Molecular Basis of Cell Cycle Control: p300 and pRb

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Abstract

The retinoblastoma protein (pRb) and p300 are transcriptional co-repressor and co-activator respectively, playing key roles in regulating the cell cycle. Both molecules are targeted by a number of viral oncoproteins, suggesting that the functional inactivation of these molecules is required for oncogenicity. Furthermore, p300 has an intrinsic histone acetyltransferase activity (HAT), which is crucial to its function. Here, studies were performed to understand how acetylation and de-acetylation may influence cell cycle regulation. I demonstrated that pRb is a substrate of p300 HAT activity. Furthermore, adenovirus E1A re-directs p300 HAT activity and enhances pRb acetylation. Acetylated pRb preferentially binds to proto-oncoprotein MDM2, indicating the functional relevance of this modification in regulating pRb function. Acetylation of pRb also influences its phosphorylation pattern, implying a certain level of 'cross-talk' between these two post-translational modification events.

In an attempt to understand the underlying mechanism of p300-mediated transcriptional co-activation, I investigated its interaction with a newly identified binding-partner NAP-2 (nucleosome assembly protein 2). First, the domains of interaction between these two proteins were studied. Subsequently, both NAP-2 and p300 were found to augment in transcriptional regulation. Overall, the results suggested a mechanism by which p300 and NAP family proteins co-operate in chromatin-remodeling related mechanisms in regulating transcription.

Finally, a series of pRb B pocket mutants were created, one of which was significantly compromised in binding to LXCXE peptide motif, including histone

deacetylases (HDACs). pRb's growth suppressive function was correlated to its intrinsic repressive activity on transcription. At least in part, this intrinsic repressive activity was described by its association with HDACs. Using this mutant, I analysed the necessity of HDAC association for pRb mediated G1 arrest. Surprisingly, the results suggested that HDAC may be dispensable for such function. Therefore, it re-addresses the question of the functional significance of the pRb-HDAC interaction.

Dedication

This thesis is dedicated with love and respect to my family,

especially to my mother Lee Tak-Yun.

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Abbreviations

A	Alanine
Ac	Acetylated
Ad	Adenovirus
AdML	Adeno-major late
AML	Acute myeloid leukemia
β-gal	β-galactosidase
bp	Base pair
BWS	Beckwith-Wiedeman Syndrome
C	Cysteine
cAMP	Cyclic adenosine mono-phosphate
CAT	Chloramphenicol acetyltransferase
CBP	CREB-binding protein
CDK	Cyclin dependent kinase
CDKI	CDK inhibitors
C/EBP	CCAAT-box/enhancer-binding protein
CH	Cysteine/histidine-rich domain
CK	Casein kinase
CMV	Cytomegalovirus
CNS	Central nervous system
CR	Conserved region (in the adenovirus E1A protein)
CRE	cAMP-response element
CREB	CRE-binding protein
CtIP	CtBP interacting protein

dCAF-1	Drosophila chromatin assembly factor-1 containing fraction
d.p.c.	days post-coitum
DHFR	Dihydofolate reductase
DMEM	Dulbecco's modificatied eagle's medium
DMSO	Dimethylsulphoxide
dNTP	Deoxy-nucleoside triphosphate
DTT	Dithiothreitol
E	Glutamic acid
EDTA	Ethylene diamine tetra-acetic acid
EKLF	Erythroid Kruppel-like factor
ER	Oestrogen
FACS	Fluorescence activated cell scanning
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FL	Full length
GR	Glucocorticoid receptor
GST	Glutathione-S-transferase
MEFs	Mouse embryonic fibroblasts
HA	Hemagglutinin protein (derived from influenza virus)
HAT	histone acetyl-transferase
HBS	HEPES-buffered saline
HBP	HMG-box protein
HDAC(s)	Histone deacetylase(s)
HEPES	N-[2-Hydroxethyl]piperazine-N'-[2-ethanesulfonic acid]
HMG	High mobility group protein

HNF	Hepatocyte nuclear factor
HPVs	Human papilloma virus
IB	Immunoblotting
ICE	Interleukin 1β-converting enzyme
INF β	Interferon β
IP	Immunoprecipitation
IPTG	Isopropyl-ß-D-thiogalactopyranoside
IR	ionizing irradiation
IVT	*In vitro* translated
JMY	Junction-mediating and regulatory protein
K	Lysine
KIX	Kinase-inducible domain
L	Leucine
LB	Luria-Bertani medium
LOH	Loss of heterozygosity
LT	large T antigen
Luc	Luciferase
MAPKs	Mitogen-activated protein kinases
MDM 2	Murine double minute 2
MEFs	Mouse embryonic fibroblasts
MLL	Mixed lineage leukemia
M.O.I.	Multiplicity of infection
MOZ	Monocytic-leukemia zinc-finger
Mut	Mutant
MyoD	Myogenic HLH transcription factor

NAP	Nucleosome assembly protein
NLS	Nuclear localization signal
NP-40	Nonidet P-40
P	proline
PP	pocket protein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
P/CAF	p300/CBP-associated factor
PCR	Polymerase chain reaction
PMSF	Phenylmethysulfonyl fluoride
PNS	Peripheral nervous system
Pol	RNA polymerase
PR	Progesterone
pRb	Retinoblastoma protein
Q	Glutamine
R	Arginine
RAR	Retinonic acid receptor
RTS	Rubinstein-Taybi syndrome
RXR	Retinoid X receptor
S	Serine
SCF	Skp-Cullin-F box
SDS	Sodium dodecyl sulphate
SRC-1	Steroid receptor co-activator 1
STAT	Signal transducer and activator of transcription
SV40	Simian virus 40

T	Threonine
TAD	*Trans*activation domain
TAF	TBP-associated factor
TAF-1	Template activating factor-1
TBP	TATA binding protein
TCF	T-cell factor
TGFβ	Transforming growth factor β
TNF	Tumour necrosis factor
TSA	Trichostatin A
TK	Thymidine kinase
TR	Thyroid hormone
Tris	Tris(hydroxymethyl)methylamine
Tween 20	Polyoxyethylene sorbitan monolaurate
UBF	Upstream binding factor
UV	Ultraviolet light
VP16	Virion protein 16 *trans* activation domain
W	Tryptophan
wt	Wild type
WT	Wilm's tumour
w/v	Weight per volume
YY1	Ying yang 1
(+/+)	Wild type
(+/-)	Heterozygous mutant
(-/-)	Homozygous mutant

Publications

The following publications were submitted during the course of the work presented in this thesis.

