharp bends within the 8bp spacer region, thereby positioning the cleavage and strand re-ligation sites into the Cre active site. A set of nucleophilic attacks catalysed by Cre Tyr324 results in strand exchange and recombination (Guo et al., 1997). Cre-mediated recombination between two directly repeated LoxP sites results in excision of the DNA between them as a covalently closed circle, whereas inversion will occur when the LoxP sites are in the opposite direction (Lewandoski &
Martin, 1997) (Figure 1.17B). Cre can also mediate integration of a LoxP-containing plasmid to a chromosomal region bearing a LoxP site (Sauer & Henderson, 1990; Sauer et al., 1992).

The Cre/LoxP system has been adapted for activating and inactivating genes in mammalian cells (Sauer, 1993) and in transgenic animals (Lakso et al., 1992; Gu et al., 1994; Kuhn et al., 1995), in a tissue-specific (Gu et al., 1994; Nelson et al., 1998; Selbert et al., 1998; Zhu et al., 2001) and temporally defined manner (Kuhn et al., 1995; Feil et al., 1996; Zhang et al., 1996). Rb and p53 have recently been inactivated in the cerebellum of mice bearing floxed (flanked by loxP sequences) Rb and p53 alleles, and expressing Cre recombinase under the control of GFAP, a cytoskeletal protein that is primarily expressed in the adult CNS (Marino et al., 2000).

Figure 1.17: Cre-mediated recombination of LoxP flanked (Floxed) DNA sequences

A

![Diagram of Cre-mediated recombination of a floxed gene.](image)

Model describing Cre-mediated recombination of a floxed gene. A. The sequence of the LoxP site showing the spacer region and inverted repeats. B. Excision: The sequence to be excised is flanked by 2 directly repeated LoxP sites. Cre recombinase brings the LoxP sites together in a parallel manner and mediates recombination. The floxed sequence is excised as a circular product containing one LoxP site while the deleted allele retains the other LoxP site. Inversion: The sequence to be inverted is flanked by 2 inverted repeats of LoxP. Cre mediates recombination resulting in inversion of the floxed sequence.
Figure 1.17: Cre-mediated recombination of LoxP flanked (Floxed) DNA sequences

B  *Excision*

Floxed allele

Floxed allele

Intermediate

Deleted allele

Recombined allele

Inversion

Floxed allele

Intermediate

Inverted allele
1.5 Project Aims

Overview of the p53-p21-Rb Network

The p53-p21-RB pathway is usually thought of in terms of a linear pathway that is activated in response to cellular stress such as DNA damage, whereby activation of p53 leads to induction of p21, which in turn activates RB, resulting in cell cycle arrest. However, the individual components of this pathway can affect each other via additional pathways. p53, p21 and RB are therefore part of a complex signaling network that could be referred to as the p53-p21-RB network. The figure below summarises the various pathways linking together these proteins.
The overall aim of this thesis was to investigate the roles of p53, p21, and RB in regulation of hepatocyte proliferation and apoptosis.

Specific aims were:

1. To establish and evaluate a system that allowed deletion of Rb-floxed alleles in primary mouse hepatocytes.
2. To study the consequences of Rb-, p53-, and p21-deficiency for hepatocyte proliferation, apoptosis, polyploidisation, and response to DNA damage.
Chapter 2
Materials and Methods

2.1 Primary Hepatocyte Culture

2.1.1 Transgenic mice

Generation of the \( p53^{−/−} \) (Purdie et al., 1994), \( p21^{−/−} \) (Deng et al., 1995), \( Rb \)-floxed (Vooijs et al., 1998), DNA Ligase1-floxed (Bentley et al., 1996), and Flox-STOP-\( \beta geo \) (Mao et al., 1999) mice used in the experiments described in this thesis, has been previously described. \( p53^{−/−} \) mice are outbred on a mixed background, segregating for 129, Ola and Balb/c, \( p21^{−/−} \) mice are NIH Black Swiss, and \( Rb \)-floxed mice are outbred 129/Ola:C57BL/6 males and FVB/N females. \( p53^{−/−}, p21^{−/−}, \) and \( Rb \)-floxed mice were crossed together to obtain all possible combinations of genotypes. Flox-STOP-\( \beta geo \) mice were from a C57BL/6 background and DNA Ligase1-floxed mice from a Balb/c background. Table 2.1 briefly summarises the genetic alteration these mice contained and the consequence of that alteration.

<table>
<thead>
<tr>
<th>Transgenic Mice</th>
<th>Genetic alteration</th>
<th>Consequence of alteration</th>
</tr>
</thead>
<tbody>
<tr>
<td>( p53^{−/−} )</td>
<td>Exons 2-6 replaced with a neo gene</td>
<td>Complete absence of p53 protein</td>
</tr>
<tr>
<td>( p21^{−/−} )</td>
<td>Exon 2 replaced with a neo gene</td>
<td>Complete absence of p21 protein</td>
</tr>
<tr>
<td>( Rb^{\text{floxed}} )</td>
<td>Exon 19 flanked by two LoxP sites</td>
<td>No functional consequences until Cre-mediated recombination at LoxP sites excises exon 19, producing a truncated, non-functional protein</td>
</tr>
<tr>
<td>DNA Ligase1( ^{\text{floxed}} )</td>
<td>Exon 27 flanked by two LoxP sites, or deleted</td>
<td>Heterozygous for DNA ligase 1 until Cre-mediated recombination at LoxP sites excises exon 27, resulting in complete absence of DNA Ligase 1 protein</td>
</tr>
<tr>
<td>Flox-STOP-( \beta geo )</td>
<td>Stop sequence flanked by two LoxP sites</td>
<td>( \beta geo ) not expressed until STOP sequence excised by Cre recombinase</td>
</tr>
</tbody>
</table>
2.1.2 Isolation of Murine Hepatocytes

2.1.2.1 Liver perfusion
Hepatocytes were isolated from male mice aged 6-12 weeks using a two-step retrograde liver perfusion procedure described by Bellamy et al., 1997a. Before use, the perfusion apparatus was sterilised with 70% ethanol and rinsed well with dH2O. Mice were killed by cervical dislocation, dissected, and the rib-cage was removed. Care was taken not to puncture the liver, heart, thoracic inferior vena cava, hepatic portal vein, or intestines. The intestines were pushed to one side to expose the hepatic portal vein and a loose ligature was placed around the thoracic inferior vena cava. An incision was made in the right atrium of the heart, a cannula (OD 1mm, ID 0.5mm) was inserted into the thoracic inferior vena cava, and secured in position by the ligature. Oxygenated perfusion medium (Appendix) at 37°C was pumped through the cannula (8.5ml/min), causing the liver to inflate. The hepatic portal vein was cut and the liver perfused for 5 minutes. The liver was then perfused with digestion medium (Appendix) for a further 10 minutes, or until visibly digested, and removed to a petri dish containing ice-cold culture medium (Appendix). The gall bladder was removed, the capsule was cut open and hepatocytes dissociated by gently scraping cells from the supporting fibrous tissue.

2.1.2.2 Purification of Cell Suspension
The cell suspension was gently aspirated a few times through a wide-bore pipette to break up clumps, and then filtered through a 250μm mesh into an ice-cold beaker. The mesh was rinsed with ice-cold culture medium, the cell suspension transferred to a falcon tube, and centrifuged at 50g for 5 minutes at 4°C. The supernatant, containing debris and non-parenchymal cells, was removed, and the pellet resuspended in 8ml ice-cold culture medium. The hepatocyte population was further purified by isopyknic centrifugation in Percoll (Appendix), adapted from Kreamer et al., 1986, whereby damaged cells float and murine hepatocytes pellet. 5.25ml Percoll was added to the cell suspension and centrifuged at 50g for 10 minutes at 4°C. The floating layer, containing dead cells, debris, and non-hepatocytes, was
removed and the pellet, containing >99% hepatocytes was resuspended in 5ml ice-cold culture medium.

2.1.2.3 Assessment of hepatocyte viability and yield
Cell viability was assessed by the trypan blue exclusion method. Cells with damaged membranes take up the dye and can be differentiated by light microscopy from unstained cells. 20µl of the cell suspension was added to 40µl trypan blue (Sigma) and 40µl PBS. A drop of this mixture was added to each side of a tightly coverslipped Neubauer haemocytometer (chamber depth 0.1cm). The number of stained (dead) and unstained (live) cells in five 1mm² squares on each side of the haemocytometer was counted. The unstained cells were expressed as a percentage of the total to give an estimate of hepatocyte viability. The hepatocyte yield was calculated from the number of live cells in a known volume (5x10⁻⁴ cm³), allowing for the dilution factor (5) and original volume (5ml).

2.1.3 Primary Hepatocyte Tissue Culture
Hepatocytes were plated onto fibronectin-coated 2-well chamber slides and 24-well plates (Appendix), or collagen-coated 60mm plates (Appendix), depending on the downstream application, at a density of 0.13 million cells/ml, and maintained in culture medium containing 2% fetal calf serum (FCS). Cells were incubated in a humid atmosphere containing 5% CO₂/air. The medium was kept less than 2mm deep to maintain optimum oxygen tension (60mm Hg) (Saad et al., 1994), and was replaced after a 1 hour attachment period and every 24h thereafter.

2.2 Cell Biology
2.2.1 Adenovirus infection
All adenoviruses used were supplied by Dominic Rannie as a stock solution of known titre. Adenovirus infection of hepatocytes was carried out 2-3 hours after plating. An appropriate amount of virus, depending on the multiplicity of infection (MOI) required, was added to a universal tube containing culture medium heated to 37°C, and mixed well by vortexing. A minimal volume of adenovirus-containing
medium, depending on the size of the dishes (Appendix), was added to the cells for 1 hour at 37°C, during which time the dishes were rocked gently. After this time, the adenovirus-containing medium was removed and replaced with fresh culture medium. All work involving adenovirus was carried out in a microbiological safety cabinet (Class II), and any contaminated glass and plastic-ware were immersed immediately in a 1000ppm presept solution (Johnson & Johnson) for 2 hours prior to disposal. After use, the safety cabinet was UV-irradiated overnight.

2.2.2 UV-irradiation
After 66-72h in culture, hepatocytes were UV-C irradiated with 10J/m² using a Spectrolinker XL-1500 (Spectronics Corporation) after complete removal of culture medium. Medium was replaced and slides were returned to the incubator. Unirradiated controls were otherwise treated identically.

2.2.3 Evaluation of apoptosis
Apoptosis was assessed morphologically by Feulgen staining of DNA. Cells used for immunohistochemistry were cultured on 2-well chamber slides. After removal of culture medium, slides were fixed and stored in Bouin’s fixative (Appendix) at 4°C. When required, slides were allowed to thaw at room temperature and were denatured in 5M HCl for 45 minutes at room temperature. They were then stained in Schiff’s reagent (Sigma) diluted 1:3 in water for 1 hour, after which they were washed well with water and counterstained with 0.1% light green. Schiff’s stains DNA a pink-purple colour and apoptotic cells are easily recognisable by their distinct morphology, including condensed and fragmented DNA. A minimum of 500 cells was counted and apoptotic cells were expressed as a percentage of the total.

2.3 Vindelov Flow Cytometric Analysis
The vindelov technique measures DNA content of isolated nuclei rather than intact cells (Vindelov, 1985). Cells used for flow cytometry were grown on 60mm plates and washed twice with PBS after removal of culture medium. 4ml collagenase type IV (Sigma) (0.5mg/ml in medium) was added to cells and incubated at 37°C for 20
minutes. Cells were removed from the plate using a wide-bore pipette, transferred to a universal, and centrifuged at 300g for 10 minutes. The supernatant was removed, the pellet was resuspended in 200μl citrate buffer (Appendix), and stored at -20°C. When required, samples were thawed at room temperature. 100μl of cell suspension was transferred to a lucham tube and 450μl of a trypsin solution (Solution A, Appendix) was added, samples were mixed by inversion, and incubated at room temperature for 10 minutes to digest the cell membrane. 325μl of a solution containing trypsin inhibitor and RNase A (Solution B, Appendix) was added for a further 10 minutes to prevent excess digestion and to degrade RNA; samples were mixed by inversion. Samples were placed on ice, 250μl of a solution containing PI (propidium iodide) (Solution C, Appendix) was added for 10 minutes, and samples were mixed by inversion. DNA content was analysed by a COULTER®EPICS®XL Flow Cytometer (Beckman-Coulter Electronics). PI was excited in 488nm Argon laser light and red fluorescence emitted was detected at 620nm. A minimum of 10,000 nuclei was analysed and doublet gating ensured that only single events were counted. Since PI binds stochiometrically to DNA, the integral red fluorescence recorded by the flow cytometer for each nucleus is proportional to DNA content. The proportions of 2n, 4n, and 8n nuclei were determined by placing gates over the main area of each peak. Gates were set on the first sample and were not changed thereafter.

2.4 Cytochemical Staining

2.4.1 MTT Assay of Hepatocyte Viability

The MTT assay depends upon the reduction of the exogenous yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) tetrazolium salt (Sigma) to a purple formazan by mitochondrial succinate dehydrogenase in living cells (Mossman et al., 1983; Carmichael et al., 1987). Cells used for the MTT assay were cultured on 24-well plates and incubated with MTT (1mg/ml) (Appendix) in medium at 37°C for 3 hours. Cells were washed twice with PBS after removal of medium and left to dry completely. 150μl DMSO was added to dissolve the formazan dye,
100μl was transferred to a 96-well ELISA plate, and the absorbance read at 490nm by an ELISA plate reader (MR 5000, Dynatech). The negative control omitted MTT.

2.4.2 Histochemical detection of β-galactosidase

Cells used for β-galactosidase staining were cultured on 24-well plates and washed twice with PBS after removal of culture media. They were then fixed in 0.05% glutaraldehyde in PBS for 5 minutes at room temperature, and washed three times with PBS, the middle wash being left on for 10 minutes. Cells were then covered in X-gal stain (Appendix) and incubated overnight at 37°C in a sealed humid chamber. X-gal (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside) is a synthetic substrate, which forms a local blue precipitate upon cleavage by β-galactosidase (Miller, 1972). Positive cells were visualised under light microscopy by their blue colour. After staining, cells were stored in PBS at 4°C.

2.5 Immunocytochemistry

2.5.1 BrdU

Cells used for immunohistochemistry were cultured on 2-well chamber slides, incubated with medium containing 40μM BrdU (5-bromodeoxy-uridine) (Amersham) for 6 hours, and fixed and stored in 80% ethanol at 4°C. When required, slides were rehydrated in PBS for 10 minutes, incubated in 1% H₂O₂ for 10 minutes, and rinsed in PBS for 5 minutes. Slides were then incubated in 5M HCl for 45 minutes at room temperature, followed by washing in PBS (3x5 minutes). Cells were incubated in blocking solution (Table 2.2, Appendix) for 10 minutes and then with primary antibody (Table 2.2, Appendix) at room temperature for 1 hour. Negative controls omitted the primary antibody. Slides were washed in PBS (3x5 minutes) and incubated with HRP (horseradish peroxidase)-conjugated secondary antibody (Table 2.2, Appendix) in 0.5%PBST at room temperature for 30 minutes, followed by washing in PBS (3x5 minutes). Positively stained nuclei were visualised using DAB chromagen, according to the manufacturer’s instructions. Cells were counterstained with haematoxylin and 0.1% light green, air-dried, mounted, and
coverslipped. A minimum of 500 cells was counted, and the BrdU-positive cells are expressed as a percentage of the total.

2.5.2 p53

p53 was labelled using pAb 421 (Table 2.2). Slides were fixed in acetone/methanol (1:1) at room temperature for 5 minutes, air-dried, and stored at -70°C. When required, slides were allowed to thaw at room temperature for 1 hour, rehydrated in PBS (10 minutes), incubated in 1% H₂O₂ for 10 minutes, and rinsed in PBS (5 minutes). Slides were then incubated in blocking solution (Table 2.2, Appendix) for 10 minutes and rinsed in PBS for 5 minutes. Endogenous biotin was blocked using a kit (Vector laboratories), according to the manufacturer’s instructions. Slides were incubated with primary antibody (Table 2.2, Appendix) in a humid chamber overnight at 4°C. Negative controls omitted the primary antibody. Slides were washed in PBS (3x5 minutes) and incubated with biotinylated secondary antibody (Table 2.2, Appendix) room temperature for 30 minutes, followed by washing in PBS (3x5 minutes). HRP-conjugated Avidin-biotin complex (Dako) was used as the final labelling step, according to the manufacturer’s instructions. Positively stained nuclei were visualised using DAB (diaminobenzidine) chromagen, and cells were counterstained with haematoxylin and 0.1% light green, air-dried, mounted, and coverslipped. A minimum of 500 cells was counted, and the p53-positive cells are expressed as a percentage of the total.

Table 2.2: Antibodies for immunohistochemistry

<table>
<thead>
<tr>
<th></th>
<th>BrdU</th>
<th>p53</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking solution</td>
<td>20%NRS/0.5%PBST</td>
<td>20%NRS/0.5%PBST</td>
</tr>
<tr>
<td>Primary antibody</td>
<td>Harlan Sera-labs Rat monoclonal 1:100 in 5%NRS/0.5%PBST</td>
<td>Pab421 Oncogene Science Mouse monoclonal 1:1000 in 5%NRS/0.5%PBST</td>
</tr>
<tr>
<td>Secondary antibody</td>
<td>Sigma Rabbit anti-rat HRP 1:200 in 0.5%PBST</td>
<td>Dako Rabbit anti-mouse biotinylated 1:400 in 0.5%PBST</td>
</tr>
<tr>
<td>dilution</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.6 Immunofluorescence

2.6.1 p53 and p21

p53 was labelled using CM5 and p21 was labelled with M7202 (Table 2.3). Slides were fixed in methanol at -20°C for 5 minutes, air-dried, and stored at -20°C. When required, slides were allowed to thaw at room temperature and rehydrated in TBS (Appendix) for 5 minutes. Slides were then incubated in the appropriate blocking solution (Table 2.3, Appendix) at room temperature for 30 minutes, after which they were incubated with primary antibody (Table 2.3) at room temperature for 1 hour. Slides were washed in TBST (Appendix) (3x5 minutes), incubated with secondary antibody at room temperature for 30 minutes (Table 2.3), and again washed in TBST (3x5 minutes). Slides were mounted without drying in Dako mounting medium and covered with tin-foil. Positive cells were visualised using a fluorescent microscope (Biorad).

Table 2.3: Antibodies for immunofluorescence

<table>
<thead>
<tr>
<th>p53</th>
<th>p21</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blocking solution</strong></td>
<td></td>
</tr>
<tr>
<td>20%NGS/0.1%TBST</td>
<td>20%NRS/0.1%TBST</td>
</tr>
<tr>
<td><strong>Primary antibody</strong></td>
<td></td>
</tr>
<tr>
<td>CM5 Novacastra Rabbit polyclonal</td>
<td>M7202 Dako Mouse monoclonal</td>
</tr>
<tr>
<td>1:400 in 0.1%TBST</td>
<td>1:10 in 0.1%TBST</td>
</tr>
<tr>
<td><strong>Secondary antibody</strong></td>
<td></td>
</tr>
<tr>
<td>Alexa 488 Molecular Bioprobes Goat anti-rabbit</td>
<td>Alexa 488 Molecular Bioprobes Rabbit anti-mouse</td>
</tr>
<tr>
<td>1:200 in 0.1%TBST</td>
<td>1:200 in 0.1%TBST</td>
</tr>
</tbody>
</table>

2.7 Western blotting

2.7.1 Protein Extraction

Cells harvested for protein were cultured on 60mm plates and washed twice with ice-cold PBS after removal of culture media. 500μl of ice-cold RIPA buffer containing protease inhibitors (Appendix) was added to the cells, cells were removed from the plate using a cell-scaper (Nalge-Nunc), and transferred to a 1.5ml Eppendorf tube kept on ice. The cell suspension was aspirated through a fine syringe needle (26 gauge) to disrupt the cells and DNA, and centrifuged at 10,000g for 5 minutes at 4°C to pellet the debris. The supernatants were transferred to fresh tubes, frozen in an
ethanol/dry ice bath or liquid nitrogen, and stored at -70°C. The protein concentration of each sample was determined using the Bio-Rad Protein Assay (Bio-Rad) in accordance with the manufacturer’s instructions.

2.7.2 Protein Electrophoresis
SDS-polyacrylamide gel electrophoresis was accomplished on precast Tris-glycine gels in a XCELL II™ tank (Novex) in accordance with the manufacturer’s instructions. 0.3 volumes of 4x SDS Sample Loading buffer (Novex) were added to a volume of sample containing 20µg of protein. Samples were boiled for 5 minutes, loaded onto the gel along with a molecular weight marker (Invitrogen) and electrophoresed at 100V in running buffer (Novex) at room temperature for 1 hour.

2.7.3 Protein Transfer
Protein transfer onto Hybond™ECL™ nitrocellulose membranes (Amersham) was accomplished in a XCELL II™ tank and blotting module (Novex) in accordance with the manufacturer’s instructions. Transfer was carried out at 30 volts for 90 minutes at 4°C in transfer buffer (Novex). Following transfer, gels were stained in GelCode (Novex) in accordance with the manufacturer’s instructions, to determine transfer efficiency and to check for equal loading between wells. Destained gels were dried in a vacuum apparatus.

2.7.4 Antibody detection
Membranes were rinsed briefly in dH2O, transferred to dishes containing blocking solution (see Appendix), and placed on a rocker. Membranes were incubated in this solution for 1 hour at room temperature. Following blocking, membranes were incubated in a similar manner with the appropriate antibody (Table 2.4) diluted in blocking solution for 1 hour at room temperature or overnight at 4°C. Negative controls omitted the primary antibody. Membranes were washed in 0.1%TBST (6x5 minutes) at room temperature and incubated with the appropriate HRP-conjugated secondary antibody (Table 2.4) diluted in blocking solution at room temperature for 1 hour. Membranes were washed in 0.1%TBST (6x5 minutes) at room temperature.
Table 2.4: Antibodies for western blotting

<table>
<thead>
<tr>
<th></th>
<th>Cre-recombinase</th>
<th>p53</th>
<th>p21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary antibody</td>
<td>No. 69050-3 Novagen</td>
<td>CM5 Novacastra</td>
<td>M19 Santa Cruz</td>
</tr>
<tr>
<td>dilution</td>
<td>Rabbit polyclonal 1:10,000</td>
<td>Rabbit polyclonal 1:400</td>
<td>Goat polyclonal 1:200</td>
</tr>
</tbody>
</table>

2.7.5 Signal detection
Visualisation was accomplished using the ECL™ Chemiluminescent detection system (Amersham) in accordance with manufacturer’s instructions. Membranes were placed protein side upwards on saran wrap and an equal mixture of reagents A and B was applied to the membrane surface for 1 minute. The solution was poured off, the membrane sealed in saran wrap and placed protein side upwards in a film cassette. ECL™ film (Amersham) was placed on top of the membrane for periods of 1 minute to 1 hour, depending on signal intensity. Films were developed using an Amersham Hyperprocessor.