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Table of Contents

CHAPTER 1: INTRODUCTION **1**

THE RETINOBLASTOMA PROTEIN (PRB) 3
pRb and its family members *3*
Cell cycle regulation of pRb *7*
Anti-apoptotic function of pRb *14*
Role in terminal differentiatio *15*
Other targets of pRb *16*
Summary of pRb function *18*
P300/CBP 19
The p300/CBP protein family *20*
Transcription regulation by p300/CBP *24*
Regulation of cell cycle by p300/CBP. *29*
Regulation of p300/CBP activities *32*
p300/CBP in human diseases. *34*
Discussion *35*

CHAPTER 2: MATERIALS AND METHODS **38**

PLASMIDS 38
SITE-DIRECTED MUTAGENESIS 40
IN VITRO PROTEIN ACETYLATION ASSAY 41
EXPRESSION AND PURIFICATION OF GLUTATHIONE S-TRANSFERASE FUSION PROTEINS 42
EXPRESSION AND PURIFICATION OF HIS-TAGGED FUSION PROTEINS 43
PRODUCTION OF FLAG-P300 $^{1135\text{-}2414}$ BACULOVIRUS 44
EXPRESSION AND PURIFICATION OF FLAG-P300 FROM SF9 CELLS 44
TISSUE CULTURE AND TRANSFECTION 45
WESTERN BLOT AND IMMUNOPRECIPITATION 46
IN VITRO-PULLDOWN ASSAYS 47
CELL CYCLE ANALYSIS 48
MASS SPECTROSCOPY AND PEPTIDE SEQUENCING 48

CHAPTER 3: ACETYLATION CONTROL OF THE RETINOBLASTOMA TUMOUR SUPPRESSOR PROTEIN **50**

INTRODUCTION 50
RESULTS 53
pRb, p107 and E2F-1 are acetylated in vitro *53*
pRb is acetylated in vivo *53*
Domain Mapping of Acetylation sites in pRb *55*
pRb acetylation by p300 is enhanced by E1A *56*
Acetylation of pRb enhances pRb/MDM2 interaction *58*
Lys873/874 are important in regulating pRb phsophorylation by CDKs *59*
DISCUSSION 61

CHAPTER 4: FUNCTIONAL INTERACTION OF A NUCLEOSOME ASSEMBLY PROTEIN-2 AND P300 ..... **95**

INTRODUCTION 95
RESULTS 99
NAP-2 binds to the CH3 domain in p300 ..... *99*
Both the central and C-terminal region of NAP-2 bind to p300 ..... *100*

The N and C-terminal regions of NAP-2 can mediate homo- or hetero-dimerisation of NAP family members *101*
NAP-2 inhibits p300-dependent histone acetylation *102*
NAP-2 and p300 co-operate in transcriptional activation *102*
DISCUSSION 104

CHAPTER 5: MUTAGENESIS OF THE PRB POCKET REVEALS THAT THE CELL CYCLE ARREST FUNCTION ARE SEPARABLE FROM LXCXE BINDING 123

INTRODUCTION 123
RESULTS 126
Mutational analysis of a conserved lysine patch in B domain of pRb *126*
The pRb 6A mutant is compromised in binding to LXCXE-motif containing proteins *126*
The pRb 6A represses E2F-1 transcription *127*
The pRb 6A induces G1 arrest in SAOS2 cells *128*
DISCUSSION 129

CHAPTER 6: SUMMARY 143

ACETYLATION: MORE THAN THE CHROMOSOME 143
PRB ACETYLATION BY P300 REVEALS ADDITIONAL LEVEL OF CONTROL 146
E1A REDIRECTS NORMAL ACETYLATION PATTERNS 147
P300 IN TRANSCRIPTION 148
RB/HDAC: CELL CYCLE ARREST OR NOT? 150

REFERENCES 153

APPENDIX:

PEPTIDE ANALYSIS REPORT FROM MICROMASS: REPORT 1.

PEPTIDE ANALYSIS REPORT FROM MICROMASS: REPORT 2.

Table of Figures

Figure 1.1 pRb integrates negative and positive signals with the cell cycle clock. *4*

Figure 1.2 The pocket protein family and the functional domains in pRb. *7*

Figure 1.3 Pocket proteins have multiple modes of regulation in controlling E2F DNA-binding sites. *12*

Figure 1.4 pRb activity is controlled by phosphorylation and dephosphorylation during the cell cycle. *13*

Figure 1.5 Schematic representation of p300/CBP. *23*

Table 2.1 Primers for PCR. *39*

Table 2.2 Primers used for site-directed mutagenesis. *41*

Figure 3.1 Purification of Flag-p300/pCAF and table of summary of the *in vitro* acetylation results. *66*

Figure 3.2 The pRb, p107 and E2F-1 are acetylated *in vitro.* 68

Figure 3.3 The retinoblastoma protein is acetylated *in vivo.* 70

Figure 3.4 Analysis of an *in vitro* acetylated peptide derived from pRb B pocket. 74

Figure 3.5 Characterisation of the pRb 713KA and 713KR mutants. 78

Figure 3.6 The C-terminal region of pRb is the major site of acetylation. 80

Figure 3.7 E1A enhances pRb acetylation, and inhibits E2F-1 and p53 acetylation by p300 *in vitro.* 82

Figure 3.8 E1A requires binding to both pRb and p300 to enhance pRb acetylation by p300 *in vitro.* 85

Figure 3.9 Acetylation of pRb enhances pRb binding to oncoprotein MDM2. 87

Figure 3.10 Acetylation at the pRb C-terminus (K873/874) influences pRb phosphorylation by cdk complexes. 90

Figure 3.11 Summary of pRb acetylation. 93

Figure 4.1 NAP-2 binds to the CH3 domain in p300. 108

Figure 4.2 NAP-2 binds to the CH3 domain in p300. 111

Figure 4.3 Domains in NAP-2 interact with p300, and NAP-2 oligomerisation domains. 113

Figure 4.4 NAP is not acetylated *in vitro*. 115

Figure 4.5 NAP-2 inhibits histone acetylation by p300 *in vitro*. 117

Figure 4.6 NAP-2 augments the p300-dependent transcription factor E2F-1. 119

Figure 4.7 A hypothetical model of p300 and NAP co-operate in transcriptional regulation. 121

Figure 5.1 Mutagenesis of pRb B pocket lysine residues. 132

Figure 5.2 pRb 6A mutant has significantly reduced ability in binding to LXCXE-motif containing proteins. 135

Figure 5.3 Transcriptional repression mediated by pRb and the mutants. 139

Figure 5.4 pRb and pRb 6A arrest SAOS2 cells in G1 phase of the cell cycle. 141

Chapter 1: Introduction

One classical approach to study cell cycle regulation is through the understanding of how viral oncoproteins disrupt normal cell cycle control. Three small DNA tumour viruses, adenovirus (Ad), simian virus 40 (SV40), and human papillomaviruses (HPVs), all encode genes which are capable of activating cell cycle functions in quiescent cells (reviewed in 167, and references therein). In this thesis, I have focused on two cellular polypeptides, pRb and p300, which are targets of Ad E1A (182, reviewed in 42 and 118). In SV40, the large T antigen (LT) binds to pRb and p300 (reviewed in 118). In HPVs, E7 and E6 were shown to interact with pRb and p300 respectively (reviewed in 118).

A converging theme is that different viral oncoproteins evolve similar strategies to interact with pRb and p300, suggesting these two proteins play an intimate part in cell cycle regulation. In the early nineties, through the studies of cellular targets of viral proteins, E2F was identified to be important for pRb function (reviewed in 43, 62, 91, 118, 175 and references therein). This finding opened up an exciting era of E2F research, pinpointing its crucial role in transcriptional regulation of the cell cycle.

After the cloning of p300 in 1994 (44), sequence homology comparison immediately identified p300 as 'a transcriptioinal adaptor protein for certain complex transcriptional regulatory elements' (44). The real explosion of the field came in late 1996 when p300 was identified to be a *bona fide* histone acetyltransferase (12, 128). p300/CBP was also found to interact with an ever increasing list of transcription factors, including various hormonal receptors (e.g estrogen receptor (ER), Retinoic acid receptor (RAR), glucocorticoid receptor (GR), etc.), differentiation factors (e.g. MyoD, c-MyB, GATA-1), and cell cycle regulators (e.g. p53, E2F, NFκB, Mdm2) (reviewed in 147, and reference therein). Furthermore,

p300/CBP interacts with RNA helicase A, which in turn binds to RNA polymerase II (Pol II), suggesting p300/CBP could be part of the basal transcriptional machinery (124).