2.8 Manipulation of DNA

2.8.1 Calcium Chloride preparation of Competent Cells
100ml LB medium (Appendix) containing 50μg/ml Ampicillin was inoculated with a single E.coli TB1 (New England Biolabs) clone and grown at 37°C in an orbital incubator at 225rpm until the OD₆₀₀ was approximately 0.125 units. The culture was split into two and harvested at 4000rpm for 10 minutes at 4°C. The supernatant was removed, the pellets placed on ice, and resuspended in 2.5ml of ice-cold 0.1M CaCl₂. The bacteria were centrifuged at 4000rpm for 10 minutes at 4°C, the pellets were resuspended in 2ml 0.1M CaCl₂, divided into 500μl and 20μl aliquots, and stored at -70°C.

2.8.2 Transformation of Bacteria with Plasmid DNA
A 20μl aliquot of E.coli TB1 competent cells was removed from storage at -70°C and thawed on ice. Plasmid DNA was added directly onto the cells and mixed by
pipetting. The mixture was incubated on ice for 30 minutes and heat shocked at 42°C for 40 seconds. 80µl of LB was added and the bacteria were incubated overnight at 37°C. Bacteria were then plated onto LB-agar (Appendix) plates containing appropriate antibiotics to select for transformants.

2.8.3 Large-scale Preparation of Plasmid DNA

Large-scale preparation of plasmid DNA was carried out using a Plasmid Maxi Kit (QIAGEN) according to the manufacturers instructions. A single bacterial clone was picked from a LB-agar plate using a sterile p200 pipette tip and transferred to a conical flask containing 250ml of LB, supplemented with the appropriate antibiotics. The bacteria were grown at 37°C for 12-16 hours in an orbital incubator at 225rpm, harvested by centrifugation at 10,000g for 10 minutes at 4°C (Sorvall GSA rotor, Centrikon H-40IB), resuspended, lysed, and neutralised in manufacturer-supplied solutions. The protein precipitate thus formed was removed by centrifugation at 12,000g for 30 minutes at 4°C (Sorvall GSA rotor, Centrikon H-40IB). Supernatants were applied directly to QIAGEN-tip columns that contain DNA-binding resin. Columns were washed with the supplied buffers and DNA was eluted from the column with the supplied elution buffer. DNA was precipitated by the addition of 0.7 volumes of isopropanol and collected by centrifugation at 12,000g for 30 minutes at 4°C (Sorvall ss-34 rotor, Centrikon H-40IB). The DNA pellet was washed with 70% ethanol, air-dried for 5 minutes, resuspended in TE buffer (Appendix), and stored at -20°C.

2.8.4 Restriction Digest Analysis of DNA

A suitable amount of DNA, depending on the downstream application, was mixed with 0.1 volumes of 10X reaction buffer (supplied by the manufacturer, New England Biolabs) and 0.1 volumes of 10X Bovine Serum Albumin (BSA) (manufacturer’s instructions). 0.1 volumes of restriction endonuclease were added to the reaction mix and any remaining volume was made up to 50µl with dH2O. The digests were incubated for 1-24hrs at the temperature recommended by the manufacturer. Digested fragments were visualised on agarose gels.
2.8.5 Agarose gel Electrophoresis of DNA

The agarose gel concentration used varied depending on the size of the DNA molecules to be resolved. TBE gels were prepared by boiling an appropriate weight of agarose (Sigma) in TBE buffer (Appendix) until dissolved, and adding 0.1mg/ml ethidium bromide (Appendix). DNA samples were mixed with one-sixth volume of 6X loading buffer (Promega) and loaded directly onto the gel. Molecular weight markers (100bp or 1Kb ladder, Promega) were also diluted in loading buffer and loaded onto the gel. Electrophoresis was carried out in 1X TBE at 50-100V and DNA was visualised with UV light (Herolab, gel documentation equipment).

2.8.6 Extraction of DNA Fragments from Agarose gels

The QIAEX II Gel Extraction Kit (Qiagen) was used for the purification of DNA fragments from agarose gels. DNA was digested with appropriate endonucleases, electrophoresed on an agarose gel, and visualised with UV light. Bands were excised from the gel with a scalpel, the gel slice was weighed, and an appropriate volume of solubilisation buffer and DNA-binding resin were added according to the manufacturers instructions. After solubilisation and adsorption of the DNA to the resin, the sample was centrifuged at 10,000g for 30 seconds. The resin pellet was washed and air-dried for 15 minutes. To elute the DNA, the pellet was resuspended in 20µl of 20mM Tris pH8.0 at room temperature for 5 minutes before centrifuging at 10,000g for 30 seconds. DNA samples were stored at -20°C.

2.8.7 Southern Blotting

2.8.7.1 DNA Extraction

Cells harvested for DNA were cultured on 60mm plates and washed twice with PBS after removal of culture media. 500µl of tail lysis buffer (Flowgen) was added to the cells, cells were removed from the plate using a cell-scaper (Nalge-Nunc), and transferred to a 1.5ml Eppendorf tube. 200µl of protein precipitation solution (Flowgen) was added, followed by the addition of 700µl phenol/chloroform/isoamyl alcohol mixture (Appendix). Tubes were shaken vigorously and centrifuged at 10,000g for 10 minutes at room temperature. Two phases form, and the top layer containing the DNA was transferred to a fresh tube. DNA was precipitated by the
addition of 500μl of isopropanol and collected by centrifugation at 10,000g for 5 minutes at 4°C. The DNA pellet was washed with 70% ethanol, air-dried for 5 minutes, resuspended in TE buffer, and stored at -20°C.

2.8.7.2 Preparation of DNA
10μg of genomic DNA was digested with the appropriate restriction enzyme. Following digestion, the DNA samples were subjected to electrophoresis on a 0.8% agarose gel at 40 volts overnight. Transfer of DNA to the nylon membrane was improved by partial depurination of the DNA by soaking the gel in 0.25M HCl for 10 minutes. The gel was then rinsed in deionised water and the DNA was transferred immediately.

2.8.7.3 DNA Transfer
DNA was transferred from the gel onto a positively charged nylon membrane (Zeta-Probe® GT, Bio-Rad). 0.4M sodium hydroxide was used as the transfer buffer and transfer was allowed to proceed overnight. After transfer, the membrane was rinsed in 2X SSC, air-dried, and baked in a vacuum oven at 80°C for 30 minutes. Membranes were stored between sheets Whatman 3MM paper at room temperature until required.

2.8.7.4 Prehybridisation
Hybridisation buffer (Appendix) was preheated to 68°C to ensure all components were in solution. 10mls of this, containing 500μl denatured carrier salmon sperm DNA (10mg/ml), was transferred to a hybridisation tube (Hybaid) containing the nylon membrane, and membrane and buffer were incubated at 68°C for 1 hour in a hybridisation oven (Hybaid).

2.8.7.5 Generation of Radiolabelled Probes
50ng of probe DNA in 11μl of dH2O (gel extracted restriction fragment) was radiolabelled with 32P-dCTP using High Prime enzyme (Boehringer Mannheim). 4μl of enzyme and 5μl of 32P-dCTP were incubated with 11μl of probe DNA at 37°C for 20 minutes. In order to separate labelled probe from unincorporated nucleotides,
20µl of carrier salmon sperm DNA (10mg/ml) was mixed with radiolabelled probe and added to a Sephadex G50 column (Pharmacia). Unincorporated nucleotides were eluted with 400µl TE buffer and the purified probe was eluted with a further 400µl TE. The quality of radiolabelling was assessed by Geiger Counter comparison of the counts/minute from the column with that of the purified probe. The probe was used only if it had incorporated >50% of radionucleotides.

2.8.7.6 Hybridisation
Following prehybridisation, radiolabelled probe was denatured by boiling for 5 minutes, and added to the hybridisation tube. Hybridisation was allowed to proceed overnight at 68°C. The membrane was washed in SSC/SDS solutions of increasing stringency (Appendix 1) for 20 minutes each, removed from the hybridisation tube, sealed in a plastic bag, and exposed to X-ray film (Kodak) in an autoradiography cassette at -70°C for 2-5 days, depending on signal strength. Autoradiography films were then developed (Amersham Hyperprocessor).

2.9 PCR reactions
PCR reactions were carried out in 0.5ml thin-walled microcentrifuge Eppendorf tubes in a standard thermocycler (Hybaid). All reagents were obtained from Life Technologies. Reactions were overlaid with autoclaved parrafin oil. PCR products were visualised on agarose gels. Primers, master mixes, and thermocycler protocols are summarised in Table 2.8 A-C.
### Table 2.9: PCR reactions

#### A. PCR Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
<th>Product size</th>
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<tbody>
<tr>
<td><strong>p53</strong></td>
<td></td>
<td></td>
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<tr>
<td>Exon 6 Intron 7 Neo</td>
<td>GTGGTGGTACCTTATGAGCC CAAAGAGCGTGTTGGGCTGTG CATCGCCTTTCTATACGCCTTC</td>
<td>642bp wt 510bp deleted</td>
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<tr>
<td></td>
<td>GTGGTGGTACCTTATGAGCC CAAAGAGCGTGTTGGGCTGTG CATCGCCTTTCTATACGCCTTC</td>
<td></td>
</tr>
<tr>
<td><strong>p21</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 2 Exon 3 Neo</td>
<td>TTCTTTGTTTTTCAGCCACAG GCAGCGTATATCCAGGAGACG CATCGCCTTTCTATACGCCTTC</td>
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<td></td>
<td>TTCTTTGTTTTTCAGCCACAG GCAGCGTATATCCAGGAGACG CATCGCCTTTCTATACGCCTTC</td>
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<tr>
<td><strong>Rb</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rb 18 Rb 19</td>
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<td>680bp wt 748bp floxed 300bp deleted</td>
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<tr>
<td></td>
<td>GGCGTGTGGCCATCAATG AACTCAAGGGAGACCTG</td>
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#### B. Master Mix Recipes

<table>
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</thead>
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<tr>
<td>dH₂O</td>
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<td>17.25µl</td>
<td>19.75µl</td>
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<td>10X PCR buffer</td>
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<td>5µl</td>
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<td>W1 detergent</td>
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<td>2.5µl</td>
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<tr>
<td>DMSO</td>
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<td>2.5µl</td>
<td>2.5µl</td>
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<tr>
<td>Mg (50mM)</td>
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<td>2µl</td>
<td>1.75µl</td>
</tr>
<tr>
<td>dNTP (1.25mM)</td>
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<td>8µl</td>
<td>8µl</td>
</tr>
<tr>
<td>Primer (10µM)</td>
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<td>2.5µl each</td>
<td>2.5µl each</td>
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<td>Taq polymerase</td>
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<td>0.5µl</td>
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<tr>
<td>DNA</td>
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</table>

#### C. Thermocycler Protocols

<table>
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<td>94°C 5min</td>
<td>94°C 5min</td>
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<tr>
<td>Melting</td>
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<td>94°C 1min</td>
<td>94°C 1min</td>
</tr>
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<td>Annealing (35 cycles)</td>
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</tr>
<tr>
<td>Cycles</td>
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<td>35</td>
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</tr>
</tbody>
</table>
2.10 Statistical Analyses

Analyses were carried out using Minitab for Windows v 13.0. For counts of BrdU positivity, p53 positivity, apoptosis, abnormal nuclei, and ploidy, the proportion of affected cells was arcsine transformed and differences between means were evaluated by ANOVA. Differences were taken to be significant at p<0.05. Satisfactory homogeneity of variances was determined with Bartlett’s test. Where a significant difference between means was identified by ANOVA, the differences between individual means was analysed further with Bonferroni simultaneous tests for making multiple comparisons. For MTT data, the differences between medians were analysed with the Kruskal-Wallis test.
Chapter 3

Deletion of *Rb*-floxed alleles in primary mouse hepatocytes using the Cre/LoxP system of inducible gene targeting

Introduction

Mice homozygous for a mutation in the *Rb* gene die between day 13.5-14.5 of gestation, due to defects in haematopoiesis and the nervous system (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992), thereby precluding further study of the consequences of *Rb* inactivation. However, with the advent of inducible gene targeting, the problem of embryonic lethality can be overcome. It is now possible to achieve activation or inactivation of genes in a tissue-specific and temporally defined manner using the Cre/LoxP system of inducible gene targeting (Kellendonk *et al.*, 1999). This system requires the generation of a floxed target gene and regulatable expression of Cre recombinase. Cre excises DNA flanked by two directly repeated LoxP recognition sites (floxed sequence).

Generation of the *Rb*-floxed mice used in these experiments has been described (Vooijs *et al.*, 1998). These mice are homozygous for the *Rb*-floxed allele, in which exon 19 is flanked by LoxP sequences. In the absence of Cre, they express a functional RB protein and are indistinguishable from wild-type mice. Cre-mediated excision of exon 19 results in a frameshift leading to the removal of 300 amino acids from the carboxy terminus and truncation of the RB protein. A similar mutation has been shown to be functionally equivalent to a null allele (Clarke *et al.*, 1992). The aim of this chapter was to express Cre recombinase in cultures of primary hepatocytes isolated from *Rb*-floxed mice, in order to mediate deletion of *Rb*-floxed sequences and thereby inactivate the *Rb* gene.

3.1 Adenovirus-mediated Cre deletion of *Rb*-floxed alleles in primary mouse hepatocytes

Expression of Cre could be achieved by several methods, including transient transfection of hepatocytes with Cre-encoding plasmids or Cre protein. Both techniques were tested, however, only relatively low transfection efficiencies were
achieved, using both lipofection and electroporation, as determined by transfection with a LacZ plasmid which expresses β-galactosidase. Electroporation also resulted in high levels of cell death, and is therefore unsuitable as a method of transfection for hepatocytes. The use of a replication-defective recombinant Cre-expressing adenovirus, AdCre1, obtained from M. Anton and FL. Graham (Anton & Graham, 1995), was therefore considered as an alternative approach.

AdCre1 is rendered replication-defective by replacement of the El region, encoding genes essential for replication, with a Cre recombinase expression cassette. Such recombinant viruses are able to infect a wide variety of cell types, both proliferating and non-proliferating, at a high efficiency, and only very rarely become integrated into the genomes of mammalian cells (Davidson et al., 1993; Ragot et al., 1993; Rosenfeld et al., 1991, 1992; Zabner et al., 1994). Cre expression is under the control of the constitutive promoter, human CMV, resulting in high levels of Cre expression in infected cells. The following sections describe the work carried out in order to evaluate the utility of AdCre1 for expression of Cre recombinase and deletion of Rb-floxed alleles in primary mouse hepatocytes.

3.1.1 Determination of the optimum MOI for adenoviral infection of primary mouse hepatocytes

To determine the efficiency of adenoviral infection, wild-type hepatocytes were infected with AdLacZ, a recombinant adenovirus that is El-deleted and expresses the reporter gene β-galactosidase, also obtained from M. Anton and FL. Graham (Anton & Graham, 1995). Hepatocytes were infected with AdLacZ over a range of MOI (0, 4, 8, 12, 16, 20). 24 and 48 hours after infection the cells were fixed and β-galactosidase activity was visualised histochemically by x-gal staining. 24 hours after infection with MOI 20, the majority of cells were stained blue, indicative of β-galactosidase activity and successful infection with AdLacZ (Figure 3.1.A). 48 hours after infection, >95% of cells infected with every other MOI were also stained blue. At lower MOI, no obvious cytotoxic effects were observed compared with uninfected controls, however, at higher MOI cell viability appeared to be increasingly compromised. To confirm high levels of adenoviral infection at low MOI, hepatocytes were infected with AdLacZ over a narrower range of MOI (0, 4, 6,
8, 10, 12). As before, MOI 4 was sufficient to infect >95% of hepatocytes without any obvious adverse affect on cell viability, while MOI 12 appeared severely damaging to cells (Figure 3.1.B).

In addition to morphological assessment of cell viability following adenoviral infection, the MTT assay of mitochondrial metabolic activity was performed on cells infected with AdLacZ, AdCre and AdΔ170-3 (Ad70) at MOI 6 and MOI 20. Like AdLacZ and AdCre, Ad70 is E1-deleted and therefore replication-deficient, but does not express any heterologous proteins and is referred to as empty (Sallenave et al., 1998). Cells infected at MOI 6 with any of the three viruses did not differ significantly from the uninfected controls (Figure 3.1.C). However, at MOI 20 AdLacZ resulted in a significant decrease in hepatocyte metabolic activity compared with uninfected controls or cells infected with AdCre or Ad70 (Figure 3.1.C). Therefore, both morphological and metabolic analyses indicate that at low MOI (MOI 4-10), neither adenoviral infection nor overexpression of Cre or β-galactosidase adversely affects hepatocyte viability. However, high levels of β-galactosidase, but not Cre expression (MOI 12-20), is damaging to cells. Based on these experiments, all subsequent adenovirus infections of hepatocytes were carried out at MOI 6, and Ad70 was used as a control instead of AdLacZ.
Figure 3.1: Adenoviral infection of primary mouse hepatocytes

A) β-gal staining of hepatocytes following infection with AdLacZ MOI 4 and MOI 20
β-gal-expressing cells are stained blue. Original magnification x40 (except MOI 4 24h after infection, x100). 24 hours following infection with AdLacZ MOI 4, approximately 50% of cells are stained blue. This increases to >95% at 48 hours following infection. >95% of cells infected with MOI 20 are stained blue at both 24 and 48 hours following infection. Cytotoxicity of AdLacZ is evident at 48 hours following infection with MOI 20.

B) β-gal staining of hepatocytes following infection with AdLacZ MOI 4 and MOI 12
β-gal-expressing cells are stained blue. Original magnification x40. >95% of cells infected with AdLacZ either MOI 4 or MOI 12 are stained blue at 48 hours following infection. Cytotoxicity of AdLacZ is evident following infection with MOI 12.

C) MTT assay of hepatocyte viability following infection with MOI 6 and MOI 20
Hepatocytes infected with AdLacZ, AdCre or AdDL-70 were compared with uninfected controls. Results are the average of triplicates, expressed as mean ± standard error. Infection at MOI 6 with any of the three viruses does not affect hepatocyte viability. Infection with AdLacZ MOI 20 decreases hepatocyte viability compared with uninfected controls or cells infected with AdCre or AdDL-70 MOI 20.
Figure 3.1: Adenoviral infection of primary mouse hepatocytes

A) 24h after infection  
   48h after infection

MOI 4

MOI 20

B) MOI 4  
   MOI 12

C) MOI 6  
   MOI 20

![Graph showing OD at 490nm over hours after infection for different MOIs and conditions]
3.1.2 AdCre-mediated deletion of Rb-floxed alleles in primary mouse hepatocytes

Having demonstrated that adenoviral infection of hepatocytes was highly efficient, the next step was to demonstrate that AdCre was expressing Cre recombinase, and that this was accompanied by recombination of Rb-floxed alleles. Homozygous Rb-floxed hepatocytes were infected with AdCre and harvested for protein and genomic DNA extraction. Cre expression was detected by western blotting 6 hours after infection with AdCre and remained high through to 96 hours (Figure 3.2.A). Recombination of Rb-floxed alleles was analysed by both PCR and Southern blotting. Genomic DNA was amplified with primers Rb19 and Rb18 (see chapter 2, Table 2.8), yielding a 748bp product for the Rb-floxed allele and a 300bp product for the recombined allele (Figure 3.2.B). Appearance of the recombined allele occurred as early as 8 hours after infection with AdCre, and >95% recombination was obtained within 24 hours of infection, demonstrating that the cells were expressing functional Cre recombinase. However, this PCR reaction was not quantitative and a Southern blot was performed in order to assess the efficiency of Cre-mediated recombination. Genomic DNA was digested with the restriction enzyme PstI and hybridized with a radiolabelled 450bp PstI-PvuII probe comprising exon 18 of Rb (Clarke et al., 1992), isolated from pHAl53 (Vooijs et al., 1998). This probe hybridizes with a 5kb fragment corresponding to the Rb-floxed allele and a 4.5kb fragment corresponding to the recombined allele (Vooijs et al., 1998). The recombined allele was observed 12 hours after infection with AdCre, and >95% recombination was obtained within 24 hours of infection (Figure 3.2.C).

It has been suggested that recombination in floxed/null or floxed/wt cells would be more efficient than in floxed/floxed cells, as only one allele needs to be recombined (Stec et al., 1998). Although recombination in floxed/wt cells was not tested, evidence from floxed/null cells (LIG1^Flox/-) suggested that this was not the case. Ligase1-floxed/null mice were obtained from David Melton (Bentley et al., 1996). Hepatocytes isolated from Ligase1-floxed/null mice were infected with AdCre and harvested for genomic DNA extraction. Recombination efficiency was quantified by Southern blot. Genomic DNA was digested with the restriction enzyme EcoR1 and hybridized with a radiolabelled 1.2Kb EcoR1-HindIII fragment.
of murine Lig1 DNA (Bentley et al., 1996). This probe hybridizes with an 8.5Kb fragment corresponding to the Lig1-floxed allele, a 6.7Kb fragment corresponding to the null allele, and a 3.4Kb fragment corresponding to the recombined allele (Bentley et al., 1996). The recombined allele was observed 12 hours following infection with AdCre, and >95% recombination was obtained within 24 hours of infection (Figure 3.2.D). Hence, in both floxed/null (LIG1\textsuperscript{Flox/-}) and floxed/floxed (Rb\textsuperscript{Flox/Flox}) cells, recombination was complete within 24 hours of AdCre infection, suggesting that homozygosity of the floxed sequence does not affect recombination efficiency.

**Figure 3.2: AdCre-mediated deletion of Rb-floxed alleles**

A) **Western blot of Cre recombinase**
Cre expression was detected with anti-Cre antibody No. 69050-3 (Novagen) on a 12% tris-glycine gel. Lanes 1-8: 20μg of protein samples harvested from Rb\textsuperscript{Flox/Flox} hepatocytes at the indicated times following infection with AdCre. Cre expression is initially observed at 6 hours, and remains high through to 96 hours following infection, as indicated by the presence of the 38kDa band.

B) **PCR detection of recombined Rb-floxed alleles**
1.5% agarose/ethidium bromide gel. Lane 1: molecular weight markers. Lanes 2-6: 10μg genomic DNA samples harvested from Rb\textsuperscript{Flox/Flox} hepatocytes at the indicated times following infection with AdCre. The 300bp band indicative of the presence of the recombined allele is observed 8 hours following infection with AdCre. >95% recombination is obtained within 24 hours of infection.

C) **Southern blot of recombined Rb-floxed alleles**
Lanes 1-6: 10μg genomic DNA samples harvested from Rb\textsuperscript{Flox/Flox} hepatocytes at the indicated times following infection with AdCre. The 4.5kb band indicative of the presence of the recombined allele is observed 12 hours following infection with AdCre. >95% recombination is obtained within 24 hours of infection.

D) **Southern blot of recombined Lig1-floxed allele**
Lanes 1-11: 10μg genomic DNA samples harvested from LIG1\textsuperscript{Flox/-} hepatocytes at the indicated times following infection with AdCre. The 3.4kb band indicative of the presence of the recombined allele is observed 12 hours following infection with AdCre. >95% recombination is obtained within 24 hours of infection.
Figure 3.2: AdCre-mediated deletion of Rb-floxed alleles

A

Hours after infection
0  6  18  24  32  48  72  96
38kD Cre recombinase

B

Hours after infection
MW  0  8  24  32  42  72
748bp Rb-floxed
300bp recombined

C

Hours after infection
0  12  18  24  48  72
5kb Rb-floxed
4.5kb recombined

D

Hours after infection
0  4  8  12  16  20  24  28  32  40  44
8.5kb Lig1-floxed
6.7kb Lig1−−
3.4kb recombined
3.1.3 Ad-Cre recombination in polyploid cells

In mice, hepatocyte polyploidy is a feature of normal liver growth, and can range from 2n to 32n depending on age (Medvedev et al., 1986; Kudryavtsev et al., 1993). Murine hepatocytes used in these experiments contain mainly diploid (~45%) and tetraploid (~45%) nuclei, with some octaploid nuclei (~10%), with 80% of hepatocytes being binuclear. To confirm the high level of recombination observed in Rb-floxed hepatocytes, and to determine whether the degree of polyploidisation affects the efficiency of recombination, ROSA 26 Flox-STOP-βgeo reporter mice were used. These mice have a floxed STOP sequence inserted between the proviral βgeo gene (LacZ-neomycin fusion protein) and its promoter, preventing transcription of the fusion protein (Mao et al., 1999). Cre-mediated excision of the STOP sequence allows expression of LacZ.

Hepatocytes isolated from Flox-Stop-βgeo mice were infected with AdCre. 48 hours after infection the cells were fixed and β-galactosidase activity was visualised histochemically by x-gal staining. >95% of cells stained blue, indicative of β-galactosidase activity and successful Cre-mediated recombination (Figure 3.3). LacZ expression was observed regardless of the cell ploidy, as assessed by nuclear size, indicating that AdCre is capable of mediating effective recombination in polyploid cells.
Figure 3.3: AdCre recombination in polyploid cells

- Uninfected
- AdCre

β-gal staining of Flox-Stop-βgeo hepatocytes following infection with AdCre MOI 4

β-gal-expressing cells are stained blue. Original magnification x40. >95% of cells infected with MOI 4 are stained blue at 48 hours following infection with AdCre, indicative of successful recombination. LacZ expression is observed regardless of the cell ploidy, as assessed by nuclear size, indicating that AdCre is capable of mediating effective recombination in polyploid cells.

3.2 Analysis of the effects of Cre expression and adenoviral infection in primary mouse hepatocytes

LoxP sites are of a sufficiently large size that they are unlikely to naturally occur in mammalian genomes. However, in vitro studies have shown that Cre is capable of catalyzing recombination between DNA sequences, surprisingly divergent from LoxP, naturally present in E. coli (Sternberg et al., 1981), yeast (Sauer, 1996) and mammalian genomes (Thyagarajan et al., 2000). These endogenous DNA sequences, termed pseudo-LoxP sites, can recombine with each other but not with the wild-type LoxP site (Sauer, 1996). Conversely, recombination between pseudo-LoxP sites might also compromise the utility of Cre/LoxP technology in mammalian cells.

For example, Cre expression in developing spermatids induced gross chromosomal rearrangements in spermatozoa, possibly at pseudo-LoxP sites, resulting in male sterility (Schmidt et al., 2000). Cre expression in MEFs caused extensive chromosomal aberrations, accompanied by G2/M arrest and inhibition of growth (Loonstra et al., 2001). It was suggested that such chromosomal abnormalities were the result of double strand break (DSB) repair via
nonhomologous end joining, and not illegitimate Cre-mediated recombination of pseudo-LoxP sites. Cre toxicity is dependent on its recombinase activity, and prolonged low-level expression of Cre (Loonstra et al., 2001), or use of a self-excising retroviral Cre expression vector (Silver & Livingston, 2001), allows recombination without concomitant toxicity. These findings suggested that careful examination of the effects of Cre expression, beyond its ability to mediate efficient recombination, was required before any attempt at phenotypic analysis of the conditional mutation. Similarly, careful analysis of the effects of adenoviral infection itself was also required, as studies in both primary cells and cell lines showed that infection with high titres of adenovirus led to growth inhibition and apoptosis (Teramoto et al., 1995, 1999; Jault et al., 1995; Brand et al., 1999). The following sections describe the work carried out in order to characterise the effects of Cre expression and adenoviral infection on hepatocytes, before analysing the consequences of Rb-deficiency.

3.2.1 p53 stabilisation
DNA damage is a potent activator of p53, and the presence of a single double strand break is sufficient to induce a p53 response (Huang et al., 1996), such as induction of growth arrest to prevent the replication of damaged DNA, or apoptosis to eliminate the aberrant cells. It is possible that Cre-mediated recombination, which involves the creation of DNA strand breaks, could trigger p53 stabilisation accompanied by growth arrest or apoptosis. Hepatocytes isolated from wild-type and Flox-STOP-βgeo mice were used to determine whether Cre expression could induce a p53 response. p53 stabilisation was quantified by immunocytochemistry. There were no significant differences in p53 immunopositivity between AdCre- or Ad70-infected cells (P = 0.92, ANOVA) (Figure 3.4.A), indicating that DNA breaks induced during Cre-mediated recombination do not induce p53 stabilisation.
Figure 3.4: Effects of adenoviral infection and Cre expression on primary mouse hepatocytes

A) p53 stabilisation in hepatocytes following infection with AdCre and Ad70
p53 stabilisation was detected by immunocytochemistry with anti-p53 antibody pAb421 (Oncogene Sciences), and the results are expressed as mean ± standard error of the mean for duplicate cultures from 1 Flox-STOP-βgeo mouse. The experiment was performed in wild-type and Flox-STOP-βgeo hepatocytes, twice with similar results. There were no significant differences in p53 immunopositivity between AdCre- or Ad70-infected cells, indicating that Cre expression does not induce p53 stabilisation.

B) Apoptosis in hepatocytes following infection with AdCre and Ad70
Apoptosis was assessed morphologically by Feulgen staining of DNA and the results are expressed as mean ± standard error of the mean for duplicate cultures from 1 Flox-STOP-βgeo mouse. The experiment was performed in wild-type and Flox-STOP-βgeo hepatocytes, twice with similar results. There were no significant differences in rates of apoptosis between uninfected, Ad70- or AdCre-infected cells, suggesting that neither adenoviral infection nor Cre expression induces apoptosis.

C & D) DNA synthesis in hepatocytes following infection with AdCre and Ad70
DNA synthesis was assessed by BrdU incorporation into hepatocyte DNA and the results are expressed as mean ± standard error of the mean for duplicate cultures from 1 Flox-STOP-βgeo mouse. The experiment was performed in wild-type and Flox-STOP-βgeo hepatocytes, twice with similar results. AdCre, but not Ad70, caused an increase in the proportion of S phase cells around 48 hours, but not over the time-course as a whole, suggesting that Cre expression, but not adenoviral infection, accelerates entry into S phase.

E) Nuclear abnormality in hepatocytes following infection with AdCre and Ad70
Nuclear abnormality was assessed morphologically and the results are expressed as mean ± standard error of the mean for duplicate cultures from 1 Flox-STOP-βgeo mouse. The experiment was performed in wild-type and Flox-STOP-βgeo hepatocytes, twice with similar results. The proportion of cytologically abnormal nuclei was similar in uninfected, Ad70- and AdCre-infected wild-type and Flox-STOP-βgeo cells, suggesting that neither Cre expression nor adenoviral infection induces nuclear abnormality.
Figure 3.4: Effects of adenoviral infection and Cre expression on primary mouse hepatocytes

A. % p53 positive cells

B. % Apoptotic cells

C. % BrdU positive cells

D. % BrdU positive cells

E. % abnormal cells

- uninfected
- Ad70
- AdCre
3.2.2 Apoptosis

Hepatocytes isolated from wild-type and Flox-STOP-βgeo mice were used to determine whether Cre expression could induce apoptosis, in the absence or presence of LoxP sites. There were no significant differences in rates of apoptosis between uninfected, Ad70-infected, or Cre-expressing wild-type (P = 0.554, ANOVA), or Flox-STOP-βgeo cells (P = 0.659, ANOVA) (Figure 3.4.B), suggesting that Cre expression, Cre-mediated recombination, or adenovirus infection do not induce apoptosis.

3.2.3 Proliferation

In the adult liver the majority of hepatocytes are quiescent (Fausto et al., 1986), and upon isolation for primary culture they are stimulated to enter the cell cycle in a near synchronous manner (Bellamy et al., 1997a). Hepatocytes isolated from wild-type mice and Flox-STOP-βgeo mice were used to assess the effect of Cre expression and adenoviral infection on DNA synthesis, in the absence or presence of LoxP sites. Hepatocytes were incubated with medium containing 40μM BrdU, and DNA synthesis was assessed by immunodetection of cells that had incorporated BrdU into newly synthesized DNA.

Expression of Cre caused a significant increase in BrdU positivity at 48h in Flox-STOP-βgeo hepatocytes, compared with uninfected (P< 0.0001, ANOVA) and Ad70-infected (P< 0.0001, ANOVA) controls. However, there were no significant differences in mean BrdU over the time-course as a whole, suggesting that Cre expression accelerates entry into S phase by shortening G1, without affecting the overall rate of DNA synthesis (Figure 3.4.C). The experiment was repeated concentrating on time-points around 48 hours, and again Cre expression caused an increase in S phase cells but Ad70 did not (Figure 3.4.D). The increased proportion of cells in S phase at 48 hours occurred in the absence (wild-type) or presence (Flox-STOP-βgeo) of LoxP sites, suggesting that this effect of Cre is not a consequence of recombination at LoxP sites.
3.2.4 Nuclear and mitotic abnormality

Hepatocytes isolated from wild-type mice and Flox-STOP-βgeo mice were used to determine whether Cre expression or adenoviral infection caused gross nuclear and mitotic abnormalities, in the absence or presence of LoxP sites, respectively. Abnormal mitosis was not observed, and the proportion of cytologically abnormal nuclei was similar in uninfected, Ad70-infected, and Cre-expressing wild-type, and Flox-STOP-βgeo cells (P = 0.164, ANOVA) (Figure 3.4.E).

Discussion

The aim of this section of work was to establish a system that allowed deletion of Rb-floxed alleles in primary mouse hepatocytes. The feasibility of using adenovirus to introduce Cre recombinase into hepatocytes, the efficiency of Cre-mediated recombination, the effects of adenoviral infection, and the effects of Cre expression beyond its ability to mediate recombination, were thoroughly investigated.

Adenoviral infection of primary mouse hepatocytes is highly efficient

The efficiency of adenovirus infection of hepatocytes was determined using AdLacZ, a recombinant adenovirus that is E1-deleted and expresses the reporter gene β-galactosidase. Wild-type hepatocytes were infected with AdLacZ over a range of MOI, and >95% of cells were shown to express β-galactosidase even at the lowest MOI used ie. MOI 4. At high MOI (12-20), AdLacZ appeared severely damaging to cells, however, this cytotoxic effect was not observed following infection with AdCre or Ad70. Since the adenoviral constructs used were similar, this suggests that high level expression of β-galactosidase, but not Cre expression or adenoviral infection itself, is toxic to hepatocytes. However, as the relative level of expression of Cre or β-galactosidase was not quantified, it is possible that the level of expression of the exogenous protein rather than the protein itself, is the cause of toxicity. Indeed the murine CMV promoter driving LacZ expression in AdLacZ is a much stronger promoter in mouse cells than the human CMV promoter upstream of Cre in the AdCre construct (Addison et al., 1997). This apparent hepatotoxicity of LacZ is an important observation considering that AdLacZ is often used as a control or reporter vector for both in vitro and in vivo liver studies, where lysis of LacZ-expressing cells
could lead to or exacerbate immune responses and hepatitis development. Use of alternative vectors for liver studies would therefore be advisable, and Ad70 was employed as the control virus throughout the rest of this project.

**AdCre-mediated deletion of Rb-floxed alleles is highly efficient**

Expression of Cre recombinase was detected within 6 hours of infection of hepatocytes with AdCre, and this was rapidly accompanied by recombination of Rb-floxed alleles 8 hours post-infection as determined by PCR, and 12 hours post-infection as determined by Southern blot. Recombination was >95% complete within 24 hours of AdCre infection. Recombination occurred efficiently in polyploid cells, as assessed by LacZ expression in Flox-Stop-βgeo hepatocytes infected with AdCre, regardless of nuclear size. Recombination also occurred as efficiently in floxed/floxed (Rb$_{Flox/Flox}$) cells as in floxed/null (LIG1$_{Flox/}$) cells, suggesting that homozygosity of the floxed sequence does not affect the efficiency of recombination.

**Adenoviral infection does not affect proliferation or apoptosis, but Cre recombinase accelerates S phase**

Adenoviral infection at high MOI has been shown to inhibit growth and induce apoptosis both in primary cells and cell lines (Katayose *et al.*, 1995; Teramoto *et al.*, 1995; Teramoto *et al.*, 1999). In the present study, a low MOI (MOI 6) was used and did not lead to growth inhibition, p53 stabilisation, apoptosis, or nuclear or mitotic abnormalities. Interestingly, infection with AdCre, but not Ad70, caused an increase in the proportion of cells in S phase around 48 hours, but not at other time-points. This suggests that Cre expression, and not adenoviral infection, was responsible for the acceleration of S phase. Interestingly, this Cre effect was found both in the presence and absence of LoxP sites, suggesting that it was not merely a consequence of recombination at LoxP sites.

Cre expression has led to chromosomal aberrations and growth inhibition in both primary cells and cell lines (Loonstra *et al.*, 2001; de Alboran *et al.*, 2001; Silver & Livingston, 2001), and chromosomal rearrangements in spermatids of transgenic mice leading to male sterility (Schmidt *et al.*, 2000). The mechanism of this Cre effect is not clear but it has been shown that Cre is capable of catalyzing
recombination between pseudo-LoxP sites (Sternberg et al., 1981; Sauer, 1996; Thyagarajan et al., 2000). It has also been suggested that such chromosomal abnormalities were the result of Cre-induced DNA damage followed by repair via nonhomologous end joining (Loonstra et al., 2001). Cre expression in hepatocytes was not accompanied by gross nuclear or mitotic abnormality, p53 stabilisation, growth inhibition or apoptosis.

Low level or transient expression of Cre has been shown to allow efficient recombination without concomitant side effects (Loonstra et al., 2001; Silver & Livingston, 2001). Different cell types seem to vary in their susceptibility to Cre toxicity (de Alboran et al., 2001), and the length of exposure to Cre may be an important determinant of Cre toxicity. In MEFs, obvious abnormalities began to appear one week after introduction of Cre (Silver & Livingston, 2001). Although hepatocytes were not monitored for Cre toxicity beyond one week in culture, the experiments performed during the rest of the project were conducted over a period of 5 days only.

Thus, the first objective of this project, to establish and evaluate a system that allows deletion of Rb-floxed alleles in primary mouse hepatocytes, was successfully achieved. Infection of hepatocytes with AdCre mediates rapid and efficient recombination of Rb-floxed alleles. Expression of Cre recombinase accelerated the onset of S phase in hepatocytes, and this effect of Cre must be taken into account when analysing the consequences of Rb-deficiency. This finding highlights the need to include proper controls, Cre-treated cells without LoxP sites and cells bearing floxed sequences other than the gene of interest, when using the Cre/LoxP system to analyse gene function.
Chapter 4

Roles of p53, p21, and RB in regulation of Proliferation and Polyploidisation in primary mouse hepatocytes

Having successfully obtained Rb-deficient hepatocytes in culture, the consequences of Rb-, p53-, and p21-deficiency for hepatocyte cell cycle regulation were investigated in terms of proliferation and polyploidisation. For ease of reading, AdCre-treated cells shall hereafter be referred to as Rb\(^+\), and uninfected cells shall be referred to as wild-type.

4.1 Activation of the p53 pathway in Rb-deficient and p21-deficient hepatocytes

Inactivation of Rb leads to activation of the p53 pathway in a variety of transgenic mice and cell types (Howes et al., 1994; Morgenbesser et al., 1994; Pan & Griep, 1994; Symonds et al., 1994; Macleod et al., 1996), and in some cell types this is accompanied by an induction of p21 (Macleod et al., 1996). p53 and p21 protein levels were analysed in Rb\(^+\) hepatocytes by immunocytochemistry and western blotting. Wild-type cells were shown to express low levels of p53. However, Rb-deficiency led to a dramatic increase in p53, as determined by p53 immunocytochemistry (Figure 4.1.A). As shown earlier, adenoviral infection did not induce p53. Western blot analysis confirmed this increase in p53 protein levels and revealed a coincident induction of p21 (Figure 4.1.B). Analysis of p21 in p53\(^+\)/Rb\(^+\) cells revealed that p21-induction upon loss of Rb is largely p53-dependent (Figure 4.1.C). p53 protein levels were also increased in p21\(^+\) cells, as determined by immunofluorescence (Figure 4.1.D).
Figure 4.1: Activation of the p53 pathway in Rb-deficient and p21-deficient hepatocytes

A) Induction of p53 in Rb-deficient hepatocytes
p53 stabilisation was detected by immunocytochemistry with anti-p53 antibody pAb421 (Oncogene Sciences), and the results are expressed as mean ± standard error of the mean for duplicate cultures from one Rb-floxed mouse. Rb-deficiency led to a dramatic increase in p53 stabilisation compared with wild-type cells. Adenoviral infection did not induce p53 stabilisation. The experiment was performed twice with similar results.

B) Induction of p53 and p21 in Rb-deficient hepatocytes
p53 and p21 protein levels were detected by western blotting, with anti-p53 antibody CM5 (Novocastro) and anti-p21 antibody M19 (Santa Cruz), on 10% and 14% tris-glycine gels, respectively. Lanes 1-8: 20μg of protein samples harvested from Rb-/- and wt hepatocytes at the indicated times post-plating. Rb-deficiency led to an increase in p53 and p21 protein levels compared with wild-type cells. The experiment was performed three times with similar results.

C) Induction of p21 in Rb-deficient hepatocytes is p53-dependent
p21 protein levels were detected by western blotting as described in B. Upregulation of p21 in Rb-/-, but not Rb-/+p53-/- hepatocytes, indicated that induction of p21 upon loss of Rb is p53-dependent. The experiment was performed twice with similar results.

D) Induction of p53 in p21-deficient hepatocytes
p53 was detected by immunofluorescence using anti-p53 antibody CM5 (Novocastro), and a secondary antibody conjugated with Alexa 488 (Molecular Bioprobes), a very stable fluorophore. Original magnification x400. The exposure time for wild-type cells (0.96 seconds) was 3 times the length of that for p21-/- cells (0.32 seconds). p21-/- cells showed increased levels of p53 compared with wild-type cells. The experiment was performed twice with similar results.
Figure 4.1: Activation of the p53 pathway in Rb-deficient and p21-deficient hepatocytes

A

![Graph showing activation of the p53 pathway](image)

**Hours post-plating**

B

<table>
<thead>
<tr>
<th>wt</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>96</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rb+/-</td>
<td>Rb-/-</td>
<td>Rb+/+</td>
<td>Rb-/-</td>
<td></td>
</tr>
</tbody>
</table>

**B**

![Western blots](image)

C

<table>
<thead>
<tr>
<th>48</th>
<th>72</th>
<th>96</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Rb+/+</td>
<td>Rb-/-</td>
<td>Rb+/+</td>
<td>Rb-/-</td>
</tr>
</tbody>
</table>

**C**

![Western blots](image)

D

<table>
<thead>
<tr>
<th>wt</th>
<th>72h</th>
<th>p21-/-</th>
</tr>
</thead>
</table>

**Exposure time 0.96 seconds**

**Exposure time 0.32 seconds**
4.2 Proliferation

4.2.1 Proliferation in Rb-deficient hepatocytes

Inactivation of Rb leads to aberrant proliferation in a variety of cell types (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992; Lee et al., 1994; Symonds et al., 1994; Macleod et al., 1996), although the consequences of Rb-inactivation for hepatocyte proliferation have not been well documented. p53 (Bellamy et al., 1997a) and p21 were previously shown to regulate hepatocyte proliferation (Wu et al., 1996; Timchenko et al., 1997). To understand better the mechanisms controlling hepatocyte growth, in particular the role of p53, p21, and RB, the consequences of p53−, p21−, and Rb-deficiency for hepatocyte proliferation were investigated.

As mentioned earlier, in the adult liver the majority of hepatocytes are quiescent (Fausto et al., 1986). Upon isolation for primary culture, they are stimulated to enter the cell cycle in a near synchronous manner (Bellamy et al., 1997a). During the experiments described in this thesis, BrdU labeling is used as a measure of DNA synthesis and proliferation, and these terms are used interchangeably. However, it is acknowledged that DNA synthesis does not necessarily culminate in mitosis or cell division. Rb−/− cells entered S phase earlier, and maintained a higher level of DNA synthesis throughout culture compared with wild-type cells (Figure 4.2.A). In agreement with earlier results, adenoviral infection had no effect on DNA synthesis.

To determine whether the increased rate of DNA synthesis in Rb−/− cells was dependent on serum, hepatocytes were cultured in the absence and presence of serum. The level of DNA synthesis in both wild-type and Rb−/− cells was higher when serum was added to the culture medium than when it was omitted, indicating that Rb-deficient cells retain some level of serum-responsiveness (Figure 4.2.B). However, the level of DNA synthesis was consistently higher in Rb−/− cells than wild-type, regardless of the presence of serum. In fact, Rb−/− cells cultured in the absence of serum underwent much higher rates of DNA synthesis than wild-type cells cultured in the presence of serum (Figure 4.2.B). This is similar to findings for p53−/− hepatocytes (Bellamy et al., 1997a).
Figure 4.2: Proliferation in Rb-deficient hepatocytes

DNA synthesis was assessed by BrdU incorporation into hepatocyte DNA and the results are expressed as mean ± standard error of the mean for duplicate cultures from one Rb-floxed mouse. The experiment was performed several times with similar results. A: Cells cultured with serum. Rb-deficiency led to a dramatic increase in DNA synthesis compared with wild-type cells. Adenoviral infection did not affect DNA synthesis. B: Cells cultured without serum. Cells undergo decreased rates of DNA synthesis in the absence of serum. Rb-deficiency increased DNA synthesis compared with wild-type cells, regardless of whether serum was present in the medium or not. SFM: serum-free medium
4.2.2 Proliferation in hepatocytes deficient in p53, p21, or Rb

The levels of DNA synthesis in p53\(^{-/-}\), p21\(^{-/-}\), and Rb\(^{-/-}\), hepatocytes were compared. p53\(^{-/-}\), p21\(^{-/-}\), and Rb\(^{-/-}\) cells all entered S phase earlier, and exhibited far more elevated levels of DNA synthesis throughout culture, than wild-type cells (Figure 4.3.A-B). This suggests that each of these genes is required for regulation of hepatocyte proliferation. It is important to note that this experiment shows that basal levels of p53 and p21 are required to regulate hepatocyte growth, which is in contrast to their more usually described role in mediating cell cycle arrest when induced in response to a variety of cellular stresses. Accelerated entry into S phase was particularly dramatic in p21\(^{-/-}\) cells, where the percentage of BrdU-positive cells increased from 2.5% to 54% over a period of 24h. During the same period, BrdU-positivity increased in p53\(^{-/-}\) cells from 0 to 25%, and in Rb\(^{-/-}\) cells from 0 to 37%. Thus, p21 may play a critical role in regulating the timing of the G1-S transition.