Whilst our knowledge on pRb and p300 keeps accumulating, key questions remain as to why viral oncoproteins interact with these two cellular polypeptides. Ad E1A, for example, sequesters both pRb and p300 via an N-terminal transformation sensitive domain (118). Mutagenesis studies suggested that E1A requires binding to both pRb and p300 for efficient transformation. The first model suggests that binding of E1A (or other viral proteins, e.g. SV40 LT) to pRb and p300 simply inactivates their cellular functions. Studies on pRb support this simple model. For example, binding of E1A to pRb disrupts pRb/E2F complex, thereby de-regulating E2F-dependent transcription (reviewed 43, 118, 169). However, a similar model may not be sufficient to explain the interactions with p300. *p300*$^{-/-}$ mice have defects in cellular proliferation (189). Furthermore, p300 is a versatile transcription co-activator (147). It is unlikely viral proteins simply abolish all p300 functions. Therefore, a second model suggests that viral oncoproteins may activate or re-direct a normal cellular activity in dis-regulating cell cycle control. For example, E1A may recruit p300 to activate its growth promoting functions. Since E1A was demonstrated to form a ternary complex between pRb and p300 (173), a third possibility also exists that viral oncoproteins mediate the assembly of novel complex(es) to run havoc with the cell cycle.

In the following chapters, I will first review current literature on pRb and p300. In chapter three, evidence will be presented that pRb can be a substrate of p300 HAT activity, suggesting a direct functional interaction between these two cellular polypeptides. In chapter four, work will be presented on a nucleosome assembly protein which was found to associate with p300, pointing towards another level of p300-mediated transcriptional control. In chapter five, the functional interaction between pRb and HDACs will be explored. Finally, I will summarise the key findings, and discuss the possible implications.

The Retinoblastoma Protein (pRb)

The retinoblastoma protein is a 110KDa nuclear phosphoprotein. It is a classical tumour suppressor. Functional inactivation of both copies of the *Rb* gene is an invariant feature of both sporadic and familial retinoblastomas (reviewed in 134). Young children with a germ-line mutation in one *Rb1* allele have a 95% chance of developing a retinoblastoma tumour in their eyes. Mutation in the *Rb1* allele also predisposes patients to develop other tumours, such as osteosarcomas and fibrosarcomas. Almost two-thirds of the secondary tumours arising in patients with retinoblastoma are mesenchymal in origin. Dryja, Friend and Weinberg (40) cloned the 4.7Kb cDNA corresponding to the *Rb1* gene. The *Rb1* gene contains 27 exons, spanning across 180kb on chromosome 13. Most mutations in *Rb1* lead to premature termination of translation.

pRb and its family members

The pRb, p107 and p130 proteins form the 'pocket protein (PP)' family that is crucial to cell cycle regulation (reviewed in 99, 134, 174 and 181). pRb and p107/p130 have five highly conserved domains. pRb shares between 30-35% sequence identity with p107/p130. Most of the conserved sequences lie within the so-called 'pocket region' (amino acid 379-792), which is composed of two subdomains, known as the 'A' and 'B' boxes. Viral oncoproteins, SV40 LT, ad E1A and 'high risk' HPVs E7 all target the pocket domain, displacing cellular proteins which interact with the pocket, thereby leading to loss of pocket protein function.

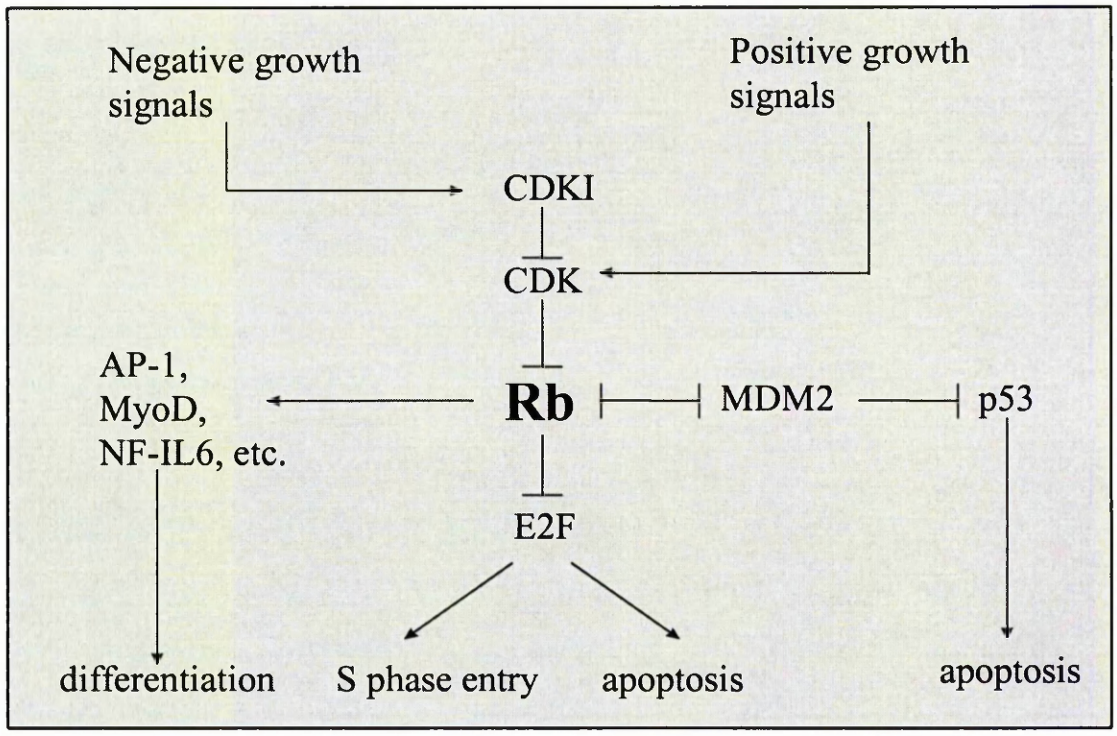


Fig. 1.1 pRb integrates negative and positive signals with the cell cycle clock.

Mitogenic signals activate cyclin-dependent kinase activity and inactivate pRb function by phosphorylation. In reverse, inhibitory signals, such as TGFβ and contact inhibition mobilise CDK inhibitors (CDKI) to maintain active pRb in its hypophosphorylated form. pRb has at least two distinct activities in tumour suppression: by inactivating downstream E2F activity, and by augmenting transcription activities of various transcription factors for cellular differentiation. MDM2 acts as a mediator bridging together the p53 and pRb pathways.

At least three distinct protein-binding activities of pRb have been described to be important for its function: the large pocket (amino acid 395-876) binds to E2F (discussed later), the small pocket (amino acid 379-792) binds to the LXCXE (L = leucine, C = cysteine, E = glutamic acid, X = any amino acid) peptide, and the C pocket binds to tyrosine kinase c-Abl and oncoprotein MDM2 (176, 183 and reviewed in 174). Viral oncoproteins e.g. E1A, E7, SV40 LT and some cellular proteins e.g. HDACs, cyclin D, possess an LXCXE motif in their protein sequence that enables them to bind to the pocket. The three dimensional structure of the pRb A/B pocket bound to an LXCXE containing peptide from E7 has been solved (94). The peptide binds to a highly conserved groove on the B box portion of the pocket, and the A box is required for the stable folding of the B box. The A/B box interface is highly conserved, suggesting an additional protein-binding region. Furthermore, both E2F and E7 peptides occupy distinct sites in the pocket. HDACs, which contain an LXCXE-like motif, may form a trimeric complex with pRb and E2F (19, 103). c-Abl can bind to pRB when the pocket region is occupied by E2F (183). Taken together, the evidence suggests a model for pRb as a 'molecular matchmaker' in mediating protein complex formation (reviewed in 174).