Having established that the rate of DNA synthesis was greatly increased, and given the role of p53, p21, and RB in regulating various checkpoints, it was of interest to determine whether or not the mutant cells were capable of continuing through the cell cycle into mitosis, or became S phase- or G2-arrested. The rate of mitosis in cultures of wild-type cells is very low, typically peaking at 1%. Rb\(^{-/-}\), p53\(^{-/-}\), and p21\(^{-/-}\) cells all exhibited a higher mitotic index than wild-type cells (Figure 4.4.A-B). At 48h, mitosis was observed only in p21\(^{-/-}\) cells, in accordance with the particularly high levels of DNA synthesis observed in these cells at this time. Therefore, the increased level of DNA synthesis in Rb\(^{-/-}\), p53\(^{-/-}\), and p21\(^{-/-}\) cells is accompanied by an increase in mitosis.
Figure 4.3: DNA synthesis in hepatocytes deficient in \(p53\), \(p21\), or \(Rb\)

A

<table>
<thead>
<tr>
<th></th>
<th>48h</th>
<th>72h</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Rb^{-/})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(p53^{-/})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(p21^{-/})</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
DNA synthesis was assessed by BrdU incorporation into hepatocyte DNA. **A:** BrdU positive nuclei stained brown, BrdU negative nuclei counterstained blue. Original magnification x100. **B:** BrdU positive cells are expressed as mean ± standard error of the mean for duplicate cultures from the relevant transgenic mice. $Rb^{-/-}$, $p53^{-/-}$, and $p21^{-/-}$ cells all exhibited higher rates of DNA synthesis than wild-type cells. $p21^{-/-}$ cells showed a particularly dramatic acceleration into S phase at 48h. The experiment was performed several times with similar results.
Figure 4.4: Mitosis in hepatocytes deficient in p53, p21, or Rb

Mitosis was assessed morphologically by Feulgen staining of DNA. 

A: Photos of wild-type, Rb<sup>−/−</sup>, p53<sup>−/−</sup>, and p21<sup>−/−</sup> cells at 48h. Original magnification x200. Mitotic cells display condensed chromatin and are stained dark pink (see arrows).

B: Mitotic cells are expressed as mean ± standard error of the mean for duplicate cultures from the relevant transgenic mice. Rb<sup>−/−</sup>, p53<sup>−/−</sup>, and p21<sup>−/−</sup> cells all exhibited higher rates of mitosis than wild-type cells. The experiment was performed three times with similar results.
4.2.3 Proliferation in hepatocytes deficient in combinations of p53, p21, and Rb

The above results suggested a variety of potential pathways involved in regulation of hepatocyte growth (Figure 4.5). RB, p53, and p21 might all regulate S phase via independent pathways (Figure 4.5.A). They could also act in a mutually dependent pathway, such that inactivation of one of the genes involved could effectively inactivate the pathway (Figure 4.5.B). p53 is induced in Rb−/− cells, resulting in induction of p21, and therefore both p53 and p21 may act to limit proliferation (Figure 4.5.C). Similarly, p53 is induced in p21−/− cells, and both p53 and RB could act to limit proliferation (Figure 4.5.D). Thus, in hepatocytes, the p53-p21-RB pathway may consist of multiple independent and interdependent axes (summarised in Figure 4.5.E). To further investigate these possibilities, DNA synthesis was analysed in hepatocytes deficient in more than one of these genes.

Rb-floxed mice were interbred with p53−/− and p21−/− mice to produce double mutant Rb^{Flox/Flox}p53−/−, Rb^{Flox/Flox}p21−/−, p53−/−p21−/−, and triple mutant Rb^{Flox/Flox}p53−/−p21−/− mice (hereafter referred to as Trpl−/−). The percentage proliferation for each genotype is presented both as a time-course (Figure 4.6.A-G) and as the mean percentage proliferation over 120h (Figure 4.6.H).
Figure 4.5: Multiple potential pathways regulating hepatocyte growth

A

\[
\begin{array}{c}
p53 \quad p21 \quad RB \\
1 \quad 2 \quad 3
\end{array}
\]

S phase

3 independent pathways

B

\[
\begin{array}{c}
p53 \downarrow \\
4
\end{array}
\]

\[
\begin{array}{c}
p21 \downarrow \\
4
\end{array}
\]

\[
\begin{array}{c}
RB \downarrow \\
4
\end{array}
\]

S phase

1 interdependent pathway

C

\[
\begin{array}{c}
p53 \downarrow \\
4
\end{array}
\]

\[
\begin{array}{c}
p21 \downarrow \\
4
\end{array}
\]

RB

S phase

Rb-deficiency pathway

D

\[
\begin{array}{c}
p53 \rightarrow \\
6
\end{array}
\]

\[
\begin{array}{c}
p21 \rightarrow \\
6
\end{array}
\]

RB

S phase

p21-deficiency pathway

E

\[
\begin{array}{c}
p53 \downarrow \\
4
\end{array}
\]

\[
\begin{array}{c}
p21 \downarrow \\
4
\end{array}
\]

RB

S phase

Summary of Independent and interdependent pathways
Figure 4.6: Proliferation in hepatocytes deficient in combinations of p53, p21, and Rb
Figure 4.6: Proliferation in hepatocytes deficient in combinations of p53, p21, and Rb

DNA synthesis was assessed by BrdU incorporation into hepatocyte DNA.

A-G: Time-course showing the percentage of DNA synthesis for each genotype.

H: Mean percentage proliferation for each genotype over first 120h. This is defined as the area under the proliferation curves (A-G) divided by the time period of 120h. The experiment was performed twice with similar results.
From the above figures, the following conclusions can be drawn:

1. The difference ‘b’ between \( Rb^{+/-}p53^{+/-} \) and \( Trp^{+/-} \) cells suggests that p21 can still inhibit proliferation in the absence of Rb and p53.

2. The difference ‘c’ between \( p53^{+/-}p21^{+/-} \) and \( Trp^{+/-} \) cells suggests that RB can still inhibit proliferation in the absence of p21 and p53.

3. The insignificant difference ‘a’ between \( Rb^{+/-}p21^{+/-} \) and \( Trp^{+/-} \) cells (ANOVA \( p=0.915 \)) suggests that p53 cannot inhibit proliferation in the absence of p21 and Rb.

4. The difference ‘d’ between \( p21^{-/-} \) and \( Trp^{+/-} \) is not significantly different from the difference ‘c’ between \( p53^{+/-}p21^{-/-} \) and \( Trp^{+/-} \) (ANOVA \( p=0.918 \)), suggesting that the ability of p53 to inhibit proliferation is dependent on p21.

5. The difference ‘f’ between \( Rb^{+/-} \) and \( Trp^{+/-} \) is much greater than the sum of ‘a’ + ‘b’, suggesting that p53 and p21 can inhibit proliferation to a greater extent when present together than when alone.

6. The difference ‘e’ between \( p53^{+/-} \) and \( Trp^{+/-} \) is greater than the sum of ‘b’ + ‘c’, suggesting that RB and p21 can inhibit proliferation to a greater extent when present together than when alone.

7. The difference ‘d’ matches the difference ‘c’, suggesting that p53 and RB cannot inhibit proliferation to a greater extent when present together than when alone.

A variety of pathways could therefore be seen to be important for proliferation control in hepatocytes. These include: 1) a p21-dependent, RB-independent, p53-independent pathway; 2) an RB-dependent, p21-independent, p53-independent pathway; 3) a p21-RB-interdependent, p53-independent pathway; 4) a p53-p21-interdependent, RB-independent pathway. The particular combination of pathways suggested to inhibit proliferation, and the reduction in percentage proliferation attributable to each, is presented for each genotype in Table 4.1. As it is important to
take into account the changes in expression of p53 and p21 that occur upon loss of Rb, a simplified summary of the proliferation results, protein levels of p53, p21, and RB, and pathways suggested to be present in each genotype is presented in Table 4.2.

**Table 4.1: Potential pathways inhibiting proliferation in each genotype**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Potential pathways present</th>
<th>% Inhibition of proliferation</th>
<th>% Proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>RB-dependent</td>
<td>12.7</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>p21-dependent</td>
<td>11.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(extra due to) p21-RB-interdependent</td>
<td>5.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(extra due to) p53-p21-interdependent</td>
<td>17.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>46.9</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rb&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>p21-dependent</td>
<td>11.7</td>
<td>19.7</td>
</tr>
<tr>
<td></td>
<td>(extra due to) p53-p21-interdependent</td>
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</tr>
<tr>
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<td>12.7</td>
<td>19.8</td>
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<tr>
<td></td>
<td>p21-dependent</td>
<td>11.7</td>
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</tr>
<tr>
<td></td>
<td>(extra due to) p21-RB-interdependent</td>
<td>5.2</td>
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<tr>
<td></td>
<td><strong>29.6</strong></td>
<td></td>
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</tr>
<tr>
<td>p21&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>RB-dependent</td>
<td>12.7</td>
<td>36.6</td>
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<tr>
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<td>36.7</td>
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<tr>
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<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>49.5</td>
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</table>

The figures given here for percentage proliferation are the same as those plotted in Figure 4.6.H. Percentage inhibition of proliferation for a given genotype is defined as the percentage proliferation in the Trpl<sup>1/-</sup> minus that for the genotype.
Table 4.2: Summary of proliferation, protein levels, and potential pathways present in hepatocytes deficient in combinations of p53, p21, and Rb

| Genotype/Prohibition | wt | < Rb<sup>-/-</sup> = p53<sup>-/-</sup> < p21<sup>-/-</sup> = Rb<sup>-/-</sup>p53<sup>-/-</sup> = p53<sup>-/-</sup>p21<sup>-/-</sup> < Rb<sup>-/-</sup>p21<sup>-/-</sup> = Trp<sup>/-/-</sup> |
|----------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| Protein levels       | Basal p21        | High p21         | Basal p21        | High p21         | Basal p21        | High p21         | Basal p21        | High p21         |
| p21-RB:              | RB               | RB               | RB               | RB               | RB               | RB               | RB               | RB               |
| Potential Pathways   | p21-RB           | p53-p21          | p21-RB           | p21             | RB               | p21             | RB               | -                |

**p21-RB:** p21-RB pathway; encompasses p21-dependent (p21), RB-dependent (RB), and p21-RB-interdependent pathways

**p53-p21:** p53-p21 pathway; encompasses p21-dependent (p21) and p53-p21-interdependent pathways

**Wild-type cells:** low levels of proliferation may be due to both p21-RB and p53-p21 pathways being intact

- **Rb<sup>-/-</sup>:** similar levels of proliferation in Rb<sup>-/-</sup> and p53<sup>-/-</sup> cells may be due to the presence of the p21-p53 or p21-RB pathway, respectively. This suggests that these pathways can inhibit proliferation to the same extent. However, Rb<sup>-/-</sup> cells contain high levels of p53 and p21, and this pathway may therefore be more effective in Rb<sup>-/-</sup> cells than in cells which contain basal levels of p53 and p21 (e.g. wild-type cells). Much higher levels of proliferation in p21<sup>-/-</sup> cells than Rb<sup>-/-</sup> or p53<sup>-/-</sup> cells may be due to complete inactivation of the p53-p21 pathway and partial inactivation of the p21-RB pathway

- **p21<sup>-/-</sup>:** similar levels of proliferation in these cells may be due to the presence of either the RB-dependent or p21-dependent pathway only. This suggests that either basal levels of p21 or RB inhibit proliferation to the same extent, and that p53 has no effect on the ability of RB to inhibit proliferation i.e. no p53-RB-interdependent pathway

- **Rb<sup>-/-</sup>p21<sup>-/-</sup>:** Highest levels of proliferation in these cells due to loss of both p21-RB and p53-p21 pathways. Comparison with Rb<sup>-/-</sup> cells suggests that p53 can inhibit proliferation in the presence of p21 only
The above pathways can be grouped into two major pathways that can operate independently of each other: 1) a p21-RB pathway that encompasses the p21-dependent, the RB-dependent, and the p21-RB-interdependent pathways; 2) a p53-p21 pathway that encompasses the p21-dependent and the p53-p21-interdependent pathways. Both of these pathways are expected to be present in wild-type cells and proliferation is therefore lowest in these cells (Table 4.2), however, the relative contribution of each pathway to proliferation control is difficult to ascertain. Proliferation levels are the same in Rb−/− and p53−/− cells suggesting that the p53-p21 and p21-RB pathways can inhibit proliferation to the same extent. However, Rb−/− cells are peculiar in that they contain high levels of p53 and p21 (Table 4.2). The p53-p21 pathway may therefore be more effective in Rb−/− cells than in wild-type cells, which contain basal levels of p53 and p21. Both p53−/− and wild-type cells contain basal levels of p21 and RB, suggesting that the p21-RB pathway might be as effective in p53−/− cells as in wild-type cells. Therefore, it is possible that the p21-RB pathway contributes more to proliferation control in wild-type cells than the p53-p21 pathway.

Proliferation levels in p21−/− cells are much greater than those in Rb−/− or p53−/− cells, illustrating the critical importance of p21 in regulation of hepatocyte proliferation. This could be due to complete inactivation of the p53-p21 pathway and partial inactivation of the p21-RB pathway in p21−/− cells (Table 4.2). Similar levels of proliferation in p21−/−, Rb−/−p53−/−, and p53−/−p21−/− cells suggests that basal levels of p21 or RB can inhibit proliferation to the same extent. They also suggest that high levels of p53 have no effect on the ability of RB to inhibit proliferation (Table 4.2). The highest level of proliferation is observed in Rb−/−p21−/− and Trp−/− cells, perhaps due to loss of both the p53-p21 and p21-RB pathways. High levels of p53 cannot inhibit proliferation in Rb−/−p21−/− cells in contrast to Rb−/− cells. This suggests that the ability of p53 to inhibit proliferation is strictly dependent on p21 (Table 4.2).

As was stated above, the results suggest that there may be two major pathways that affect hepatocyte proliferation: a p53-p21 pathway and a p21-RB pathway (each consisting of both independent and interdependent pathways) (Figure 4.7, 1 and 2). p53 appears to inhibit S phase through p21 (1); p21 may inhibit S
phase through RB (2), or independently of RB and p53 (3); RB may inhibit S phase independently of p53 and p21 (4). The involvement of p21 in both of the potential major pathways suggests a critical role for p21 in regulation of hepatocyte proliferation.

Figure 4.7: Proposed regulation of Hepatocyte Proliferation

- p53
- p21
- RB
- S phase
4.3 Polyploidisation

4.3.1 Polyploidisation in Rb-deficient hepatocytes

Hepatocyte polyploidisation is a normal physiological process, and hepatocyte ploidy in normal mouse liver can range from 2n to 32n depending on age (Medvedev et al., 1986). Hepatocytes deficient in p53, p21, or Rb were found to contain enlarged nuclei compared with wild-type cells. To determine whether this increased nuclear size reflected an increase in DNA content, and to gain further insight into the cell cycle defects described above, nuclei from wild-type cells and cells deficient in Rb were subjected to Vindelov flow cytometric analysis after 48, 72, and 96 hours in culture (Figure 4.8). The percentage of diploid nuclei decreased over time in both wild-type (uninfected and Ad70) and Rb~'~ cells; the percentage of tetraploid and octaploid nuclei increased. The increase in the percentage of nuclei with tetraploid or octaploid DNA content was presumed not simply to represent G2 arrest as they continued to take up BrdU and were thus in S phase. This indicates that hepatocytes become progressively more polyploid in culture. At 120h the diploid and tetraploid populations were smaller, and the octaploid population greater, in Rb~'~ cells than in wild-type. Furthermore, a small percentage of Rb~'~ nuclei exhibited a 16n DNA content. This suggested that loss of Rb accelerates polyploidisation in hepatocytes. Adenoviral infection (Ad70) had no effect on polyploidisation (Figure 4.8).
Figure 4.8: Polyploidisation in Rb-deficient hepatocytes

Rb-floxed hepatocytes, infected or not with Ad70 or AdCre, were subjected to flow cytometric analysis of DNA content at various times during culture. The results are expressed as mean ± standard error of the mean for triplicate cultures from one Rb-floxed mouse. The experiment was performed three times with similar results. A decrease in diploid nuclei was accompanied by an increase in tetraploid and octaploid nuclei in both wild-type and Rb<sup>−/−</sup> hepatocytes, indicating that hepatocyte ploidy increases over time. The increase was much greater in Rb<sup>−/−</sup> hepatocytes, which exhibited a dramatic increase in the proportion of octaploid nuclei, and a small fraction of nuclei with a 16n DNA content. Adenoviral infection (Ad70) did not affect polyploidisation.
Figure 4.9: Polyploidisation in Rb-deficient hepatocytes

Flow cytometric analysis of DNA content and S phase in wild-type and Rb<sup>−/−</sup> cells after 120h in culture. The results are expressed as mean ± standard error of the mean for triplicate cultures from one Rb-floxed mouse. The experiment was performed three times with similar results.

A: A decrease in diploid and tetraploid nuclei, accompanied by an increase in octaploid and 16n nuclei, was observed in Rb<sup>−/−</sup> cells compared with wild-type.

B: Rb<sup>−/−</sup> cells exhibited much higher levels of S phase compared with wild-type, in agreement with measurement of S phase by BrdU incorporation.

C: In order to discount the varying levels of S phase between wild-type and Rb<sup>−/−</sup> nuclei, the values for individual 2n, 4n, 8n, and 16n populations were normalised with respect to these populations combined, excluding S phase populations. The result was similar to A, where S phase was not discounted.

D: Levels of S phase arising from diploid, tetraploid, and octaploid populations. A higher rate of DNA synthesis was observed in Rb<sup>−/−</sup> nuclei for each ploidy class.
To confirm the above findings, and to determine the proportion of cells in S phase, the 120h time-point was repeated since this was the time of greatest change in hepatocyte ploidy. As before, there was a decrease in the diploid and tetraploid populations concomitant with an increase in the octaploid population in \( Rb^{+/−} \) cells compared with wild-type (Figure 4.9.A). A small fraction of 16n \( Rb^{+/−} \) nuclei was also detected. S phase activity was estimated by measuring the percentage of cells between bands defining the 2n, 4n, 8n, and 16n peaks. A higher percentage of \( Rb^{+/−} \) nuclei were in S phase than wild-type (Figure 4.9.B). This is in agreement with the result obtained by BrdU incorporation. However, differences in the absolute values between the two techniques could be due to a combination of discrepancies: 1) BrdU incorporation is a more sensitive measure of DNA synthesis than flow cytometry. 2) BrdU incorporation was measured over a period of 6 hours, whereas FACS measures DNA synthesis at a particular moment in time only. 3) The BrdU results refer to the number of cells in S phase, whereas the FACS results refer to the number of nuclei in S phase.

In order to discount the varying levels of S phase between wild-type and \( Rb^{+/−} \) nuclei, the values for individual 2n, 4n, 8n, and 16n populations were normalised with respect to these populations combined, excluding S phase populations (Figure 4.9.C). Although the absolute values were different, the overall result remained the same, whether including S phase populations or not (compare Figure 4.9.A and 4.9.C). DNA synthesis was observed in nuclei of all ploidy classes in both wild-type and \( Rb^{+/−} \) nuclei, and a higher rate of DNA synthesis was observed in \( Rb^{+/−} \) nuclei for each ploidy class (Figure 4.9.D). Therefore, the results indicate that loss of \( Rb \) increases both DNA synthesis and polyploidisation in hepatocytes.
4.3.2 Polyploidisation in hepatocytes deficient in Rb, p53, or p21

DNA content in p53/<sup>−/−</sup>, p21/<sup>−/−</sup>, and Rb/<sup>−/−</sup>nuclei was analysed in triplicate by Vindelov flow cytometry after 120h in culture. As was previously found for Rb/<sup>−/−</sup>nuclei, there was a decrease in the diploid and tetraploid populations, concomitant with an increase in the octaploid population in p53/<sup>−/−</sup> and p21/<sup>−/−</sup>nuclei compared with wild-type (Figure 4.10.A). The increase in octaploid nuclei was similar in Rb/<sup>−/−</sup> and p53/<sup>−/−</sup>nuclei, but greater in p21/<sup>−/−</sup>nuclei. This is in accordance with the increased rate of DNA synthesis in these cells. At 120h, the rate of DNA synthesis in p21/<sup>−/−</sup> cells was lower than that in p53/<sup>−/−</sup> cells. This is in agreement with the BrdU results (compare Figure 4.10.A with Figure 4.6.A). The results were normalised as above, discounting S phase, and again, the overall result remained similar (Figure 4.10.B). Differences in the proportion of S phase nuclei between genotypes were consistent across each ploidy class (Figure 4.10.C). Thus, increased ploidy in the mutant cells may not be due to direct involvement of RB, p53, or p21 in regulation of polyploidisation itself, but simply a consequence of increased proliferation accelerating the normal polyploidisation process. If this were true, then polyploidisation should continue to increase in double and triple mutant cells with the increase in proliferation.
Flow cytometric analysis of DNA content and S phase in wild-type, p53<sup>−/−</sup>, p21<sup>−/−</sup>, and Rb<sup>−/−</sup> nuclei after 120h in culture. The results are expressed as mean ± standard error of the mean for triplicate cultures from the relevant transgenic mice. The experiment was performed twice with similar results. A: A decrease in diploid and tetraploid nuclei, accompanied by an increase in octaploid nuclei, as well as an increase in S phase, was observed in p53<sup>−/−</sup>, p21<sup>−/−</sup>, and Rb<sup>−/−</sup> nuclei compared with wild-type. Levels of S phase as determined by FACS are consistent with levels of S phase determined by BrdU incorporation. B: Values were normalised as before, discounting S phase, and the results were similar to A. C: Differences in the proportion of S phase nuclei were consistent across each ploidy class.
4.3.3 Polyploidisation in hepatocytes deficient in combinations of p53, p21, and Rb

To determine whether polyploidisation was simply a function of the proliferation rate, polyploidisation in single mutants (Figure 4.11.i.A) was compared with that in double (Figure 4.11.i.B-D) and triple mutant cells (Figure 4.11.i.E-G). The results were normalised as above, discounting S phase. Combined deletion of Rb and p53 led to an increase in octaploid nuclei compared with single mutants (Figure 4.11.i.B). Combined deletion of Rb and p21 led to a much greater increase in octaploid nuclei than deletion of either gene alone (Figure 4.11.i.C). Combined deletion of p53 and p21 slightly increased the percentage of octaploid nuclei compared with nuclei deficient in p53, but not p21 (Figure 4.11.i.D). Furthermore, combined deletion of p53, p21, and Rb increased the ploidy level compared with combined deletion of Rb and p53 (Figure 4.11.i.E), or p53 and p21 (Figure 4.11.i.F). Therefore, the level of ploidy in double and triple mutants increased with proliferation, as was the case for single mutants. This strongly suggested that the level of proliferation is an important determinant of the degree of polyploidisation, although a direct role for RB, p53, or p21 in regulation of polyploidisation cannot be ruled out. Indeed, despite having a similar level of proliferation, the level of ploidy was different for Rb<sup>−/−</sup>p21<sup>−/−</sup> cells and Trp<sup>−/−</sup> cells (ANOVA p<0.05) (Figure 4.11.i.G). The ploidy and proliferation data are summarised in Figure 4.11.ii.A-B. Figure 4.11.ii.C is a plot of ploidy against proliferation and clearly shows that ploidy increases with proliferation. It also shows that while the level of proliferation is similar, the level of ploidy is different, for Rb<sup>−/−</sup>p21<sup>−/−</sup> and Trp<sup>−/−</sup> cells.