Despite the close similarities among the family members, only *Rb* was shown to be mutated in tumour cells. Neither *p107* nor *p130* were frequently found to be mutated in naturally occurring tumours. In order to study the specific cellular function of pRb, p107 and p130, knock out mice in *Rb*, *p107* and *p130* have been developed. The phenotypes indicated both distinct and over-lapping functions among the family members (32, 33, 73, 93, 95 and reviewed in 99 and 120). The absence of pRb in mice causes embryonic lethality, although $p107^{-/-}$ and $p130^{-/-}$ mice survive to term, possibly as a result of functional redundancy between p107 and p130. This is consistent with the phenotype of the $p107^{-/-}$:$p130^{-/-}$ mice which exhibit

embryonic lethality. Interestingly, $Rb^{+/-}$ mice do not suffer from retinoblastoma, but develop tumours of the pituitary and thyroid origin. This difference may be attributable to the physiology of humans and mice. Bilateral, multifocal retinal dysplasia is indeed observed in $Rb^{+/-}$:$p107^{-/-}$ mice. This suggests that pRb and p107 may share overlapping functions in controlling cellular homeostasis in the murine retina, and loss of both is required for tumour formation. $p107^{-/-}$:$p130^{+/-}$ and $p107^{+/-}$:$p130^{-/-}$ mice were developed to investigate whether p107 and p130 possess tumour suppression function. However, analysis of such animals did not reveal any obvious tumour phenotype. An alternative explanation is that unlike the mutations in pRb, mutations in p107 and/or p130 may not be advantageous for tumourgenesis. Therefore mutations in other components of the pathway, such as p16 (86), which is indeed frequently found to be mutated in human tumour(s), may contribute to the inactivation of p107/p130 tumour suppression function, without abrogating other essential functions of p107/p130.

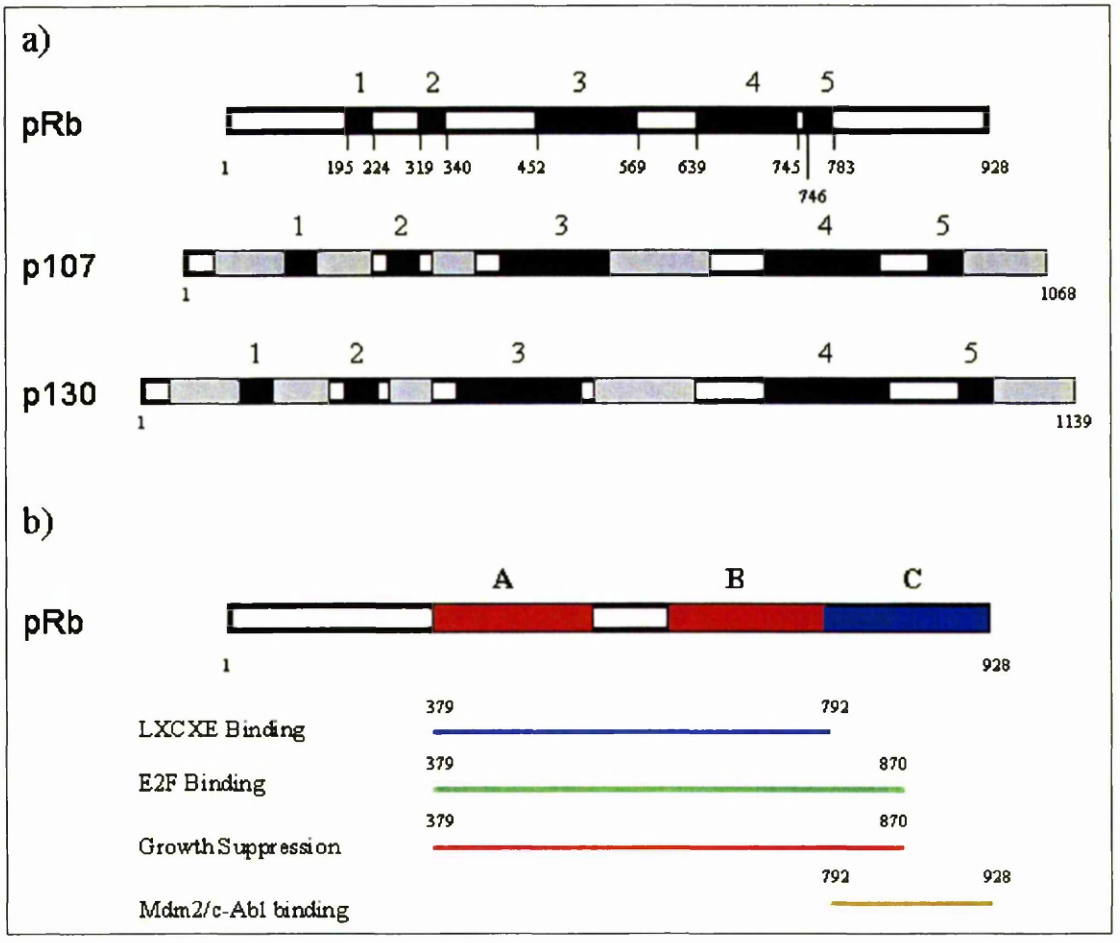


Fig. 1.2 The pocket protein family and the functional domains in pRb.

a) A schematic organisation of pRb, p107 and p130 is shown. Regions conserved among the three pocket proteins are highlighted in black. Shaded areas indicate additional regions of similarity between p107 and p130.

b) Functional and biochemical domains within pRb are indicated that are required for interacting with the LXCXE motif, E2F, MDM2/c-Abl, and functionally required for growth suppression. The A and B domains constitute the pocket domain of pRb, and the C pocket is indicated.

Cell cycle regulation of pRb

pRb/E2F

The pRb protein is ubiquitously expressed in most cycling and resting cells. pRb acts as a negative regulator for cellular proliferation, sequestering a variety of nuclear proteins involved in cell cycle progression (reviewed in 175). The most studied pRb target is the family of transcription factors known as E2F (reviewed in 43, 91). E2F consists of a heterodimer of an E2F protein bound to a DP partner. Together, they regulate the timing and levels of expression of many genes involved in cell cycle progression (reviewed in 62, and references therein). E2F target genes encode proteins involved in DNA replication (for example DNA polymerase α, thymidine kinase (TK), dihydrofolate reductase (DHFR) and cdc6), chromosomal replication (for example replication origin-binding protein HsOrc1, MCM proteins) and cell cycle regulation (for example cyclin A, E and D1, p107, cdc2, E2F1, 4 and 5 and $p19^{Arf}$). One important feature of E2F DNA-binding site is that in many promoters (including B-myb, E2F-1, cyclin E, cyclin A, ORC1, cdc2, cdc6), E2F DNA-binding sites contribute to transcription repression, presumably through the assembly of the E2F/PP repression complex on these promoters. Indeed, *in vivo* footprinting experiments obtained on cdc2 and B-myb promoters demonstrated E2F DNA-binding site occupation during G0 to early G1, when the E2Fs are in transcriptional repressive complexes with the PPs (162, 199). A few activating E2F DNA binding sites were also identified, which include p107, DHFR and TK (reviewed in 62, and references therein).

A simple working model for how pRb regulates E2F function suggests that during G0/G1, hypophosphorylated pRb binds to E2F, inactivating it and thereby preventing cell cycle progression (reviewed in 115, also see later). Cyclin D/cdk4/6 and cyclin E/cdk2 progressively phosphorylate pRb at late G1 to early S phase. In S phase, pRb phosphorylation

is maintained by cyclin A/cdk2. Progressive phosphorylation of pRb results in reduced affinity for E2Fs, the release of free E2F and thereafter induction of E2F transcription.

Different PPs have a preference for binding to different E2F family members (reviewed in 62, and references therein). The E2F family now consists of six members (E2F-1 to 6). E2F-1, 2 and 3 exhibit high affinity binding towards pRb, but bind weakly to p107/p130. In contrast, E2F-4 and 5 show greater specificity for p107/p130, but also bind pRb. Complexes between the pRb family members and E2F form at different phases of the cell cycle. In general, p130/E2F complexes are mainly found in quiescent or differentiated cells, p107/E2F complexes predominate in S phase cells, and pRb/E2F complexes are most evident during G1/S phase transition, but also exist in quiescent or differentiated cells (69, 117 and reviewed in 43 and 62). Using chromatin immunoprecipitation assays, it was found that repression on E2F-responsive promoters was associated with the recruitment of E2F-4 and p130, together with low level of histone acetylation (190). In late G1 phase, the E2F-4/p130 complex is replaced by E2F-1 and E2F-3, together with enhanced level of acetylation of histones H3 and H4. Taken together, it seems that distinct E2F heterodimers recruit enzymes to deacetylate or acetylate core histones, therefore repressing or activating E2F-responsive genes (190).

pRb can modulate E2F transcription activity in at least two distinct ways (reviewed in 43). Firstly, pRb binds to the transcription activation domain in E2F and directly inhibits E2F-mediated transcription. Using *in vitro* transcription and footprinting assays, Ross *et. al.* (138) demonstrated that pRb blocks the recruitment of the transcription initiation complex by E2F. Secondly, pRb binds to HDACs (19, 103, and 106), which is believed to deacetylate histones on the promoter, and actively repress transcription via chromatin remodelling. Zhang *et. al.* (197) demonstrated that active repression of the pRb/E2F complex is important in mediating G1 arrest triggered by transforming growth factor-β (TGFβ), p16 and contact

inhibition. Interestingly, a trimeric complex of pRb-HDAC-hSWI/SNF has recently been demonstrated (196). The hSWI/SNF complex also has chromatin remodelling activity, implying a third mechanism for pRb modulation of E2F transcription activity. Furthermore, the pRb-hSWI/SNF complex can exist in the absence of HDAC, and this complex is sufficient to repress cyclin A and cdc2 genes, but not cyclin E or E2F-1 genes (196). Therefore, pRb-HDAC-hSWI/SNF and pRB-hSWI/SNF could be two distinct complexes, acting at different phases of the cell cycle and regulating the expression of different sets of gene.

Apart from regulating E2F transcription, pRb and its family members also regulate E2F protein stability, cellular localisation and apoptotic function (reviewed in 43, 62). E2F-1 is actively degraded by the ubiquitin-proteosome pathway through an SCF-like complex (107). E2F-1 mutants which cannot be degraded by the SCF pathway drive cell cycle progression into S phase followed by apoptosis. Unphosphorylated pRb stabilised E2F-1 and protects it from degradation (61, 63). E2F-1 mediated apoptosis is both p53-dependent and -independent (130). Ecotopic expression of E2F-1 causes apoptosis in SAOS2 cell, which is a $p53^{-/-}$ cell line, suggesting this apoptotic mechanism is p53-independent (65, 130). The apoptotic function seems to be specific to E2F-1 since overexpression of E2F-2 and E2F-3 failed to induce apoptosis, even though they induced the expression of E2F target genes and drove cells into S phase (35). Phillips *et. al.* (131) demonstrated that E2F-1 mediated apoptosis can at least in part occur through a death receptor-dependent mechanism by inhibiting activation of anti-apoptotic signals including NF-κB. Analysis of the E2F-1 mutants indicates that the apoptotic function is independent of its transcriptional activation domain, but requires DNA binding (65, 130). Over-expression of pRb overcomes E2F-1 mediated apoptosis.

While E2F-1, 2 and 3 protein levels change according to the cell cycle, being peak at late G1 to early S phase and degrading after S phase; E2F-4 and 5 protein levels stay relatively constant in different phases of the cell cycle (96, 107). From extracts of tissue culture cells, it is E2F-4 which contributes the bulk of E2F DNA-binding activity. E2F-4 is both nuclear and cytoplasmic in quiescent cells, but relocates almost entirely to the cytoplasm when the cells reach S phase. Expression of DP-2, p107 and p130 was reported to drive nuclear localisation of E2F-4 (36, 98, 105).

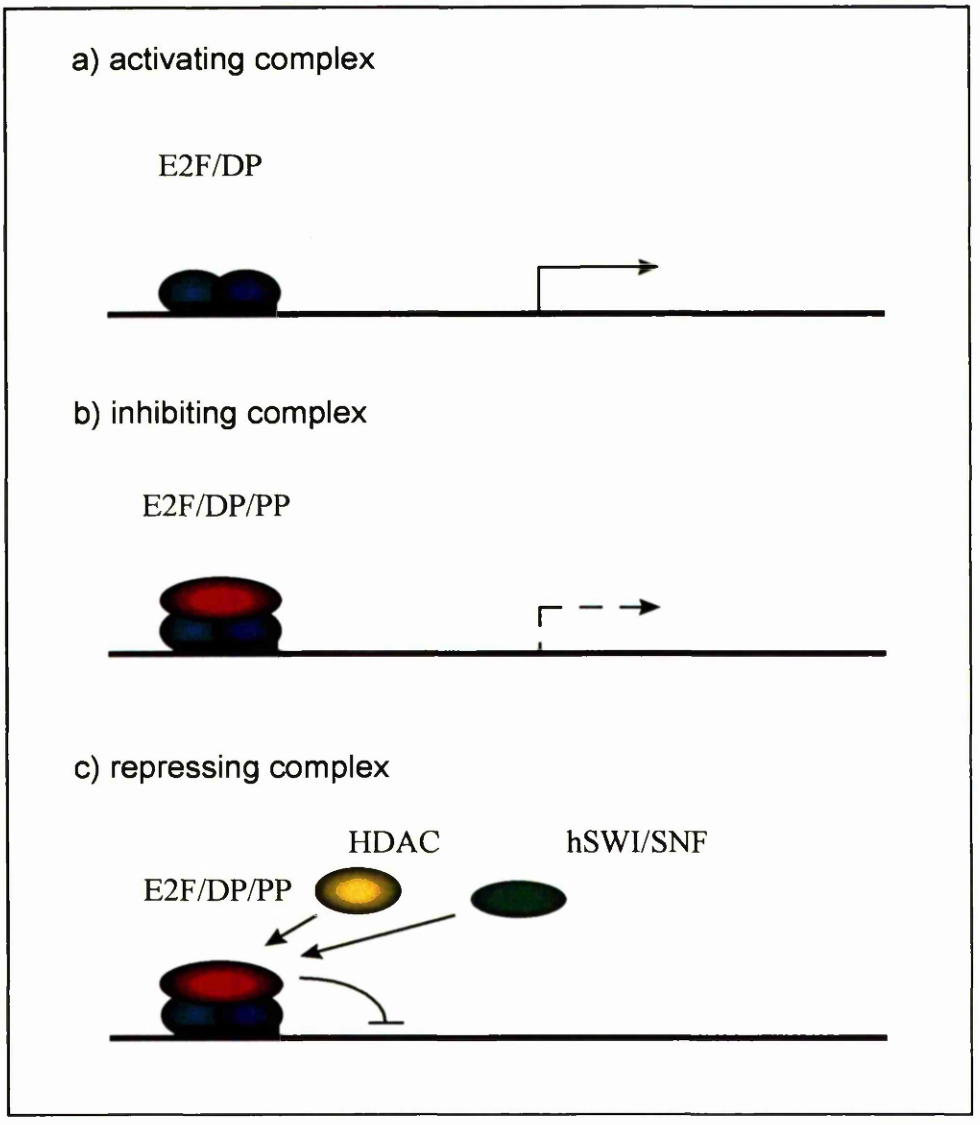


Fig 1.3 Pocket proteins have multiple modes of regulation in controlling E2F DNA-binding sites.