4.4 Nuclear and mitotic abnormalities in hepatocytes deficient in p53, p21, or Rb

Cells deficient in p53, p21, or Rb were found to contain micronuclei and cytologically abnormal nuclei that were lobulated. The proportion of abnormal nuclei in Rb<sup>−/−</sup>, p53<sup>−/−</sup>, Rb<sup>−/−</sup>p53<sup>−/−</sup>, and wild-type cells was determined morphologically (Figure 4.12.A). Regardless of genotype, nuclear abnormality was low after 24 and 48 hours in culture, indicating that this phenomenon did not occur in vivo, and was perhaps a result of the strong proliferative signal provided in vitro (Figure 4.12.B). However, while nuclear abnormality remained low in wild-type cells throughout
culture, it increased dramatically in mutant cells between 72 and 120 hours of culture. The proportion of abnormal nuclei in \( Rb^{-/-} \) and \( p53^{-/-} \) cells was similar, and much greater still in \( Rb^{-/-}p53^{-/-} \) cells. In contrast to the normal mitotic process observed in wild-type hepatocytes (Figure 4.13.A), cells deficient in \( Rb \) or \( p53 \) exhibited abnormal mitoses, with cells containing multiple spindle poles, which may hinder the correct segregation of chromosomes (Figure 4.13.B). Thus, increased nuclear abnormality coincided with increased rates of DNA synthesis, abnormal mitoses, and polyploidisation.

**Figure 4.11: Polyploidisation in hepatocytes deficient in combinations of \( p53, p21, \) and \( Rb \)**

i) Flow cytometric analysis of DNA content in hepatocytes deficient in combinations of \( p53, p21, \) and \( Rb \), after 120h in culture. The values presented were normalised as before. A: see legend for Figure 4.6. B: Combined deletion of \( Rb \) and \( p53 \) led to an increase in octaploid nuclei compared with nuclei deficient in \( Rb \) or \( p53 \) alone. C: Combined deletion of \( Rb \) and \( p21 \) led to a much greater increase in octaploid nuclei than deletion of either gene alone. D: Combined deletion of \( p53 \) and \( p21 \) slightly increased the percentage of octaploid nuclei compared with nuclei deficient in \( p53 \), but not \( p21 \). E: Combined deletion of \( p53, p21 \) and \( Rb \) led to a greater increase in octaploid nuclei than that caused by deletion of \( Rb \) and \( p53 \). F: Combined deletion of \( p53, p21, \) and \( Rb \) led to a greater increase in octaploid nuclei than that caused by deletion of \( p53 \) and \( p21 \). G: Combined deletion of \( p53, p21 \) and \( Rb \) did not increase the percentage of octaploid nuclei compared with cells deficient in \( Rb \) and \( p21 \).

ii) Summary of polyploidisation (A) and proliferation (B) results in hepatocytes deficient in combinations of \( p53, p21, \) and \( Rb \). C: Plot of proliferation against ploidy. A, B, C: An increase in ploidy correlated with an increase in proliferation in all genotypes. \( Rb^{-/-}p21^{-/-} \) cells and \( Trp^{+/-} \) showed similar levels of proliferation but different levels of ploidy. ✪
Figure 4.11.i: Polyploidisation in hepatocytes deficient in combinations of *p53*, *p21*, and *Rb*
Figure 4.11.ii: Summary of polyploidisation and proliferation in hepatocytes deficient in combinations of p53, p21, and Rb
Figure 4.12: Nuclear abnormalities in hepatocytes deficient in p53 or Rb

A: Normal BrdU-positive nuclei indicated by thin arrows, abnormal BrdU-positive nuclei indicated by thick arrows. Original magnification: Left panel x630; Right panel x400. Nuclear abnormality was observed in both BrdU-positive and -negative nuclei. BrdU-positive nuclei chosen to show nuclear abnormality as it is easier to discern abnormal morphology compared with BrdU-negative nuclei.

B: The proportion of abnormal nuclei in hepatocytes isolated from the various transgenic mice. The experiment was performed twice with similar results.
Figure 4.13: Mitotic abnormalities in hepatocytes deficient in p53, p21, or Rb

A

Normal Mitotic process

<table>
<thead>
<tr>
<th>Stage</th>
<th>Image</th>
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<tbody>
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<td>Anaphase</td>
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<tr>
<td>Cytokinesis</td>
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Bipolar mitosis

Tripolar mitosis

B

Mitotic Abnormalities in mutant cells

<table>
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<tr>
<th>Cell Type</th>
<th>Image</th>
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<tbody>
<tr>
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<td><img src="mutant_cells.png" alt="Image" /></td>
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<tr>
<td>Rb&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<tr>
<td>p21&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<tr>
<td>p53&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td><img src="mutant_cells.png" alt="Image" /></td>
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A: Normal mitotic process as observed in hepatocytes. Hepatocytes can undergo normal tripolar mitoses accompanied by cytokinesis. Original magnification x630.

B: Examples of mitotic abnormalities observed in mutant cells, including multipolar spindle formation. Rb<sup>−/−</sup>p53<sup>−/−</sup> and Rb<sup>−/−</sup> cells are BrdU-positive, p21<sup>−/−</sup> and p53<sup>−/−</sup> cells stained with Feulgen's reagent. Original magnification x630.
Discussion

The aim of this section of work was to investigate the roles of p53, p21, and Rb in regulation of hepatocyte proliferation and polyploidisation by using cells deficient in one or more of these genes.

Activation of the p53 pathway in Rb-deficient and p21-deficient hepatocytes

Inactivation of Rb in hepatocytes resulted in p53 stabilisation and p53-dependent induction of p21. This is consistent with findings in the CNS of Rb\(^{-}\) mouse embryos (Macleod \textit{et al.}, 1996). Rb\(^{-}\) cells are expected to contain dysregulated levels of E2F-1, which have been shown to induce accumulation of p53 in some cell types (Hiebert \textit{et al.}, 1995; Pan \textit{et al.}, 1998; Tsai \textit{et al.}, 1998). E2F-1 induces expression of p19\textsuperscript{ARF} (DeGregori \textit{et al.}, 1997; Bates \textit{et al.}, 1998; Zhu \textit{et al.}, 1999), and p19ARF (hereafter referred to as ARF) promotes p53 stabilisation and activation by inhibiting the ability of MDM2 to target p53 for degradation (Kamijo \textit{et al.}, 1998; Pomerantz \textit{et al.}, 1998; Zhang \textit{et al.}, 1998; Honda \textit{et al.}, 1999). Therefore, ARF seemed a good candidate for the observed induction of p53 in hepatocytes. However, attempts to show upregulation of ARF in Rb\(^{-}\) hepatocytes proved inconclusive, perhaps due to the low level of ARF expression in hepatocytes (data not shown). Similarly, attempts to show upregulation of ARF in the CNS of Rb\(^{-}\) embryos proved negative, and it was suggested that this might have been due to the very low level of ARF expression in this tissue (Tsai \textit{et al.}, 2002). Importantly, ARF was subsequently shown to be dispensable for p53-induction in the CNS (Tsai \textit{et al.}, 2002), indicating that, upon Rb-inactivation, other pathways to p53-induction are involved, although these have not yet been elucidated.

Like Rb\(^{-}\) cells, p21\(^{-}\) cells contained increased levels of p53. While induction of p53 in p21\(^{-}\) lung epithelial cells has also been observed by others in this department (Renald Blundell, pers. comm.), the mechanism has not yet been determined. However, given the role of p21 in positive regulation of RB activity, it is reasonable to assume that loss of p21 might result in RB-inactivation, and that induction of p53 upon loss of RB or p21 is likely to occur via the same pathway.
Future work might involve investigation of the pathways leading to p53 stabilisation upon loss of Rb and p21.

**Regulation of Proliferation by p53, p21, and RB**

Analysis of cells deficient in p53, p21, or Rb indicated that each of these genes is involved in regulation of hepatocyte proliferation. Analysis of cells deficient in various combinations of all three genes provided insight into the extent of their interdependence in regulation of proliferation. The results suggest that both independent and interdependent pathways may be involved in regulating hepatocyte proliferation. These potential pathways include: 1) an RB-dependent pathway that is independent of p21 and p53; 2) a p21-dependent pathway that is independent of RB and p53; 3) a p21-RB-interdependent pathway that is independent of p53; 4) a p53-p21-interdependent pathway that is independent of RB.

These individual pathways can be grouped into two major pathways that may operate independently of each other: 1) a p21-RB pathway that encompasses the RB-dependent pathway, the p21-dependent pathway, and the p21-RB-interdependent pathway; 2) a p53-p21 pathway that encompasses the p21-dependent pathway and the p53-p21-interdependent pathway. The extent of the interdependence between these two major pathways could not be resolved here.

Loss of both Rb and p21 was previously shown to cause greater growth dysregulation than loss of either Rb or p21 alone (Brugarolas et al., 1998; Jiang et al., 2000). Loss of p21 was shown to accelerate tumour development in Rb+/− mice (Brugarolas et al., 1998). This is consistent with the present study's suggestion that RB and p21 may regulate proliferation both independently and interdependently of each other.

Although the downstream targets of RB-dependent and p21-dependent regulation of proliferation were not investigated here, they are likely to include E2Fs, PCNA, and cyclin-cdk5. The activities of these are required for proliferation, and they can be inhibited by RB and p21 independently of each other (see chapter 1, sections 1.1.3.a and 1.3.3.a for details). The ability of RB to inhibit proliferation requires inhibition of RB phosphorylation, which can be regulated by CKIs other than p21, such as p16 and p27 (Koh et al., 1995; Lukas et al., 1995; Medema et al., 1995).
1995; Sherr & Roberts, 1995). It would be interesting to investigate the role of p16 and p27 in regulation of RB phosphorylation in hepatocytes to determine if they contribute to the ability of RB to inhibit proliferation independently of p21. The ability of p21 to inhibit RB phosphorylation (Brugarolas et al., 1999), and the ability of RB to regulate p21 expression (Decesse et al., 2001), may contribute to a p21-RB-interdependent pathway.

RB and p53 were previously shown to cooperate to prevent tumourigenesis (Howes et al., 1994; Pan & Griep, 1994; Symonds et al., 1994; Williams et al., 1994; Marino et al., 2000), and in some cases, inhibition of tumourigenesis was due to p53-dependent apoptosis caused by inactivation of RB (Howes et al., 1994; Pan & Griep, 1994; Symonds et al., 1994). These findings are consistent with those presented here, in that loss of Rb and p53 led to additive defects in proliferation control. This was not due to loss of p53-dependent apoptosis, however, as apoptosis induced upon loss of Rb is p53-independent in hepatocytes (see chapter 5, section 5.3).

Despite high levels of p53 in \( p21^+ \) cells, loss of p53 did not increase proliferation, suggesting that p53 does not inhibit proliferation independently of p21. Given that p53 can regulate p21 expression, and that the ability of p53 to inhibit proliferation appears to be dependent on p21, it seemed reasonable to expect that p53 might inhibit proliferation via induction of p21. While this is true for \( Rb^+ \) cells, where p53 induced p21, it is not the case for wild-type cells. Therefore, in wild-type cells, regulation of proliferation by p53 does not appear to involve transcriptional regulation of p21. p53 has previously been shown to inhibit proliferation (Del Sal et al., 1995) and induce apoptosis (Caelles et al., 1994; Wagner et al., 1994; Haupt et al., 1995) in a manner that does not require p53 transcriptional activity. It has been proposed that p53 may use either transcriptional activation or direct protein-protein signaling to regulate proliferation and apoptosis depending on the cell type or cellular context (Levine, 1997). Therefore, p53 may inhibit proliferation via transcriptional regulation of p21 in \( Rb^+ \) cells but via direct protein signaling to p21 in wild-type cells. The mechanism of p53-mediated direct protein signaling is not yet known.
In the normal liver, the majority of hepatocytes are quiescent and consequently proliferation is low. In contrast, hepatocyte proliferation in vitro is much higher as hepatocytes are exposed to mitogenic factors including growth factors and serum, and this coupled with the isolation procedure, provides a strong proliferative stimulus that is usually absent in vivo. It would therefore be interesting to reproduce in vivo, the in vitro experiments performed here, using mice with liver-specific knock-outs of p53, p21, and Rb, to determine whether the pathways suggested here to be important for proliferation control in vitro, also control proliferation in vivo. Alternatively, the high rate of hepatocyte proliferation in vitro may more closely resemble the process of liver regeneration rather than homeostasis, and therefore it would be of interest to determine if the pathways controlling in vitro proliferation are also important for control of regeneration.

Given the roles of p53, p21, and RB in regulating hepatocyte proliferation, it is possible that increased proliferation that results upon loss of these proteins contributes to the development of hepatocellular carcinoma (HCC). RB is thought to be inactivated in most HCCs, either by Rb mutation (Murakami et al., 1991) or inactivation of p16 (Hui et al., 1998). In addition, expression of SV40 TAg in mouse liver, which inactivates both RB and p53, induced HCC, which was not suppressed by overexpression of p53. This implicated inactivation of RB in the development of HCC (Gillet et al. 2000) and suggested that RB might be a target for the treatment of HCC. p53 mutation is found in 42% of HCCs (Tannapfel et al., 2001) and is associated with decreased expression of p21 (Hui et al., 1998). This suggests that, as in inhibition of hepatocyte proliferation, p53 might act upstream of p21 to inhibit the development of HCC. Given that p21 (Wu et al., 1996; Hui et al., 2002), but not p53 (Gillet et al., 2000), strongly inhibits hepatocyte proliferation when overexpressed in mouse liver, suggests that p21, rather than p53, might be an effective target for the treatment of HCC.

The ability of p53 to inhibit tumour formation (Donehower et al., 1992; Purdie et al., 1994) does not appear to involve p21 (Brugarolas et al., 1995; Deng et al., 1995), suggesting that functions of p53 other than those involved in proliferation are important for its tumour-suppressing properties. Indeed, it has been shown that regulation of apoptosis (Howes et al., 1994; Pan & Griep, 1994; Symonds et al.,
and genomic stability (Livingstone et al., 1992; Yin et al., 1992; Harvey et al., 1993; Blount et al., 1994; Donehower et al., 1995) contributes to the tumour suppressing function of p53. Therefore, although p53 may require p21 to inhibit hepatocyte proliferation, it may inhibit the development of HCC independently of p21.

**Regulation of Polyploidisation by p53, p21, and RB**

Polyploidisation in hepatocytes is a normal physiological process that increases with age. However, the mechanism and biological significance of polyploidisation is poorly understood. It is thought to occur through repeated rounds of acytokineti
cmitosis to produce a binuclear cell, followed by nuclear fusion in a subsequent cell cycle. It involves modulation of both centrosome replication, and the normal couplings of S phase to M phase, and M phase to cytokinesis (Brodsky & Uryvaeva, 1977). Polyploidisation may play a role in the liver by allowing it to preserve its normal size without having to undergo mitosis, thereby preventing propagation of DNA damage and aneuploidy. However, polyploidisation may also allow cells to bypass cell cycle arrest and apoptosis, permitting their abnormal survival, which may result in malignancy. Endoreduplication is often associated with genomic instability, aneuploidy and neoplastic transformation.

*In vitro*, hepatocytes are exposed to strong proliferative stimuli that are usually absent *in vivo*. Therefore, increased proliferation could account for the greatly accelerated rate of polyploidisation observed *in vitro* compared with that *in vivo*, where hepatocytes are largely quiescent. Likewise, increased ploidy observed in each of the mutant genotypes examined with respect to wild-type could simply result from acceleration of the polyploidisation process due to the increased proliferation in these mutant cells. However, a direct role for p53, p21, and RB in regulation of polyploidisation cannot be ruled out. Indeed, cells lacking p53, p21, or Rb are capable of undergoing DNA synthesis in the absence of mitosis (Minn et al., 1996; Di Leonardo et al., 1997; Khan & Wahl, 1998; Notterman et al., 1998; Stewart et al., 1999; Ciciarello et al., 2001; Tsuiki et al., 2001). This phenomenon, known as mitotic slippage, results in polyploidisation (Hoyt et al., 1991; Khan & Wahl, 1998).
Therefore, it is possible that mitotic slippage may account for part of the increase in polyploidisation observed in the mutant genotypes compared with wild-type.

In exception to the pure increased proliferation $\Rightarrow$ increased ploidy relationship, $Rb^{+/p21}^{+/}$ cells contained a higher proportion of octaploid nuclei than $Trp^{+/}^{+/}$ cells, despite exhibiting similar levels of proliferation. Therefore, in these cells, it is possible that some other factor besides increased proliferation is responsible, at least in part, for the increase in ploidy. The presence of wild-type p53 in $Rb^{+/p21}^{+/}$ cells singled it out as a potential candidate for regulation of polyploidisation in the absence of $Rb$ and $p21$.

Overexpression of p53 in the absence of any exogenous stress has been shown to induce G2 arrest in a variety of cells types (Agarwal et al., 1995; Stewart et al., 1995; Flatt et al., 2000; Park et al., 2000). Regulation of the G2/M transition by p53 involves inhibition of cdc2, which is essential for entry into mitosis (Nurse, 1991). The transition is simultaneously inhibited by three transcriptional targets of p53 (see chapter 1, Figure 1.10). p21 inhibits cdc2 kinase activity directly, although less efficiently than other cdks (Harper et al., 1995), and indirectly via inhibition of cdk2 activity, which is required for activation of cdc2 (Guadagno & Newport, 1996; Moro et al., 1997). GADD45 dissociates cdc2 from its regulatory subunit cyclin B1 (Wang et al., 1999b; Zhang et al., 1999), and 14-3-3$\sigma$ sequesters cdc2 in the cytoplasm where it cannot induce mitosis (Hermeking et al., 1997; Chan et al., 1999). In addition, p53 represses transcription of cdc2 and cyclin B, a process that may involve p21 and RB (Flatt et al., 2000; Krause et al., 2000; Taylor et al., 2001). p53 can mediate G2 arrest independently of its effects on cdc2 activity via downregulation of Topoisomerase II, which is required during the G2/M transition where it orchestrates the higher order compaction of chromatin to form highly condensed mitotic chromosomes (Withoff et al., 1996; Wang et al., 1997b).

It was previously demonstrated that $\gamma$-irradiated cells containing high levels of p53 and inactive RB and p21 (which in this respect resemble the $Rb^{+/p21}^{+/}$ hepatocytes used here), did not arrest in G1. However, they did arrest temporarily at the G2/M transition and this was followed by polyploidisation (Bulavin et al., 1999). Similarly, $Rb^{+/p21}^{+/}$ hepatocytes express high levels of p53 that are perhaps capable
of inducing a G2/M delay, but neither a stable G2 arrest nor G1 arrest. Therefore, high levels of p53 may effectively block mitosis but not S phase in the absence of Rb and p21, and thereby contribute to the high level of ploidy in Rb^−/p21^−/− cells.

The significance of increased ploidy in mutant cells observed here is unclear. It may exacerbate the severe defects in S phase already present in these cells, and perhaps contribute to nuclear and mitotic abnormalities. Alternatively, regulation of polyploidisation may represent an integral part of proliferation control in dysregulated cells, whereby DNA synthesis proceeds but cells do not undergo mitosis. Disruption of p53, p21, or Rb leads to loss of proliferation control, which may, in part, be counterbalanced by an increase in ploidy limiting the multiplication of cells. This may be particularly important for p53, whose inability to inhibit proliferation independently of p21 may be counterbalanced by its ability to increase polyploidisation independently of p21.

**Nuclear and Mitotic Abnormalities in cells deficient in p53, p21, or Rb**

Nuclear and mitotic abnormalities in mutant cells coincided with high levels of proliferation and ploidy. This is consistent with findings in Rb^−/− chimeras, which displayed enlarged liver cells, and Purkinje neurons with pleomorphic, polyploid nuclei (Williams et al., 1994; Lipinski et al., 2001). Following DNA damage, cells lacking p53 or p21 were unable to undergo cytokinesis and exhibited abnormal and multilobulated nuclei with abnormal numbers of centrosomes (Bunz et al., 1998), or underwent transient G2 arrest, after which they reentered S phase in the absence of mitosis and became polyploid with nuclear abnormalities (Waldman et al., 1996). Similarly, hepatocytes deficient in p53, p21, or Rb exhibit abnormal, multilobulated nuclei and become highly polyploid and multinucleated. This may be due to defects in, or uncoupling of, S phase, mitosis, and cytokinesis.

p53 (Fukasawa et al., 1996; Mussman et al., 2000; Tarapore et al., 2001), p21 (Mussman et al., 2000), and RB (Meraldi et al., 1999) each regulate centrosome duplication, which is required for bipolar spindle formation and correct segregation of chromosomes during mitosis (Hyman & Karsenti, 1996), and is tightly coupled with DNA replication (Figure 4.14) (for review, see Tarapore & Fukasawa, 2000).
p53, p21, and RB are required for the coordinated regulation of centrosome and DNA duplication, essential for maintenance of genomic instability. p53 may regulate centrosome duplication independently of p21 and RB via GADD45.

If proliferating cells fail to coordinate centrosome duplication with DNA replication, this can lead to the formation of monopolar or multipolar spindles and abnormal segregation of chromosomes, which can cause a change in ploidy, as well as aneuploidy, and thus contribute to cancer formation. Centrosome hyperamplification is commonly observed, and is the major contributing factor for chromosome instability in human tumours (Pihan et al., 1998; Lingle et al., 1998; Carroll et al., 1999). Alternatively, defects in spindle formation could lead to an increase in ploidy via mitotic slippage. The fact that the increase in ploidy coincided with an increase in proliferation in each of the mutant genotypes examined, and that nuclear and mitotic abnormalities coincided with an increase in proliferation in the three genotypes examined for abnormalities, supports the theory that loss of coordination of DNA and centrosome duplication contributes to nuclear and mitotic abnormalities, as well as changes in ploidy, in hepatocytes.
Summary

Disruption of p53, p21, or Rb, and combinations thereof, caused an increase in hepatocyte proliferation, which was accompanied by nuclear and mitotic abnormalities and an increase in ploidy. It is hypothesised that failure to coordinate DNA synthesis and centrosome duplication could contribute to the increase in polyploidisation and nuclear and mitotic abnormalities observed in the mutant cells (Figure 4.15).