There are at least three types of E2F complex: an activating E2F complex, an inhibiting E2F complex and a repressing E2F complex (43).

Phosphorylation Control of pRb

The pRb function is controlled by phosphorylation during the cell cycle (reviewed in 115). In its active state, pRb is hypophosphorylated, which occurs in early G1 phase of the cycling cells. Hyperphosphorylated pRb, which shows retarded migration in SDS gel, is generated in late G1 to S phase, and correlates with inactivation of pRb. Phosphorylation of pRb in late G1 was carried out by a combination of cyclin/CDKs, and type 1 protein phosphatase (PP1) was found to dephosphorylate pRb in late mitosis (reviewed in 115).

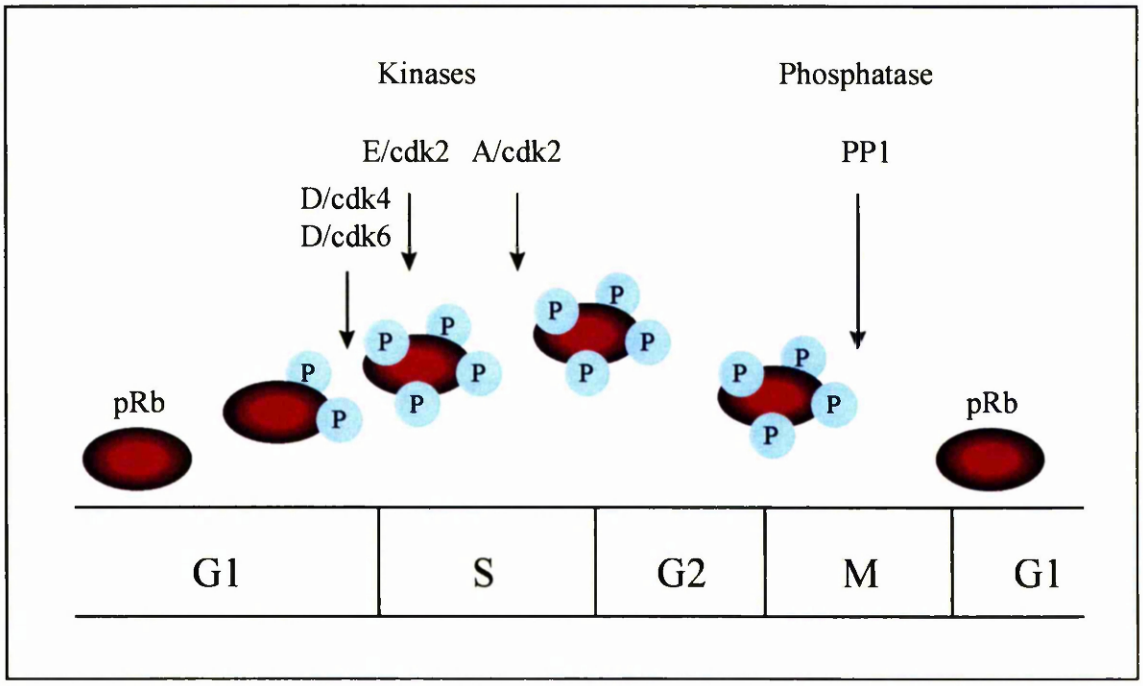


Fig 1.4 pRb activity is controlled by phosphorylation and dephosphorylation during the cell cycle.

Hypophosphorylated pRb is active in growth repression. Phosphorylation of pRb in late G1 by a combination of cyclin/CDKs converts pRb to an inactive hyperphosphorylated form. Dephosphorylation of pRb in late mitosis by type 1 protein phosphatase (PP1) restores pRb growth suppressive activity (115).

pRb contains 16 S/T-P motifs which are potential CDK phosphorylation sites. At least seven of those sites (S-249, -807, -811, and T-252, -373, -821, -826) were shown to be phosphorylated *in vivo* (84, 85 and references therein). Cyclin D- and cyclin E- activated kinase complexes phosphorylate pRb on different sites *in vitro*, neither one being sufficient to phosphorylate the full set of sites that is seen in *in vivo* (115). Phosphorylation of certain sites of pRb was demonstrated to carry specific functions. For example, phosphorylation at S807 and -811 was shown to release the interaction of c-Abl from pRb (84). The binding of pRb to E2F is regulated by a number of sites in the C-terminus of pRb, and possibly involves two serines in the linker region between the A and B boxes (85, 168).

The three dimensional structure of the pRb A/B pocket in complex with the E7 peptide identified a highly conserved lysine patch in B pocket which could function as phospho-receptor sites (94). Harbour *et al.* (59) presented evidence that phosphorylation of the pRb C-terminus by CDK4/6 causes successive intramolecular interactions between the C-terminus and the central pocket region, presumably via the lysine patch. The initial interaction is thought to disrupt HDAC binding, and therefore relieves active repression by pRb. This event then facilitates the phosphorylation of the pocket region by cyclin E/CDK2, thereby disrupting the pRb/E2F interaction. These intramolecular interactions provide a molecular basis for how phosphorylation alters pRb protein structure, and progressively inactivates its growth suppression function.

Anti-apoptotic function of pRb

As discussed above, over-expression of E2F-1 causes apoptosis in SAOS2 cells. Co-expression of pRb protects cells from E2F-1 mediated apoptosis, which depends upon the ability of pRb to bind to E2F-1. Conversely, cells lacking pRb leads to inappropriate S-phase

entry, activation of E2F responsive genes, and apoptosis (3). Several tissues in the *Rb*$^{-/-}$ mice display extensive cell death. The analysis of the *Rb*$^{-/-}$ mouse embryos versus the *E2F-1*$^{-/-}$:*Rb*$^{-/-}$ embryos also indicated that pRb suppresses E2F-1 mediated apoptosis (see below, 167, 186, 187 and reviewed in 99 and 120). Interestingly, one report suggested that pRb could promote apoptosis by forming a trimeric complex with MDM2 and p53 (64). pRb was shown to antagonise MDM2 mediated p53 degradation, stabilising p53, and restoring p53 trans-repression and apoptotic function. However, p53 *trans*activation was not rescued in those assays. Interleukin 1β-converting enzyme-like (ICE-like) protease was found to cleave off the C-terminus of pRb in response to tumour necrosis factor (TNF)- and staurosporin-induced apoptosis (135). Cleaved pRb still bound to E2F-1 and cyclin D, but lost binding to MDM2. Therefore, the pRb/MDM2 interaction is likely to affect the apoptotic function of pRb, though it is not clear if this interaction is pro- or anti- apopotosis.