There appears to be two major pathways regulating hepatocyte proliferation: a p21-RB pathway and a p53-p21 pathway. These pathways may operate independently of each other, although the extent of their interdependence was not resolved here. The involvement of p21 in both pathways suggests that p21 is of critical importance in regulation of hepatocyte proliferation. RB and p21 may inhibit proliferation independently of each other, and of p53, however, p53 cannot inhibit proliferation independently of p21. Instead, high levels of p53 may increase polyploidisation in Rb\(^+\)p21\(^-\) cells, perhaps through its ability to inhibit mitosis but not S phase independently of RB and p21. Thus, under normal growth conditions, RB and p21 may inhibit proliferation primarily at the G1/S phase of the cell cycle, whereas, at least in the absence of p21, p53 may increase ploidy by acting at the G2/M phase (Figure 4.16).
Figure 4.14: Proliferation, Nuclear/Mitotic abnormalities, and Polyploidisation in hepatocytes

i)

Aberrant DNA synthesis

Centrosome Abnormalities

Multipolar Spindle formation

Aberrant mitotic exit

S phase

Ploidy

Abnormal chromosome segregation

Failure of cytokinesis

Abnormal nuclei/ multinucleation

p53, p21, and RB are involved in regulation of DNA synthesis and centrosome duplication. In the absence of p53, p21 or Rb, these processes become dysregulated and uncoordinated, which can cause mitotic defects resulting in either aberrant mitotic exit followed by DNA synthesis and an increase in ploidy, or abnormal chromosome segregation, leading to a failure in cytokinesis, nuclear abnormality, and ploidy.
There are two major pathways suggested to regulate proliferation in hepatocytes: a p53-p21 pathway (1) and a p21-RB pathway (2). The proposed p53-p21 pathway includes a p21-dependent pathway (3) and a p53-p21-interdependent pathway (1). The proposed p21-RB pathway includes a p21-dependent pathway (3), an RB-dependent pathway (4), and a p21-RB-interdependent pathway (2). p21 (3) and RB (4) may regulate proliferation independently of each other, and of p53. p53 does not regulate proliferation independently of p21 (1), but appears to regulate polyploidisation, perhaps via the G2/M checkpoint in Rb⁻/p21⁺ cells: in the absence of Rb and p21, cells might re-enter S phase without undergoing mitosis, resulting in increased ploidy (5). Increased ploidy could also be the result of aberrant DNA synthesis in cells lacking p53, p21, or Rb, and may limit multiplication of mutant cells (6). Loss of p21 (7) or Rb (8) activates the p53 pathway, which inhibits proliferation in Rb⁻ cells (1, 3) and increases ploidy in p21⁺ cells (5). Thus, under normal growth conditions, RB and p21 may exert their control over hepatocyte growth primarily at the G1/S phase of the cell cycle, whereas, in the absence of Rb and p21, p53 may act primarily at the G2/M phase to increase ploidy.
Chapter 5

Roles of p53, p21, and RB in regulation of the DNA damage response in primary mouse hepatocytes

p53, p21, and RB are critical components of a stress response pathway (including DNA damage) where stabilisation and activation of p53 leads to transcriptional activation of p21 and inhibition of RB phosphorylation, resulting in cell cycle arrest (the p53-p21-RB pathway). This chapter investigates the role of these proteins in regulation of hepatocyte responses to DNA damage.

Introduction

The p53-p21-RB pathway plays a key role in the cellular responses to DNA damage, which include cell cycle arrest, apoptosis and DNA repair (for reviews see Albrechtsen et al., 1999; Burns & El Deiry, 1999; Lakin & Jackson, 1999; Taylor & Stark, 2001). Arrest at the G1/S (Kastan et al., 1991; Hupp et al., 1995) or G2/M (Agarwal et al., 1995; Stewart et al., 1995; Bunz et al., 1998) transition phases of the cell cycle prevents DNA synthesis and mitosis in the presence of unrepaired chromosomal alterations, and propagation of mutations that could contribute to the progressive accumulation of genomic changes necessary for neoplastic transformation to occur. A more drastic response to DNA damage is the induction of apoptosis (Clarke et al., 1993; Lowe et al., 1993), which results in permanent removal of damaged cells. The decision to undergo growth arrest or apoptosis in response to DNA damage depends on the cell type, the severity of the damage, the presence of growth and survival factors, the level of p53 expression, oncogenic activation, and the checkpoint controls operative in a cell (see Sionov & Haupt, 1999 for review). In addition to regulating cell cycle arrest and apoptosis, p53 (Ford & Hanawalt, 1995; Smith et al., 1995; Wang et al., 1995; Prost et al., 1998a, 1998b; Therrien et al., 1999; Smith et al., 2000), p21 (Li et al., 1994a; Pan et al., 1995; McDonald et al., 1996; Savio et al., 1996; Sheikh et al., 1997; Shivji et al., 1998; Wani et al., 2000; Smith et al., 2000; Stivala et al., 2001; Therrien et al., 2001; Wani et al., 2002), and RB (Therrien et al., 1999; Smith et al., 2000), have been implicated
in regulation of DNA repair, although conflicting evidence has meant that their precise roles in repair remain controversial (see Albrechtsen et al., 1999 and McKay et al., 1999 for reviews).

After DNA damage p53 becomes stabilised, and its DNA binding and transactivation activity increases, due to post-translational modifications of the p53 polypeptide that include phosphorylations and acetylations (for review see Lakin & Jackson, 1999). Activation of p53 leads to activation of its target genes, which encode proteins involved in cell cycle arrest such as p21 (Brugarolas et al., 1995; Deng et al., 1995; Bunz et al., 1998), 14-3-3s (Hermeking et al., 1997), Gadd45 (Zhan et al., 1998), and pro-apoptotic proteins such as BAX (Miyashita & Reed, 1995).

5.1 Activation of the p53 pathway in response to DNA damage in primary mouse hepatocytes

The response to both UV-C- and γ-irradiation-induced DNA damage was previously investigated in hepatocytes (Bellamy et al., 1997b). UV induced both p53 stabilisation and transactivation, leading to p53-dependent cell cycle arrest and p53-independent apoptosis. In contrast, γ-irradiation did not induce p53 stabilisation, transactivation, or apoptosis, but did induce p53-dependent cell cycle arrest. Differences in the response to UV and γ-irradiation have been demonstrated in other cell types (Al-Mohanna et al., 2001). To extend the investigation into hepatocyte responses to DNA damage, including the role of RB and p21, hepatocytes were UV-C-irradiated, in order to examine both cell cycle arrest and apoptosis.

Hepatocytes were subjected to UV-C irradiation (10J/m²) and p53 and p21 protein levels were analysed by western blotting. p53 protein was dramatically increased following irradiation, and this was accompanied by an increase in p21 protein (Figure 5.1.A). p53⁺/⁻ cells exhibited basal levels of p21 following irradiation, indicating that UV-induced p21 is p53-dependent (Figure 5.1.A). Induction of p53 after UV-irradiation was also observed by immunofluorescence (Figure 5.1.B). To determine whether activation of the p53 pathway in response to DNA damage occurs in Rb⁺/⁻ hepatocytes, p53 and p21 protein levels were analysed before and after irradiation (Figure 5.1.C). As before, unirradiated Rb⁺/⁻ cells
exhibited increased levels of p53 and p21 compared with wild-type cells. After irradiation, \(Rb^{-/-}\) cells exhibited a further increase in p53 protein levels, although it was not possible to detect a corresponding increase in p21, perhaps due to high levels of p21 already present in non-irradiated controls (Figure 5.1.C). Similar to \(Rb^{-/-}\) cells, \(p21^{-/-}\) cells were shown to express elevated levels of p53 (Figure 4.1), which increased further upon UV-irradiation (Figure 5.1.D). Thus, activation of the p53 pathway in response to DNA damage appears to be intact in \(Rb^{-/-}\) and \(p21^{-/-}\) cells.
Figure 5.1: Activation of the p53 pathway in response to DNA damage

A

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(Hours after UV)

p53

p21

B

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(Hours after UV)

p53

C

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(Hours after UV)

wt

Rb⁻/⁻
Figure 5.1: Activation of the p53 pathway in response to DNA damage

A & B) Induction of p53 and p21 in wild-type hepatocytes after UV-irradiation

A: p53 and p21 protein levels were detected by western blotting, with anti-p53 antibody CM5 (Novocastro) and anti-p21 antibody M19 (Santa Cruz), on 10% and 14% tris-glycine gels, respectively. Lanes 1-9: 20μg of protein samples harvested from wt and p53−/− hepatocytes at the indicated times post-irradiation. p53 and p21 increased following irradiation; induction of p21 was p53-dependent. The experiment was performed three times with similar results.

B: p53 was detected by immunofluorescence using anti-p53 antibody CM5 (Novocastro), and a secondary antibody conjugated with Alexa 488 (Molecular Bioprobes), a very stable fluorophore. Original magnification x400. p53 increased following UV-irradiation.

C) Induction of p53 in Rb−/− hepatocytes after UV-irradiation

p53 and p21 protein levels were detected by western blotting as described in A. p53 increased following UV-irradiation.

D) Induction of p53 in p21−/− hepatocytes after UV-irradiation

p53 was detected by immunofluorescence as described in B. Original magnification x400. p53 increased following UV-irradiation.
5.2 Cell cycle arrest in response to DNA damage in primary mouse hepatocytes

Hepatocytes were UV-C-irradiated and DNA synthesis was assessed before and after irradiation by immunodetection of cells that had incorporated BrdU. Wild-type cells underwent cell cycle arrest in response to UV that was sustained over a period of 36h following irradiation. In contrast, \( Rb^{+/−}, p53^{+/−}, \) and \( p21^{+/−} \) cells underwent a transient cell cycle arrest after which DNA synthesis was resumed (Figure 5.2). Thus it appears that \( p53, p21, \) and \( RB \) are required to maintain UV-induced cell cycle arrest in hepatocytes.

**Figure 5.2: Cell cycle arrest in response to DNA damage**

DNA synthesis was assessed by BrdU incorporation into hepatocyte DNA. Open symbols: 10J/m²
BrdU positive cells are expressed as mean ± standard error of the mean for duplicate cultures from the relevant transgenic mice. After UV, wild-type cells underwent prolonged cell cycle arrest. In contrast, \( Rb^{+/−}, p53^{+/−}, \) and \( p21^{+/−} \) cells underwent transient arrest, after which they resumed DNA synthesis.
5.3 Induction of Apoptosis in response to DNA damage in primary mouse hepatocytes

Hepatocytes were UV-C-irradiated and apoptosis was assessed morphologically before and after irradiation. The level of apoptosis in non-irradiated cells is low regardless of genotype, although Rb-deficiency caused a small but significant increase in apoptosis compared with wild-type cells (ANOVA p<0.003), whereas p53- or p21-deficiency did not (Figure 5.3.A). Loss of p53 did not reduce apoptosis in Rb+/ cells (ANOVA p<0.450), suggesting that apoptosis caused by Rb-deficiency is p53-independent (Figure 5.3.B). After irradiation, apoptosis was induced to a similar extent in wild-type, Rb+, and p21+ cells, and to a much lesser extent in p53+ cells, suggesting that UV induces both p53-dependent and -independent apoptosis in hepatocytes (Figure 5.3.C). The results also suggest that loss of either Rb or p21 does not alter the susceptibility of hepatocytes to UV-induced apoptosis (Figure 5.3.C). To further examine regulation of the apoptotic response to UV, cells deficient in more than one gene were analysed. UV induced similar levels of apoptosis in Rb+/p21+ cells as wild-type cells and cells deficient in Rb or p21 alone, consistent with the idea that neither RB nor p21 protects against UV-induced apoptosis (Figure 5.3.D). However, Rb+/p53−, p53+/p21−, and Trpl+ cells exhibited levels of apoptosis that were intermediate between that of p53− (lowest) and that of Rb− and p21− cells (highest) (Figure 5.3.E). This suggested that RB and p21 protect against UV-induced p53-independent apoptosis.
Figure 5.3: Induction of Apoptosis in response to DNA damage

Apoptosis was assessed morphologically by Feulgen staining of DNA. Apoptotic cells are expressed as mean ± standard error of the mean for duplicate cultures from the relevant transgenic mice. A: In the absence of UV-irradiation, the level of apoptosis in p53<sup>-/-</sup> and p21<sup>-/-</sup> cells was not significantly different to wild-type cells, but was higher in Rb<sup>-/-</sup> cells. B: Apoptosis caused by Rb-deficiency was p53-independent. C: After irradiation, apoptosis was induced to a similar extent in wild-type, Rb<sup>-/-</sup>, and p21<sup>-/-</sup> cells, and to a much lesser extent in p53<sup>-/-</sup> cells, suggesting that UV indues both p53-dependent and -independent apoptosis. D: Combined deletion of Rb and p21 did not further increase apoptosis compared with cells deficient in Rb or p21 alone. E: Combined deletion of Rb and p53, p21 and p53, and Rb, p21 and p53, increased the level of apoptosis compared with cells deficient in p53 alone, suggesting that loss of Rb or p21 sensitises cells to p53-independent apoptosis.
Figure 5.3: Induction of apoptosis in response to DNA damage

[Graphs showing the induction of apoptosis over time for different cell lines with varying DNA damage.]
Discussion

The aim of this section of work was to examine the role of p53, p21, and RB in regulation of the DNA damage response in hepatocytes. This pathway is critical in regulating the response to a variety of genotoxic stimuli, including UV-irradiation, γ-irradiation, and chemotherapeutic drugs. The DNA damage response varies depending on the cell type, the source and severity of the damage, the cellular environment, and the checkpoint controls operative in a cell, and involves induction of cell cycle arrest, apoptosis, and DNA repair. The present study examined hepatocyte responses to UV-C-irradiation in terms of cell cycle arrest and apoptosis.

UV-induced arrest is transient in cells deficient in p53, p21, or RB

Roles for p53 (Kastan et al., 1992; Kuerbitz et al., 1992; Bunz et al., 1998; Brugarolas et al., 1999), p21 (Brugarolas et al., 1995, 1999; Deng et al., 1995; Bunz et al., 1998) and RB (Harrington et al., 1998; Brugarolas et al., 1999) in regulation of DNA damage-induced cell cycle arrest in response to γ-irradiation have been well established. While a requirement for p21 (Loignon et al., 1997) and RB (Harrington et al., 1998) in regulation of cell cycle arrest in response to UV-irradiation has also been shown, the role of p53 is somewhat controversial. Some studies have indicated a requirement for p53 (Loignon et al., 1997; Haapajarvi et al., 1999; Geyer et al., 2000; Al-Mohanna et al., 2001), while others have indicated the opposite (Gujuluva et al., 1994; Courtois et al., 1997; Bissonnette et al., 1998; Geyer et al., 2000). It is therefore likely that the role of p53 in mediating cell cycle arrest in response to UV-irradiation is cell-type specific. This study investigated the role of p53, p21 and RB in regulation of cell cycle arrest in response to UV-irradiation in hepatocytes.

After UV-irradiation, wild-type hepatocytes underwent p53 stabilisation and p53-dependent upregulation of p21 accompanied by sustained cell cycle arrest. Induction of p21 in response to UV in hepatocytes is therefore dependent on p53, in contrast to other cell types where both p53-dependent and -independent mechanisms of p21 induction exist (Loignon et al., 1997; Haapajarvi et al., 1999). Despite activation of the p53 pathway being intact in Rb−/− and p21−/− cells, these cells, along with p53−/− cells, underwent a transient cell cycle arrest only, after which they
reentered S phase. Thus, it appears that p53, p21 and RB are not required for the initiation, but are required for the maintenance of UV-induced cell cycle arrest in hepatocytes. This is consistent with findings in other cell types (Loignon & Drobetsky, 2002), and with the idea that transcription-dependent and protein synthesis-dependent arrest induced by the p53 pathway is implemented too slowly to account for the rapid arrest seen upon genotoxic stress (Bartek & Lukas, 2001).

Rapid induction of cell cycle arrest after UV could occur via several mechanisms. Firstly, DNA damage triggers a cascade of phosphorylation events, mediated by the ATM-Chk2 (IR) and ATR-Chk1 (UV) kinase pathways (see 1.2.4.a), independently of transcription and protein synthesis. Chk2/Chk1 phosphorylate the Cdc25A and Cdc25C phosphatases (Peng et al., 1997; Sanchez et al., 1997; Chaturvedi et al., 1999). Cdc25A phosphorylation leads to its ubiquitination, resulting in persistent inhibitory phosphorylation of cdk2, inhibition of cyclin E-cdk2 activity and cell cycle arrest in G1 or S phase (Mailand et al., 2000; Costanzo et al., 2000; Falck et al., 2001). Cdc25C phosphorylation results in its binding to 14-3-3 proteins (Peng et al., 1997; Sanchez et al., 1997), which prevents its translocation to the nucleus (Lopez-Girona et al., 1999; Kumagai & Dunphy, 1999), resulting in persistent phosphorylation of cdc2, inhibition of cyclin B-cdc2 activity, and G2 arrest. Secondly, DNA damage induced by γ-irradiation (Agami & Bernards, 2000) and cisplatin (Lan et al., 2002) was shown to trigger degradation of cyclin D1, allowing redistribution of p21 from cyclin D-cdk4 complexes to cyclin E-cdk2 complexes, leading to rapid cell cycle arrest independently of the p53 pathway. A similar pathway might be induced in response to UV. Thirdly, initiation of UV-arrest could also be due to the presence of transcription-blocking DNA lesions such as cyclobutane pyrimidine dimers, resulting in global inhibition of mRNA synthesis (Ljungman, 1999), independently of the p53 pathway.

**Rb-deficiency induces p53-independent apoptosis**

In the absence of DNA damage, the level of apoptosis in hepatocytes is low, however, Rb-deficiency sensitized cells to apoptosis that was p53-independent. Rb-deficiency has been shown to cause p53-dependent apoptosis in the CNS (Macleod et al., 1996), lens (Morgenbesser et al., 1994; Pan & Griep, 1994, 1995), retina (Howes
et al., 1994), and choroid plexus (Symonds et al., 1994), and p53-independent apoptosis in the PNS and skeletal muscle (Morgenbesser et al., 1994; Macleod et al., 1996; Jiang et al., 2000), of transgenic mice. Apoptosis induced by Rb-deficiency could be due to dysregulated levels of E2F-1, which can induce p53-independent apoptosis (Phillips et al., 1997; Irwin et al., 2000; Stiewe et al., 2000; Moroni et al., 2001), loss of transrepression of pro-apoptotic genes by the RB-E2F repressor complex (Hsieh et al., 1997), or inappropriate S phase entry (Lee et al., 1994; Tsai et al., 2002). Wild-type cells were shown to rescue their Rb-deficient neighbours from apoptosis, suggesting that RB may regulate the expression of survival factors (Robanus-Maandag et al., 1994; Williams et al., 1994; Lipinski et al., 2001).

**UV-induced Apoptosis is both p53-dependent and p53-independent**

A previous study in hepatocytes suggested that p53 mediates cell cycle arrest but not apoptosis after UV-induced DNA damage (Bellamy et al., 1997). This contrasts with the present study, which suggests that p53 mediates both cell cycle arrest and apoptosis after UV. However, in the previous study, the effect of UV (10J/m²) on cell cycle arrest was examined by irradiating cells that were either in S phase or entering S phase (66h), while the effect of UV on apoptosis was assessed by irradiating cells in G1 (24h). Cells underwent cell cycle arrest that was p53-dependent but did not undergo apoptosis. In contrast, the present study examined the effect of UV (10J/m²) on cell cycle arrest and apoptosis simultaneously, by irradiating cells that were either in S phase or entering S phase (between 66-72h). Cells underwent cell cycle arrest and apoptosis that were both p53-dependent.

The apparent discrepancy between these two studies could be due to the difference in timing of irradiation, and suggests that hepatocytes in S phase are more susceptible to UV-induced apoptosis than cells in G1. Indeed, several lines of evidence support this reasoning. Firstly, it has been suggested that apoptosis can result from inappropriate S phase entry (Lee et al., 1994; Gill & Windebank, 1998; Chauhan et al., 1999; Tsai et al., 2002). Secondly, transcriptionally active E2F-1 accumulates as cells enter S phase, and E2F-1 can cooperate with p53 to induce apoptosis (Wu & Levine, 1994). Induction of p53 during S phase may therefore result in apoptosis (Gottifredi et al., 2001), but not during G1 when E2F-1 is
repressed. Thirdly, DNA damage that is sustained whilst cells are undergoing replication might be more detrimental to the cell than if incurred during G1. Indeed, when DNA replication complexes encounter DNA lesions they can cause DNA double-strand breaks (DSBs), causing more severe and perhaps irreparable damage, culminating in apoptosis (Jackson, 2001). If cells in S phase were indeed more susceptible to apoptosis, cells deficient in p21 and Rb would be expected to undergo higher levels of apoptosis compared with wild-type cells due to a higher proportion of p21<sup>-/-</sup> and Rb<sup>-/-</sup> cells than wild-type cells being in S phase at the time of irradiation. This was not the case, however.

In addition to p53-dependent apoptosis, hepatocytes underwent p53-independent apoptosis. Potential candidates for the induction of p53-independent apoptosis include p73 and E2F-1, which are induced in response to UV independently of p53 (O’Connor & Lu, 2000; Chen et al., 2001), and are capable of inducing apoptosis (Hsieh et al., 1997; Phillips et al., 1997; Agami et al., 1999; Gong et al., 1999; Yuan et al., 1999). p53-independent apoptosis increased in the absence of Rb or p21, consistent with findings that both RB (Almasan et al., 1995; Haas-Kogan et al., 1995; Li et al., 1995; McKay et al., 1998; Wallace et al., 2001) and p21 (Waldman et al., 1996; Canman et al., 1995; Polyak et al., 1996; Bunz et al., 1999; McKay et al., 1998; Bissonnette et al., 1998; Komarova et al., 2000; Wallace et al., 2001) can protect against DNA-damage-induced apoptosis, and undergo cleavage in response to DNA damage (Janicke et al., 1996; Gervais et al., 1998; Zhang et al., 1999a; Wang et al., 2001). That the anti-apoptotic function of RB and p21 is revealed in the absence of p53 only, may be due to the fact that the strong pro-apoptotic function of p53 is sufficient to override the anti-apoptotic function of RB and p21 in hepatocytes, and that cells undergoing p53-dependent and -independent apoptosis are not two different subsets of cells. Consistent with these findings, p21 was previously shown to protect cells from UV-induced p53-independent apoptosis (Bissonnette et al., 1998), but not p53-dependent apoptosis induced by γ-irradiation (Brugarolas et al., 1995; Deng et al., 1995). Alternatively, the ability of RB and p21 to protect against p53-dependent apoptosis may be masked by the fact that Rb<sup>-/-</sup> and p21<sup>-/-</sup> cells contain higher levels of p53 than wild-type cells.
In some cell types, increased cellular sensitivity to DNA-damaging agents correlates with abrogation of cell cycle arrest and DNA repair (see Smith & Fornace, 1997 for review). It would be of interest to investigate the role of the p53-p21-RB pathway in regulation of DNA repair in hepatocytes to determine how defects in repair might impinge on regulation of cell cycle arrest and apoptosis. A greater understanding of the mechanisms underlying radiation sensitivity might allow the classification of tumours into those that would respond well to radiation treatment or not. For example, \( Rb^- \) and \( p21^- \) cells are often hypersensitive to DNA-damaging agents and therefore respond well to genotoxic drugs. However, \( Rb^- \) and \( p21^- \) hepatocytes are not more sensitive to p53-dependent apoptosis than wild-type cells. Therefore treatment of liver tumours deficient in \( Rb \) or \( p21 \) with genotoxic drugs might not prove effective.