Role in terminal differentiation

A role for pRb in differentiation was suggested by the phenotype of *Rb*$^{-/-}$ mice, which show a pronounced defect in erythroid, neuronal and lens development (reviewed in 99, 120). Although loss of pRb function allows the initiation of differentiation, the embryos fail to achieve a fully differentiated state, indicating that pRb is likely to play an important role in achieving and maintaining the post-mitotic state. The aberrant cell cycle entry observed in the CNS and the PNS of the *Rb*$^{-/-}$ embryo caused elevated levels of apoptosis. This again implied a role for pRb in protecting cells from apoptosis. Indeed, *Rb*$^{-/-}$:*E2F*$^{-/-}$ mice showed reduced levels of ectopic cell cycle entry and apoptosis in both the CNS and the lens at 13.5 d.p.c. as compared to *Rb*$^{-/-}$ embryos, suggesting that these defects are in part due to de-regulated E2F-1

activity. However, this mechanism is tissue type-specific, as loss of E2F-1 has less of an effect on cell cycle entry and apoptosis in the PNS.

It is clear that pRb interacts with other non-E2F targets. This was best demonstrated in the analysis of pRb mutants that showed reduced affinity binding to E2Fs, but were still capable of augmenting MyoD transcriptional activity and inducing tissue-specific gene transcription (142). Furthermore, these mutants retained certain tumour suppressor functions. Therefore, at least part of the tumour suppression function of pRb correlates with its ability to promote tissue differentiation.

Although the pRb protein level does not change dramatically upon differentiation, pRb was found to be hypophosphorylated during cell cycle exit. Hypophosphorylated pRb augments the transcriptional activity of various transcription factors important for tissue differentiation, including MyoD for myogenesis and C/EBPα for adipogenesis (27, 57). Also, pRb was shown to bind to C/EBPα (27), NF-IL6 (28) and AP-1 (126) transcription factors, and enhance their DNA binding activity.

Other targets of pRb

Low-penetrance pRb mutants, such as substitution of tryptophan (W) for arginine (R) 661 in the B pocket of pRb (661W), which are inactivate in both E2F and LXCXE binding, still retain tumour suppressor activity. In cell based assays, the 661W mutant was shown to inhibit G1/S progression. Furthermore, C pocket mutations in full-length pRb also reduced pRb function (180). Taken together, these suggest that non-E2F targets of pRb contribute to tumour suppression.

The C-terminus of pRb interacts with the c-Abl tyrosine kinase (176, reviewed in 174). The c-Abl kinase is ubiquitously expressed in both the cytoplasm and nucleus. The

nuclear kinase activity of c-Abl is under cell cycle control, being activated during cell cycle progression. Interestingly, c-Abl can simultaneously interact with pRb when the pocket region is occupied by E2F.

The human homologue of yeast SNF/SWI2 proteins, hBrm/hBrg interact with pRb in a pocket-dependent manner (166, reviewed in 18). hBrm/hBrg has chromatin remodelling activity and co-operates with pRb in the transcriptional activation of the GR (151). Together with the observation that pRb also recruits HDACs, it suggests that pRb regulates transcription via chromatin remodelling mechanisms.

pRb interacts directly with the largest TATA-binding protein associated factor, $TAF_{II}250$, through multiple regions in each protein (143, 144). Apart from being part of the basal transcriptional machinery, $TAF_{II}250$ possesses intrinsic HAT activity and kinase activity. Mutagenesis studies suggested that $TAF_{II}250$ is a cell cycle regulated protein and its HAT activity is required for cell cycle progression (41). pRb inhibited the kinase activity of $TAF_{II}250$, but was not found to be acetylated by $TAF_{II}250$ (150). These findings point to an additional mechanism for pRb regulation of transcription by modulating the activity of the basal transcription apparatus.

Evidence also exists that pRb modulates transcription mediated by RNA polymerase I and III (reviewed in 179). During differentiation of U937 myeloid porgenitor cells, pRb becomes localised to nucleoli, which are the major sites of ribosomal gene transcription by RNA polymerase I. Immunoprecipitation experiments demonstrated that pRb can associate with transcription factor UBF1, and that this interaction compromised the transcriptional activity of UBF1, thus leading to the down-regulation of Pol I transcription (24).

Loss of pRb function also correlated with up-regulation of Pol III transcription in tumour cells. $Rb^{-/-}$ fibroblasts have elevated Pol III activity compared to $Rb^{+/+}$ fibroblasts.

Immunoprecipitation and co-fractionation experiments demonstrated that pRb interacts with TFIIIB, and TFIIIB was identified as a target of repression by pRb (90).

Summary of pRb function

The pRb tumour suppressor protein is an essential component of the cell cycle clock, integrating both positive and negative signals for cellular growth and proliferation with the transcription machinery. The pRb protein exerts its tumour suppression function by both antagonising and synergising with downstream effectors, such as E2F. pRb has two modes of action: it can inactivate E2F transcription activity, and assemble active repression complexes with E2F. Apart from E2F, pRb synergies with various factors to promote cellular differentiation. The differentiation property of pRb contributes partly to its tumour suppressor function. The pRb-c-Abl interaction and pRb-MDM2 interaction also add to its growth suppression ability, though the mechanisms remain to be elucidated.

It is also clear that pRb is a master regulator for transcription. It can both activate and repress transcription in a context-dependent manner. pRb interacts directly with HAT, HDACs, and hBrm/hBrg, all of which are classes of proteins involved in chromatin remodelling. It will be important to investigate how pRb regulates transcription in a chromatin environment. Last but not the least, pRb regulates transcription by Pol I, II and III, thereby integrating the cell cycle clock with the biosynthetic capacity of the cell in controlling cellular proliferation and cellular growth.

p300/CBP

The p300/CBP proteins are a family of transcription co-activators which mediate multiple, signal-dependent transcription events (reviewed in 75, 145). They participate in many cellular programmes, including proliferation, differentiation and apoptosis (reviewed in 52). Most of the studies on p300/CBP have focused on their ability to act as transcription co-activators. For example, they have been considered as part of the basal transcription machinery (81), as they interact with TBP (140), TFIIB (48), TFIIE, TFIIF (68) and the RNA helicase A (124). It is believed that p300/CBP can bridge DNA-binding factors with the basal transcription machinery to allow transcriptional activation (reviewed in 52, 75). Secondly, p300/CBP possesses HAT activity (13, 128), which might act directly on nucleosomes to activate transcription via chromatin remodelling related mechanisms (reviewed in 21, 54, 60, 82, 170), or could act on transcription factors, such as p53 (56, 100, 139) and E2F-1 (110, 111), and/or other factors such as TFIIE, TFIIF (68) and HMG proteins (121) to regulate transcription. More recently, p300/CBP has been implicated in the regulation of protein stability (53, 110). Finally, a role for their involvement in tumour etiology has been established by the fact that they are targets of viral oncoproteins, including Ad E1A (180, reviewed in 42, 118) and SV40 LT (45). Mutations in *p300* and *cbp* were also found in colorectal tumours, Rubinstein-Taybi Syndrome (RTS), leukaemia and epithelial malignancies (reviewed in 51). Therefore, p300/CBP may function as classical tumour suppressors.

The p300/CBP protein family

The paralogous proteins p300 and CBP were originally identified as binding proteins to E1A and the cAMP response element binding protein (CREB) respectively (31, 45, 182). Their genes are conserved in a variety of multicellular organisms from worms to humans. A number of conserved sequences were present between the two proteins, which comprise of most of the known functional domains in p300/CBP (6). These include: i) the bromodomain, which is conserved among many mammalian HATs; ii) three cysteine-histidine rich domains, also known as the CH1, CH2 and CH3 domain; iii) a KIX domain; and iv) an ADA2 domain, which shows extensive homology to a yeast transcription co-activator ADA2 (Fig 1.5). The CH1, CH3 and the KIX domains are important in mediating protein-protein interaction. A number of cellular and viral proteins bind to these regions (reviewed in 147). Crystallographic structural studies on the bromodomains of pCAF (39) and $TAF_{II}250$ (74) suggested that this region performs a unique function in recognising acetylated peptides. Given that many cellular factors are found to be acetylated by p300/CBP (see later), the bromodomain could function to recognise specific motif(s) which are acetylated. In addition, different combinations of bromodomains might recognise different patterns of acetylated nucleosomes. Both the N- and the C-terminal regions of p300/CBP possess *trans*activation functions, though the mechanism is not clear. The HAT activity resides within the central region of the protein (128). It is likely that the modular organisation of p300/CBP proteins is a crucial feature that allows them to function as a scaffold in the formation of multimeric transcription co-activator complexes. The p300/CBP protein family also contains at least two other putative members, p270 and p400, which have not yet been fully characterized (51). The p270 protein shares some common antigenic determinants with p300, and was found to be a component of the mammalian SWI/SNF complex (34).