**Summary**

Loss of \( p53 \), \( p21 \), or \( Rb \) compromised the ability of hepatocytes to maintain arrest following DNA damage, which may jeopardise effective DNA repair, allowing cells to resume cycling in the presence of damaged DNA. This defect is likely to encourage the propagation of carcinogenic mutations, genomic instability, and malignant progression.

Induction of apoptosis provides an alternative mechanism to cell cycle arrest by which damaged cells are kept in check. This apoptotic response was intact in \( Rb^- \) and \( p21^- \) cells but severely compromised in \( p53^- \) cells, and is likely to allow enhanced survival of \( p53^- \) cells bearing potentially deleterious mutations compared with cells deficient in \( Rb \) or \( p21 \), placing them at greater risk of malignant progression. Loss of \( Rb \) or \( p21 \) sensitised \( p53^- \) cells to apoptosis, perhaps helping to counteract the cell cycle arrest defect in these cells. Taken together, the results show that \( p53 \), \( p21 \), and RB are critically involved in regulation of hepatocyte responses to DNA damage, which include both cell cycle arrest and apoptosis (Figure 5.4).
UV induces p53 protein levels, which in turn induce p21 (1). RB (2), p21 (3), and p53 (4) induce cell cycle arrest. UV induces p53-dependent apoptosis (5), and p53-independent apoptosis (6). p21 (7) and RB (8) protect against UV-induced p53-independent apoptosis, and RB protects against p53-independent apoptosis in the absence of UV (9).
Chapter 6

Final Discussion

The aim of this thesis was to examine the role of p53, p21, and RB in regulation of hepatocyte proliferation and apoptosis. The significance of p53, p21, and Rb in normal cells is emphasized by the fact that most, if not all, human cancers carry mutations in these genes. Defining the precise roles of these proteins in regulation of cell proliferation and carcinogenesis has been complicated by the fact that experiments have often been carried out in tumour cells bearing mutations in additional genes. p53, p21 and RB have been extensively studied in a wide range of cell types, providing a wealth of information as to their functions. It has become obvious that these functions are often cell-type specific. Therefore, to better understand the mechanisms of liver-specific growth, and subsequently, how disruption of these mechanisms could lead to carcinogenesis, a thorough investigation of the role of p53, p21, and RB was carried out in hepatocytes. Specifically, this study employed primary mouse hepatocytes that contain inactivated alleles of p53, p21, and Rb, but do not harbour mutations in other genes. This provides a defined genetic system in which to study the specific impact of their inactivation on hepatocyte proliferation and apoptosis. Furthermore, by using cells deficient in one or more of these genes, the extent of their interdependence could also be ascertained.

Adenovirus-Cre mediated deletion of Rb-floxed sequences is highly efficient

The problem of embryonic lethality of Rb gene knock-out can be overcome using the Cre/LoxP system of conditional gene targeting. The first objective of this study was to establish and evaluate a system that allowed deletion of Rb-floxed sequences to inactivate the Rb gene in cultures of primary mouse hepatocytes. Cre recombinase-expressing adenovirus was successfully used to deliver Cre to hepatocytes. Infection efficiencies of 100% were obtained with MOI as low as 4. Infection with AdCre resulted in rapid and highly efficient (>95%) deletion of Rb-floxed sequences. Recombination was as efficient in floxed/floxed (Rb^{Flox/Flox}) cells as in floxed/null
cells, suggesting that homozygosity of the floxed sequence does not affect the efficiency of recombination. While adenoviral infection itself had no adverse effect on cell viability, proliferation or apoptosis, LacZ expression was toxic to hepatocytes. Use of alternative control and reporter vectors for in vitro and in vivo liver studies would therefore be appropriate. Expression of Cre accelerated S phase entry in hepatocytes both in the presence and absence of LoxP sites. Cre expression was previously shown to cause chromosomal abnormalities and growth inhibition (Loonstra et al., 2001; de Alboran et al., 2001; Silver & Livingston, 2001). Careful analysis of Cre-specific effects beyond its ability to mediate recombination is therefore necessary when using the Cre/LoxP system to analyse gene function.

A p21-RB pathway and a p53-p21 pathway may independently regulate hepatocyte proliferation

Having successfully inactivated the Rb gene, hepatocytes deficient in combinations of Rb, p53, and p21 were employed to investigate pathways involving these genes in regulation of hepatocyte proliferation and apoptosis, both in the absence and presence of DNA damage. In the absence of DNA damage, it appears that two major pathways may be involved in regulating hepatocyte proliferation: 1) a p21-RB pathway that can operate independently of p53 and 2) a p53-p21 pathway that can operate independently of RB. Each pathway includes both independent and interdependent axes: pathway 1 includes a p21-dependent pathway that is independent of RB, an RB-dependent pathway that is independent of p21, and a p21-RB-interdependent pathway; pathway 2 includes a p21-dependent pathway that is independent of p53, and a p53-p21-interdependent pathway. In other words, p21 may inhibit proliferation independently of RB and p53, RB may inhibit proliferation independently of p21 and p53, and p53 may inhibit proliferation independently of RB but not p21.

In the absence of DNA damage the level of hepatocyte apoptosis is of a similar level in all genotypes examined, except for Rb<sup>−/−</sup> cells. Inactivation of Rb is associated with induction of apoptosis that is p53-dependent or -independent, depending on the cell type (Morgenbesser et al., 1994; Macleod et al., 1996; Jiang et al., 2000). Despite containing high levels of p53, apoptosis due to inactivation of Rb
in hepatocytes is p53-independent. p21<sup>−/−</sup> cells also contain high levels of p53 that do not induce apoptosis. Therefore, the proliferation and apoptosis results suggest that high levels of p53 cannot induce apoptosis in Rb<sup>−/−</sup> or p21<sup>−/−</sup> cells, cannot inhibit proliferation in p21<sup>−/−</sup> cells, but can inhibit proliferation in Rb<sup>−/−</sup> cells, and that this is dependent on p21.

p53, p21, and RB regulate cell cycle arrest and apoptosis following DNA damage

Hepatocytes undergo both cell cycle arrest and apoptosis in response to UV-induced DNA damage incurred during S phase. These processes prevent the propagation of carcinogenic mutations and maintain genomic stability following DNA damage by allowing sufficient time to repair damaged DNA or by eliminating damaged cells. p53, p21, or RB are required to maintain a stable cell cycle arrest in response to UV-irradiation. Therefore, the transient cell cycle arrest observed in cells deficient in p53, p21, or Rb may not allow sufficient time for effective DNA repair. These cells may resume cycling in the presence of damaged DNA, placing them at risk of malignant progression.

UV-induced apoptosis is largely p53-dependent and RB-, p21-independent. p53<sup>−/−</sup> cells are therefore compromised in their ability to undergo both cell cycle arrest and apoptosis following DNA damage, perhaps placing them at greater risk of malignant progression than Rb<sup>−/−</sup> or p21<sup>−/−</sup> cells, in which the apoptotic response remains intact. There is a p53-independent pathway to apoptosis following UV, although this is much weaker than the p53 pathway. Loss of Rb or p21 increased the effectiveness of the p53-independent apoptotic pathway, suggesting that RB and p21 protect against p53-independent apoptosis.

As mentioned earlier, in the absence of DNA damage, Rb<sup>−/−</sup> and p21<sup>−/−</sup> cells express higher levels of p53 than wild-type cells, but do not undergo apoptosis. Following DNA damage, Rb<sup>−/−</sup> and p21<sup>−/−</sup> cells expressed even higher levels of p53 than wild-type cells, but underwent similar levels of p53-dependent apoptosis. This suggests that specific activation of p53 apoptotic activity by UV rather than an increase in p53 protein levels is required for an apoptotic response.
Aberrant proliferation is associated with increased polyploidisation and nuclear and mitotic abnormalities in hepatocytes deficient in p53, p21, or Rb

Polyploidisation in hepatocytes is a normal physiological process that increases with age, although the mechanism and purpose of this process is poorly understood. It involves modulation of both centrosome replication, and the normal couplings of S phase to M phase, and M phase to cytokinesis (Brodsky & Uryvaeva, 1977). In vitro, polyploidisation increases much more rapidly than it does in vivo. This may be due to the higher proliferation rate observed in vitro than in vivo, where hepatocytes are largely quiescent. In vitro, polyploidisation increased with the increase in proliferation in all mutant genotypes examined, suggesting that polyploidisation is indeed a function of the proliferation rate. However, other factors such as mitotic slippage, whereby cells lacking p53, p21, or Rb can undergo DNA synthesis in the absence of mitosis, may also contribute to polyploidisation. Indeed, in the absence of Rb or p21, p53 may increase polyploidisation above a level that might be expected for the level of proliferation in Rb⁻/⁻p21⁻/⁻ cells. p53 may therefore regulate polyploidisation independently of its ability to regulate proliferation. The mechanism by which p53 might regulate polyploidisation was not demonstrated here. However, it is possible that p53 regulation of the G2/M transition is involved (Taylor et al., 2001). Cells containing high levels of p53 and inactive RB and p21 were previously shown to undergo a temporary G2/M delay after which they continued DNA replication resulting in polyploidisation (Bulavin et al., 1999). It is possible that a similar situation occurs in Rb⁻/⁻p21⁻/⁻ hepatocytes containing high levels of p53, where p53 can block mitosis but not S phase in the absence of Rb and p21, resulting in high levels of polyploidisation.

Nuclear and mitotic abnormalities coincided with increased levels of proliferation and polyploidisation in mutant cells. In addition to regulating proliferation, p53, p21, and RB are involved in regulating centrosome duplication (Fukasawa et al., 1996; Meraldi et al., 1999; Mussman et al., 2000; Tarapore et al., 2001). Coordinated regulation of both centrosome and DNA replication is required for correct segregation of chromosomes. Failure to coordinate these two processes can result in nuclear and mitotic abnormalities as well as changes in ploidy. It is
therefore hypothesised that dysregulation of both proliferation and centrosome duplication could contribute to nuclear and mitotic abnormalities and changes in ploidy in cells deficient in p53, p21, or Rb.

**Implications for hepatocarcinogenesis**

Hepatocytes deficient in p53, p21, Rb, and combinations thereof, exhibit high levels of proliferation, ploidy, nuclear and mitotic abnormalities, and defective cell cycle arrest in response to DNA damage. Each of these factors is associated with the development of cancer. Despite being highly dysregulated, and containing high levels of p53 (Rb<sup>−/−</sup> or p21<sup>−/−</sup>), these cells did not undergo apoptosis in the absence of DNA damage. They may therefore continue to proliferate unchecked, becoming increasingly abnormal, and accumulating mutations that could contribute to the development of hepatocarcinogenesis.

The involvement of p21 in both of the major pathways suggested to regulate hepatocyte proliferation suggests that it might prove to be a more effective target for the treatment of hepatocarcinogenesis than RB or p53. Targeting of p53 may prove least effective given that it neither inhibits proliferation independently of p21 nor induces apoptosis in the absence of DNA damage. However, p53 may inhibit tumour development via mechanisms other than inhibition of proliferation, such as maintenance of genomic stability and induction of apoptosis following DNA damage. Genotoxic drugs have been successfully used to selectively target tumour cells that are often more susceptible to DNA damage-induced apoptosis than normal cells. Rb<sup>−/−</sup> and p21<sup>−/−</sup> cells were previously shown to be hypersensitive to DNA-damaging agents, however, Rb<sup>−/−</sup> and p21<sup>−/−</sup> hepatocytes were not more sensitive to DNA damage-induced apoptosis than wild-type cells. This suggests that genotoxic drugs may not prove effective in the treatment of hepatocarcinogenesis.
Future Work

The results presented in this thesis point to a number of important and exciting experiments. For example, reintroduction of p53, p21, or RB into the mutant cells employed here might provide an alternative method by which to examine the extent to which these proteins inhibit proliferation independently or interdependently of each other. Investigation of centrosome duplication would help to determine whether or not dysregulation of this process is associated with nuclear and mitotic abnormalities, as well as changes in ploidy, observed in mutant cells. Quantification of mitosis for each mutant genotype might help to determine whether inhibition of mitosis by p53 contributes to the increase in polyploidisation observed in Rb⁻/⁻ p21⁻/⁻ cells. It would be interesting to further investigate the mechanism of p53 stabilisation that occurs upon loss of Rb or p21, and to determine whether p53 phosphorylation or acetylation is involved. Finally, it would be of interest to determine whether or not the same pathways regulate proliferation in the absence or presence of DNA damage, and how defects in DNA repair may affect the cellular response to DNA damage.
Appendix

Solutions

Tissue Culture

Stock Solutions

**0.36mg/ml Dexamethasone**
1mg Dexamethasone (Sigma)
1ml absolute ethanol
27ml PBS
(store at -20°C in 500μl aliquots)

**10mg/ml DNase type 1**
100mg DNase type 1 (Roche)
10ml dH₂O
(store at -20°C in 500μl aliquots)

**25μg/ml EGF**
100μg EGF (Sigma)
4ml 1X BSA/Linoleic acid
(store at -20°C in 500μl aliquots)

**20X BSA/Linoleic Acid**
500mg BSA/Linoleic acid (Sigma)
5ml dH₂O
(store at -20°C in 5ml aliquots)

**1X BSA/Linoleic Acid**
250μl 20X BSA/Linoleic stock
4.75ml PBS

PBS
10X PBS (Gibco)

Percoll
20ml Percoll (Amersham Pharmacia Biotech)
2.2ml Hank’ buffered saline (Gibco)
Media

Liver Perfusion Medium
500ml Liver perfusion medium (Gibco)
500µl Gentamicin (Gibco)

Digestion medium
125ml Modified Chee's medium, pH 7.4 (Gibco: custom-made)
75mg collagenase type IV (Sigma)
500µl DNase type 1 (Roche)

Culture Medium
500ml Modified Chee's medium, pH 7.4 (Gibco: custom-made)
500µl Gentamicin (Gibco)
500µl Dexamethasone (Sigma)
5ml ITS (Gibco)
500µl EGF (Sigma)
1ml NaOH (Sigma)
10ml L-glutamine (Gibco)
10ml FCS (Sigma)

Collagen and Fibronectin coating of Slides and Plates

<table>
<thead>
<tr>
<th>Collagen</th>
<th>Fibronectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 ml 0.1M Glacial acetic acid</td>
<td>100ml PBS</td>
</tr>
<tr>
<td>100mg Collagen type 1 (Sigma)</td>
<td>1ml Fibronectin (Sigma)</td>
</tr>
</tbody>
</table>

Sterilise overnight by addition of 50ml chloroform (store at 4°C)

Fibronectin was allowed to adsorb overnight at 4°C and was aspirated prior to plating hepatocytes. Collagen was allowed to adsorb overnight at room temperature. Collagen was then aspirated, plates were washed twice with dH2O, allowed to air-dry, and stored at room temperature until required.

<table>
<thead>
<tr>
<th>Slide/Plate</th>
<th>Fibronectin/ Collagen</th>
<th>No. cells/volume plated</th>
<th>Volume used for Adenovirus infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-well chamber slides (Nunc)</td>
<td>0.85ml Collagen</td>
<td>0.13 million/1ml</td>
<td>300µl</td>
</tr>
<tr>
<td>24-well plate (Cellstar Greiner)</td>
<td>0.45ml Fibronectin</td>
<td>0.065 million/500µl</td>
<td>200µl</td>
</tr>
<tr>
<td>60mm plates (Cellstar Greiner)</td>
<td>2ml Collagen</td>
<td>0.52 million/4ml</td>
<td>1ml</td>
</tr>
</tbody>
</table>
Flow Cytometry

Citrate Buffer, pH 7.6
85.5g Sucrose
11.76g Trisodium citrate
50ml DMSO
Dissolved in 800ml dH2O, pH adjusted to 7.6, made up to 1L

Stock Solution, pH 7.6
2g Trisodium citrate (Sigma)
121mg Tris (Sigma)
1.044g Spermine tetrahydrochloride (Sigma)
2ml Nonidet P40 (Sigma)
Dissolved in 1.8L dH2O, pH adjusted to 7.6, made up to 2L

Solution A, pH 7.6
500ml stock solution
15mg Trypsin (Sigma)
(store at -20°C in 10ml aliquots, bring to room temperature before use)

Solution B, pH 7.6
500ml stock solution
250mg Trypsin inhibitor (Sigma)
50mg RNase A (Sigma)
(store at -20°C in 10ml aliquots, bring to room temperature before use)

Solution C, pH 7.6
500ml stock solution
208mg PI (Sigma)
500mg Spermine tetrahydrochloride
(store at -20°C in 10ml aliquots, bring to 0° before use)
Fixatives and Stains

Bouin’s Fixative
425ml Methanol
25ml Glacial acetic acid
50ml 40% Formalin

X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) Stain
20mM NaH₂PO₄
80mM Na₂HPO₄
1.3mM K₃Fe(CN)$_₆$
K₄Fe(CN)$_₆$
1mg/ml X-gal (Sigma)

10mg/ml MTT
50mg MTT (Sigma)
5ml PBS
Filtered and stored at 4°C

Immunocytochemistry

5M HCl
216ml stock HCl (11.6M)
dH₂O to 500ml

20% NRS/0.5% PBST (Blocking solution)
2ml normal rabbit serum (NRS)
7.95ml PBS
50μl Tween 20

5% NRS/0.5% PBST
500μl normal rabbit serum (NRS)
9.45ml PBS
50μl Tween 20

0.5% PBST
995μl PBS
50μl Tween 20
**Immunofluorescence**

20% Normal Serum/0.1% TBST (Blocking Solution)

2ml normal serum (NRS or NGS)
8ml 0.1% TBST

0.1% TBST

1ml Tween 20
TBS to 1L

**Western Blotting**

**Blocking Solution**

10g Marvel
100ml 0.1% TBST

**RIPA Buffer**

50mM NaCl
1% NP-40
12mM deoxycholate
3mM SDS
50mM Tris-HCl pH7.5
1 pellet protease inhibitor cocktail/10ml (Roche)
(store at 4°C for a few weeks)

**5X TBS**

200g NaCl
60.5g Tris
dH₂O to 4L, pH to 7.5 with HCl
dH₂O to 5L

**5X 0.1%TBST**

200g NaCl
60.5g Tris
dH₂O to 4L, pH to 7.5 with HCl
add 5ml Tween-20
dH₂O to 5L
Molecular Biology

**Dextran-sulphate hybridisation solution**

50g dextran-sulphate  
300ml dH$_2$O  
Heat to 65°C, stir  
(Store at -20°C in 30ml aliquots)

**1.25mM dNTP stocks**

125µl 100mM dATP  
125µl 100mM dCTP  
125µl 100mM dGTP  
125µl 100mM dTTP  
9.5ml dH$_2$O  
(Store at -20°C in 500µl aliquots)

**10mg/ml Ethidium Bromide**

1g ethidium bromide (Sigma)  
dH$_2$O to 100ml

**0.25M HCl**

10.8ml stock HCl (11.6M)  
dH$_2$O to 500ml

**Hybridisation Buffer**

15ml 20X SSC  
5ml 10% SDS  
30ml dextran-sulphate hybridisation solution  
(store at -20°C)

**LB medium**

10g bacto-tryptone (Difco)  
5g bacto-yeast extract (Difco)  
10g NaCl  
dH$_2$O to 1L  
Adjust the pH to 7.0 and autoclave to sterilise

**LB Agar**

10g bacto-tryptone (Difco)  
5g bacto-yeast extract (Difco)  
10g NaCl  
15g Bacto-agar (Difco)  
dH$_2$O to 1L  
autoclave to sterilise, cool to 55°C, add appropriate antibiotics, pour into sterile petri dishes
0.4M NaOH  
32g NaOH  
dH$_2$O to 2L  

**Phenol/chloroform/isoamyl alcohol (25:25:1)**  
100ml TE-saturated phenol  
100ml chloroform  
4ml isoamyl alcohol  

**10% SDS (sodium dodecyl sulphate)**  
10g SDS (Fisher)  
dH$_2$O to 1L  

**20X SSC (standard saline citrate)**  
17.53g NaCl  
8.82g sodium citrate  
dH$_2$O to 80ml, pH to 7.0 with HCl  
dH$_2$O to 100ml  

**2X SSC**  
10ml 20X SSC  
90ml dH$_2$O  

**SSC/SDS Washes**  
Wash 1: 2X SSC/1% SDS  
Wash 2: 1X SSC/1% SDS  
Wash 3: 0.5X SSC/1% SDS  
Wash 4: 0.25X SSC/0.1% SDS  
Wash 5: 0.1X SSC/0.1% SDS  

**10X TBE**  
216g Tris base  
110g Boric acid  
16.6g EDTA  
H$_2$O to 2L  

**TE Buffer**  
10ml Tris-HCl, pH 7.6  
2ml 0.5M EDTA  
dH$_2$O to 1L  

**1M Tris-HCl, pH 7.6**  
121.1g Tris base  
dH$_2$O to 800ml  
Adjust pH with HCl, dH$_2$O to 1L
Reference List


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chromosome aberrations in cerebral primitive neuroectodermal tumors with TP53 mutations. *Cytogeten Cell Genet* 83, 266-269.


Adenovirus-mediated Cre deletion of floxed sequences in primary mouse cells is an efficient alternative for studies of gene deletion

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ABSTRACT

This study evaluates the utility of Cre-expressing adenovirus for deletion of floxed genes in primary cells using primary murine hepatocytes. Adenovirus infection was very efficient, even at very low MOI (>95% infection at a MOI of 6) and did not reduce viability. High level LacZ expression was cytotoxic to hepatocytes but Cre expression had no effect on viability. Cre-mediated recombination was completed within a timespan that permits experimentation during primary culture (>95% recombination after 24 h), independently of the number of floxed alleles per cell. Recombination did not induce p53 or produce cytological nuclear abnormalities (even in polyplord cells). Contrary to expectation, deletion of DNA ligase 1 did not alter cell cycle progression, although Cre expression hastens entry to S phase from G1, independently of the presence of floxed sequences. We conclude that adenovirus-mediated deletion of floxed alleles in primary cells is a straightforward and highly efficient tool for conducting preliminary studies of conditional gene targeting. Primary cells have advantages of differentiation, relative purity and ease of experimentation within controlled conditions, while avoiding confounding problems encountered in vivo (i.e. target cell specificity, kinetics and level of recombination, and elicitation of inflammatory and immune responses). This system could help identify important phenotypic effects and design and interpret in vivo studies.

INTRODUCTION

The development of the Cre/lox system marks a milestone in mouse conditional gene targeting. In this approach, Cre recombinase excises a critical gene segment flanked by LoxP recognition sequences (floxed sequence) (1,2). However, to achieve successful tissue-specific, temporally controlled gene targeting using Cre/lox technology necessarily requires stringently regulated Cre expression in the strain with which floxed mice are mated. Indeed, while successful applications using Cre transgenic mice have been published, many failures and problems have also been reported (3). In particular, the desired tissue specificity and level and timing of induction are difficult to integrate using Cre transgenic lines: often mosaic expression, leakiness of the promoter or expression in unwanted tissues are reported and can confound the original purpose of the investigator. As an alternative to Cre transgenic mice, Cre-expressing adenoviruses have been used to overcome some of these problems (4,5). However, once again the system lacks the flexibility required for many studies: although target specificity can be achieved for some tissues by choice of the route of administration, many tissue types cannot be selectively infected. In theory, specific promoters can be inserted into the virus to achieve cell specificity, but the level of infection in vivo depends on the tissue. Finally, an important problem with this approach is the host immune and inflammatory response to adeno viral infection (6), which may remove the infected cells or itself cause tissue damage and disease, for example in the liver (7).

Therefore, whilst the benefits from in vivo study to assess the physiological function of a gene are clear, strong limitations remain to using Cre/lox technology for in vivo evaluations. The use of primary cells in culture allows baseline data and principles to be established in a regulated setting without the drawbacks of in vivo work, but still benefiting from relatively differentiated and representative cells. From the perspective of Cre/lox conditional targeting, immune responses are avoided, target cell specificity is achieved by the isolation method, the time of recombination is dictated by the time of infection, and many experiments can be achieved with a limited number of cells, reducing to a minimum the number of animals used in subsequent in vivo work. However, whilst a few studies have been published that use primary cells and Cre/lox technology, to our knowledge no systematic evaluation of the applicability to primary culture of Cre-expressing adenovirus has been reported. In the present study we show that infection of primary epithelial cells is very efficient, requiring a low multiplicity of infection (MOI) that avoids potential cytotoxicity. Furthermore, the interval from exposure to adenovirus to successful recombination is short, so the time available for biochemical or biological analysis is appropriate. Finally, we show that in vitro investigations revealed a significant effect of Cre recombinase on hepatocyte proliferation that would not readily have been detected in vivo.

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Materials and Methods

Adenovirus propagation and titration

All adenoviruses used in this study are replication-deficient (E1 deleted), serotype 5 adenovirus. AdCA35LacZ encodes for the LacZ reporter gene (Ad-LacZ) (8) and Ad-Cre for Cre recombinase (Ad-Cre) (5). Under control of the CMV promoter, the Cre recombinase virus Ad-d70-3 has no inserted transgene (Ad-T70) (9). Viruses were propagated in 293 cells and titrated using a plaque assay as previously described (10). Stock titrations were: Ad-LacZ, 1.3 x 10^10 (SEM 7.8 x 10^9); Ad-Cre, 3.1 x 10^10 (SEM 2.1 x 10^9); Ad-T70, 1.9 x 10^11 (SEM 8.3 x 10^10). Virus stocks were aliquoted and stored at -70°C.

Hepatocyte isolation, culture and adenovirus infection

Primary hepatocytes from adult mice (6–12 weeks old) were isolated by a two-step retrograde perfusion procedure as previously described (11). Hepatocytes were plated onto fibronectin-coated chamber slides (Lab-Tek) or 24-well tissue culture plates (Greiner) at 0.2–0.3 x 10^5 per cm² in serum-free modified Chee’s medium.

Adenovirus infections were performed 2–3 h after plating. Cells were incubated for 1 h at 37°C with a reduced volume of culture medium containing the virus at the appropriate concentration, then re-fed with fresh medium.

Mouse strains

Wild-type mice were from mixed backgrounds or littersmates of the floxed mice, depending on the experiment. ROSA 26 Flox-Stop-βgeo mice (12) (Flox-Stop LacZ) have a floxed stop sequence inserted between the proviral β-geo gene (Lacz-neomycin fusion protein) and its promoter, preventing LacZ expression. Upon recombination the stop sequence is excised, and β-galactosidase expressed. LIGI^Flox/– (13) bears one floxed ligase 1 allele and one null ligase 1 allele. Rp^Flox/Flox are homozygous for the Rp floxed allele, in which exon 19 is flanked by LoxP sequences (14).

MTT assay

 Cultures were incubated for 2 h in the presence of 1 mg/ml MTT [tetrazolium salt of 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide]. The medium was removed, the well dried and crystals dissolved in DMSO. Values are average ± SEM absorbance at 490 nm of triplicate experiments.

Quantification of apoptosis

Cells were stained using Feulgen staining and light green counterstain. Briefly, cells were fixed in Boom’s fixative at 4°C overnight. Slides were treated with 5 M HCl for 45 min then stained with Schiff reagent. Apoptosis was quantified according to morphology. Results are the percentage ± SEM of 500 cells counted in duplicate. Experiments were performed three times with similar results.

BrdU immunocytochemistry

Hepatocyte cultures grown on chamber slides were incubated with 40 μM BrdU (Amersham) for 6 h and fixed in 80% ethanol. Immunodetection of BrdU incorporation was performed as previously described (11) using rat anti-BrdU IgG (1/100 dilution) (Sera Labs, Sussex, UK) as primary antibody and rabbit anti-rat IgG HRP conjugate (1/100 dilution) as secondary antibody. Slides were counterstained with haematoxylin and light green. Negative controls omitted the primary antibody. BrdU incorporation was estimated by counting positive cells. Results are the percentage ± SEM of 500 cells counted in duplicate. Experiments were performed three times using cells isolated from different mice strains with similar results.

Western blotting for Cre recombinase expression

Total cell protein (2 μg) was prepared from primary hepatocytes using RIPA buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, protein inhibitor cocktail pellet) and subjected to SDS-PAGE on a 12% gel, transferred to nitrocellulose and probed with an anti-Cre rabbit polyclonal antibody (1:10 000) (no. 69050-3; Novagen) and secondary antibody donkey anti-rabbit HRP conjugate (1:2000) (sc-2313; Santa Cruz). Proteins were visualized by ECL.

Cre-mediated recombination

Cre-mediated recombination of the floxed allele was quantified by Southern blotting and PCR. Cells were lysed for DNA extraction various times after infection.

For LIGI^Flox/–, EcoRI digests were probed with a 1.2 kb EcoRI–HindIII genomic fragment. The 8.5 and 3.4 kb bands correspond to the floxed and the recombined allele, respectively. Quantification was by densometric analysis using a phosphorimager. Analysis was completed using Aida 2.0 software. Recombination was expressed as the percentage difference between the 8.5 and the 3.4 kb signals relative to the 6.7 kb band corresponding to the null allele.

For Rp^Flox/Flox, Psfl digests were probed with a 450 bp Psfl–PvuII probe isolated from pHA153 (15). The 5 and 4.5 kb bands correspond to the floxed and the recombined allele, respectively (16). PCR analysis for recombination of the Rp^Flox allele used oligonucleotides Rb19 (5’-AACAAGGGAGGACCTG-3’) and Rb18 (5’-GGGTGTTGCCATCAATG-3’) as previously described (16). Thermocycling was as follows: step 1, 4 min at 94°C; step 2, 35 cycles of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C; step 3, 10 min at 72°C. Amplified products from Rp^Flox, wild-type and recombined alleles were separated on a 1.8% agarose gel, giving bands of 748, 680 and 300 bp length, respectively.

Statistical analyses

Analyses were done with Minitab for Windows v.13.0. For counts of BrdU, p53, apoptosis and abnormal nuclei, the proportion of affected cells was arcsine transformed and differences between means were evaluated by ANOVA. Differences were taken to be significant at P < 0.05. Satisfactory homogeneity of variances was determined with Bartlett's test. Where a significant difference between means was identified by ANOVA, the differences between individual means were analysed further with Bonferroni simultaneous tests for making multiple comparisons. For MTT data, the differences between medians were analysed with the Kruskal–Wallis test.
RESULTS

Adenoviral infection of primary hepatocytes is highly efficient and has no effect on cell viability

We used an adenovirus expressing the reporter gene LacZ to assess the efficiency of infection in primary epithelial cells. We found that a MOI as low as 4 is sufficient to infect >95% of primary hepatocytes. Similar results were obtained at our centre using Clara primary lung cells (J.M.Sallenave, personal communication) and by ourselves with primary murine renal epithelial cells (infection >95% at MOI 2, >98% at MOI 6) and epithelial colon cells (infection >95% of cells growing out of isolated crypts).

The impact of adenovirus infection and gene expression on hepatocyte health and viability was assessed with the MTT assay of mitochondrial metabolic activity over a period of 120 h. Cells infected at MOI 6 with adenovirus expressing LacZ (Ad-LacZ) or expressing Cre-recombinase (Ad-Cre) were not significantly different from the uninfected control, suggesting that neither adenovirus infection nor overexpression of an exogenous protein (Cre or β-galactosidase) had a significant effect on primary hepatocytes (P = 0.102, Kruskal–Wallis test) (Fig. 1). However, at higher MOI (MOI 20) infection with Ad-LacZ significantly decreased MTT staining compared with infection with the control adenovirus or Cre-expressing adenovirus (P < 0.0001, Kruskal–Wallis test). This suggests that high levels of expression of β-galactosidase but not Cre recombinase are toxic to hepatocytes (Fig. 1).

To investigate whether Cre-mediated recombination could cause hepatocyte apoptosis, we used wild-type and Floc-Stop LacZ primary hepatocytes infected with either Ad-70 or Ad-Cre. There were no significant differences in rates of apoptosis between the adenovirus treatments and untreated controls for cells of either genotype (P = 0.659 and P = 0.554 for Floc-Stop and wild-type, ANOVA) (a representative experiment is shown in Fig. 2). Thus there is no evidence that Cre-mediated recombination causes apoptosis.

Adenovirus-mediated expression of Cre recombinase is rapid and accompanied by effective recombination

Expression of Cre recombinase was detected by western blotting 6 h after infection and remained high through to 96 h (Fig. 3). Cre-mediated recombination was tested in hepatocytes isolated from mice bearing three different floxed sequences. Firstly, with Floc-Stop LacZ primary hepatocytes, recombination excised the stop sequence, permitting expression of β-galactosidase. X-gal staining showed that >95% of exposed cells underwent recombination within 48 h after infection with Ad-Cre. Next, the kinetics of recombination were analysed with heterozygous and homozygous hepatocytes isolated from two different strains of mice (LIG1Flox/− and RbFlox/−). Recombination of the floxed LIG1 allele in primary hepatocytes heterozygous for ligase 1 (LIG1Flox/−) was quantified by Southern blotting. Recombination occurred between 12 and 24 h after infection with Ad-Cre (MOI 6), with >95% deletion of the floxed allele (Fig. 4). A similar level of recombination was observed with MOI as low as 2. Recombination in primary hepatocytes homozygous for a floxed Rb allele (RbFlox/Flox) was quantified by both PCR and Southern blotting. Again, there was >95% recombination (deletion of the floxed allele), starting as early as 6 h after infection (Fig. 5). With both genotypes, recombination was complete within 24 h after infection, suggesting that homozygosity of the floxed sequence does not affect the kinetics of recombination. Moreover, most primary murine hepatocytes are polyploid (>80% of cells) (11),
Ad-Cre infection.

after

4. DNA ligase recombination.

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tissues) and that the indicated times after

(Base and bottom, respectively)

Lanes show hours after infection with Ad-Cre at an MOI of 6.

Figure 4. DNA ligase recombination. Southern blot analysis of the LigI locus after Ad-Cre infection. (Top) Duplicate infections at increasing MOI (0–10). (Bottom) Lanes show hours after infection with Ad-Cre at an MOI of 6.

Figure 5. Kinetics of recombination of Rb fused, Southern blotting and PCR (top and bottom, respectively) showing the floxed and the recombined bands at indicated times after infection.

frequently with 4 and as many as 32 floxed alleles per cell. Hence, recombination between floxed sequences located on different chromosomes could be more likely than in diploid cells. However, no abnormal band was detected by Southern blotting in either genotype tested and LacZ expression was observed regardless of the cell ploidy, as assessed by the size of the nuclei, in Flox-Stop LacZ cells after infection with Ad-Cre.

It has also been reported that Cre recombinase may induce chromosomal abnormalities (17) that could lead to increased occurrence of abnormal mitosis and nuclear abnormalities. However, no abnormal mitoses were observed and the proportion of cytologically pleomorphic nuclei was similar between wild-type and Flox-Stop cells infected with Cre-expressing or control adenoviruses (P = 0.164, ANOVA) (Fig. 6). Taken together, these results suggest that the Cre/lox technology can be reliably used in polyploid cells (which are present in many tissues) and that the timing of recombination in vitro is appropriate for use in primary cultures even with primary cells with reduced lifespans in culture, such as the primary hepatocytes used in the present study (10–14 days).

Adenovirus-mediated expression of Cre recombinase does not stabilise p53

p53 is a key protein for induction of cell cycle arrest and apoptosis in response to even very low levels of DNA damage (18). Potentially, therefore, induction of p53 by Cre-mediated recombination, which involves the creation of DNA strand breaks, could trigger unwanted cellular responses resulting from Cre-mediated targeting. We therefore investigated whether Cre-mediated recombination induced p53. Flox-Stop hepatocytes were isolated, infected with the various adenoviruses, and p53 stabilisation quantified by immunocytochemistry. There were no significant differences in p53 immunopositivity between uninfected cells, dl70 or Cre-infected hepatocytes (P = 0.92, ANOVA) (Fig. 7), suggesting that Cre-mediated DNA breaks during recombination are not recognised as DNA damage.
Adenovirus-mediated expression of Cre recombinase accelerates entry into S phase, but DNA ligase 1 deletion has no effect on DNA synthesis

Isolation of hepatocytes from the liver stimulates them to enter the cell cycle, modelling liver regeneration. Cultured primary hepatocytes enter the cell cycle in a more or less synchronous manner, reaching an S phase peak 72 h after isolation (11). Time courses of BrdU immunopositivity 0–144 h after adenovirus infection showed no significant differences in mean BrdU between uninfected controls, adenovirus (dl-70) infection, Cre expression or Cre-mediated recombination (ANOVA). This suggests that there were no significant differences in the total number of cells entering S phase over the time course. However, the ANOVA showed a significant interaction between adenovirus treatment and time (P < 0.0001), which suggests that the different adenovirus treatments behaved differently with time. Indeed, Cre-expressing adenovirus infection appeared to increase proliferation early (48 h) after infection, compared with other treatments, but not later (see for example Fig. 8). This effect was consistently apparent in different experiments regardless of genotype (wild-type, Flox-Stop or LIGIfloxed). Indeed, post hoc statistical analysis on 48 h time points of four experiments suggested that Cre does indeed produce an early and briefly increased BrdU positivity compared with dl-70 or uninfected controls (P < 0.0001 and P < 0.0001, respectively; ANOVA with Bonferroni simultaneous tests). We performed an additional experiment to evaluate this effect, concentrating on early time points (48 h after plating), which gave similar results (data not shown). To test whether expression of Cre recombinase from a different vector had similar results, we attempted transfection (lipofection) of a plasmid encoding for Cre recombinase under control of the CMV promoter; however, no meaningful results were obtained due to a very low transfection efficiency (data not shown). The increased proportion of cells in S phase at 48 h in cells expressing Cre, but without significant differences in overall mean BrdU over the time course, suggests that Cre recombinase shortens G1 in primary hepatocytes (i.e. hastens entry into S phase), rather than increases the total number of cells entering S phase.

There was no significant difference in overall mean BrdU immunopositivity between cultures after adenovirus-induced deletion of ligase 1, in dl-70-infected cells and in uninfected controls (P = 0.668, ANOVA). Hence, DNA ligase 1 does not appear, at least in hepatocytes, to be necessary for a normal pattern of DNA synthesis after stimulation to proliferate.

DISCUSSION

Cre/lox technology using adenovirus-mediated Cre expression can be used efficiently in primary cells

We have shown here that inducible gene targeting using adenovirus delivery of Cre to primary cells is efficient, rapid and without significant cytotoxicity. Rapid accumulation of reliable preliminary data on phenotypes is therefore possible, free of many confounding factors encountered in vivo: non-specificity and a lower rate of target cell infection, the timing of recombination and deletion of infected cells due to innate and immune responses. In this way, therefore, primary cell data can define and refine in vivo experiments and possibly give insights into the in vivo phenotype by providing a controllable and flexible study system. The high level of infection at low MOI observed in the different primary cells tested here and in other reports (pancreatic acinar cells (19) and breast epithelial cells (20)) shows that this technology is applicable to many epithelial cell types. We focused on liver cells for three main reasons. First, primary hepatocytes are commonly used in a variety of toxicological and environmental investigations, as well as cell cycle studies. Second, primary hepatocytes typically exhibit a reduced lifespan in vitro (10–14 days), making it a good model to investigate feasibility of Cre recombination studies. Third, although the liver is relatively easily and specifically infected in vivo with adenovirus to achieve conditional gene targeting (4,6,21–24), the development of hepatitis due to adenoviral infection is a major confounding factor to studies of liver cell biology (6,7,25,26). It must be acknowledged that for other study purposes hepatitis may not present a major problem, for example, a recent investigation of the consequences for blood pressure of deletion of a gene expressed in the liver (floxed human angiotensinogen transgene) (6).

β-Galactosidase is toxic to hepatocytes

The present findings that LacZ expression was toxic to hepatocytes support and extend in vivo observations of liver toxicity with high doses of adenovirus expressing LacZ, but where the effect of LacZ itself could not be clearly separated from toxicity due to immune or inflammatory responses to adenovirus infection (23,27). We have shown here that neither adenovirus infection nor overexpression of Cre recombinase is significantly cytotoxic, whereas β-galactosidase expression leads to lysis of primary hepatocytes in vitro infected with adenovirus at high MOI. We have observed similar lysis in hepatocytes when β-galactosidase is expressed at a high level from a transfected (lipofection) expression plasmid (S.Prost, unpublished observation). This is an important observation because in vivo direct toxic lysis of cells that express β-galactosidase could stimulate and exacerbate immune reactions to other adenovirus-infected hepatocytes (7). Indeed β-galactosidase
is known to be very immunogenic (27,28). Although Takeuchi et al. (29) did not observe a toxic effect of β-galactosidase in primary hepatocytes infected at an MOI of 24, the human CMV promoter used in that study is a much weaker promoter in mice than the mouse CMV promoter used here (8). Taken together, the data suggest that a high concentration of β-galactosidase is toxic to hepatocytes and could induce or at least exacerbate immune responses and hepatitis development after adenovirus infection in vivo. The use of an alternative reporter gene for liver studies would therefore be merited.

**Polyplody does not reduce the efficiency of Cre-mediated recombination or precipitate inappropriate recombination events**

One concern about the Cre/lox technology is the possibility of unintended inter-allelic rearrangements when there are more than two intra-chromosomal LoxP sites; it has been reported that Cre recombinase under a strong promoter produced illegitimate chromosome rearrangements in haploid mouse spermatids (17), even without LoxP sites, suggested to be due to recombination between pseudo-LoxP sites (30). We did not formally investigate chromosomal rearrangement, but Cre expression in wild-type or Flox-Stop LucZ cells did not produce abnormal mitoses or nuclei. Furthermore, no band of unexpected size was detected by Southern blotting in either LIG1<sup>Flox</sup> or Rb<sup>h3a</sup> cells after Cre expression, suggesting that no inappropriate inter-chromosomal recombination events took place at detectable level in these cells, which are mostly polyploid (>80%). However, a low percentage of recombination involving pseudo-LoxP and LoxP sites cannot be excluded.

One possible explanation is that the distance between LoxP sites is an important determinant of the probability of recombination. Indeed, recombination between LoxP sites on different chromosomes has been shown to occur at much lower frequency than intra-allelic recombination (31). Furthermore, whilst Cre recombinase may act at some pseudo-LoxP sites in the setting of a plasmid (30), recombination has not been shown between a pseudo-LoxP and a LoxP site and the frequency and distribution of pseudo-LoxP sites in the mammalian genome has not reported (30). The degree and duration of Cre expression are also factors likely to influence the probability of illegitimate chromosome rearrangement (17). In the present study Cre recombinase was under the control of a strong CMV promoter. However, the study was carried out on cells in culture for ≤144 h, corresponding to a maximum of two cell cycles. This may not be enough to induce recombination from rare, less specific sites. In addition, the CMV promoter is rapidly switched off in hepatocytes (32,33), which is likely to reduce illegitimate recombination events.

It has also been suggested that recombination would be more efficient with Floxed/deleted mice than with homozygous Floxed animals, as only one allele needs to be recombined (34). However, the use of heterozygous Floxed/deleted mice may be impractical or undesirable if heterozygosity of the gene of interest has a phenotype (e.g., Rb). The present data suggest that even with polyploid cells that bear several copies of each allele there was no appreciable alteration of the kinetics of recombination.

Ligase 1 is not required for DNA synthesis but Cre affects proliferation, even in the absence of LoxP sites

We report for the first time the phenotype of deletion of DNA ligase 1 from adult hepatocytes. Homozygous germ line deletion of DNA ligase 1 is embryonic lethal (35,36), so conditional gene targeting strategies are necessary to investigate the role of DNA ligase in adult somatic cells. There are suggestions that ligase 1 is critical for DNA replication (36 and references therein), however, previous work in our laboratory with a mammary gland-specific Cre transgenic failed to establish any effect of ligase 1 deletion on mammary epithelial cell proliferation (13; unpublished data). Here we have tested the hypothesis that ligase 1 is important for hepatocyte DNA synthesis. The synchronised entry into the cell cycle of primary hepatocytes in culture is an ideal system to address this question. The results indicate that, as in the mammary gland, ligase 1 is not a prerequisite for DNA synthesis.

The observation that Cre expression itself, regardless of the presence of floxed sequences, hastened the onset of DNA synthesis in hepatocytes after stimulation to proliferate is intriguing. The mechanism and its consequences for other studies using Cre recombinase are not clear. However, the observation would not have been as readily made in vivo without studies specifically designed to test liver regeneration and requiring considerably more animal resources.

In conclusion, we have shown that the Cre/lox technology using adenovirus infection in primary cells is an easy, reliable study system which can provide interesting and complementary information to in vitro studies. The cell specificity is defined by the isolation technique and the timing of Cre expression by the time of infection in vitro, there are no additional adverse effects due to the infection and important preliminary data can be readily accrued. Although in vivo studies are indispensable for physiological interactions, use of primary cells may reduce the number of transgenic animals required and may highlight or help define complex phenotypes.

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