Although p300 and CBP share extensive homology, genetic and molecular analyses suggested that they perform both overlapping and unique functions. In transient transfection, most transcription factors were activated by either p300 or CBP (reviewed in 52, 75, 147). Similarly, E1A binds to CBP and CREB can use p300 for co-activation (5, 102). The *p300*$^{-/-}$, *cbp*$^{-/-}$ and compound *p300/cbp* heterozygous mice show similar phenotypes in embryonic lethality, defects in growth and in neural tube closure, suggesting certain overlapping functions between p300 and CBP during embryonic development (189). Furthermore, some *p300* and *cbp* heterozygous mice suffered early lethality. One explanation is that there is an absolute requirement for *p300* and *cbp* gene activity during early embryogenesis to carry out certain functions, and a slight drop of, for example, 0.25-fold in the total p300/CBP protein levels may leave embryoinc development unviable. Consistently, RTS, which is a haplo-insufficient syndrome where patients are heterozygous for mutations in the *cbp* allele, suggests that the loss of one functional copy of *cbp* gene is sufficient to confer a propensity for malignancy (129). Indeed, *cbp* heterozygous mice also show skeletal abnormalities reminiscent of RTS (158). An alternative but non-exclusive model that could also accommodate the above observations suggests that p300 and CBP have unique cellular functions. To this end, the *p300*$^{-/-}$ fibroblast was found to have specific defects in retinoic acid-dependent transcription, whilst retaining normal CREB function (189). Furthermore, the heterozygous *cbp* mice showed highly penetrant, multilineage defects in haematopoietic differentiation (89). These mice had an increased chance of developing haematologic malignancies at advancing age. This pathology was unique to the *cbp*$^{+/-}$ mouse, but not in the *p300*$^{+/-}$ mouse, indicating that a full complement of *cbp*, but not *p300*, is required for normal haematopoietic differentiation. Furthermore, using hammerhead ribozyme technology to specifically inactivate either p300 or CBP, it was demonstrated that the retinoic acid-induced differentiation of F9 embryonal carcinoma cells was inhibited by co-expression of a p300-inactivating ribozyme, but not a CBP-inactivating ribozyme (77).

Similarly, ionizing radiation (IR)-induced apoptosis was found to be impaired in p300-deficient cells, but not in CBP-deficient cells (193). Interestingly, ribozymes directed against either p300 or CBP can block retinoic acid-induced apoptosis of F9 cells (77). Overall, it suggests that p300 and CBP have overlapping functions, but they also perform unique tasks in certain biological programmes.

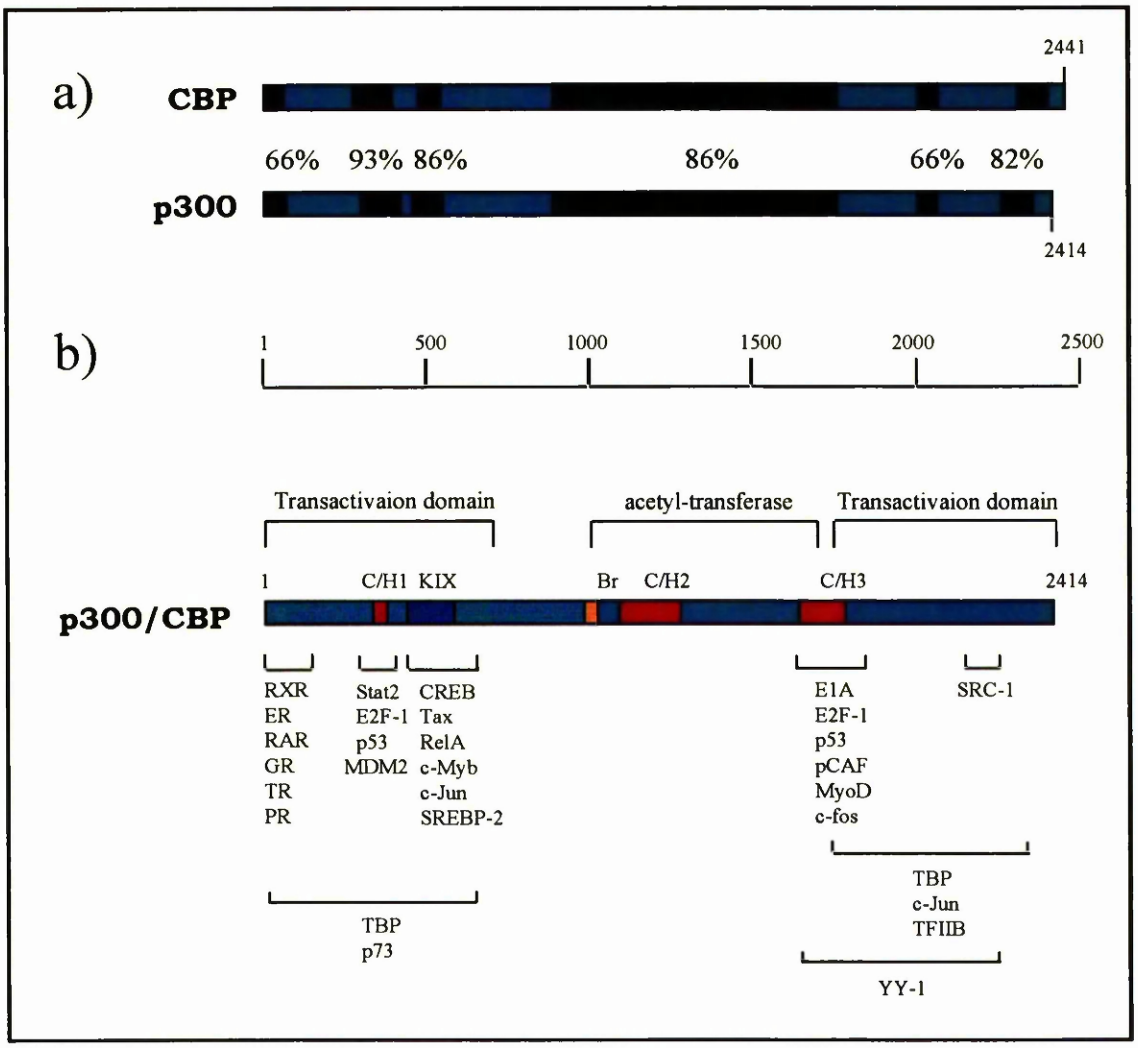


Figure 1.5 Schematic representation of p300/CBP.

a) Alignment of p300 and CBP protein. The shaded regions indicate areas of high homology, and percentage of identity between the two proteins are indicated.

b) The diagram shows various functional domains in p300, including three cysteine/histidine-rich domains, C/H1, 2 and 3 respectively, a KIX domain, and the bromodomain. Both the N- and C-terminal regions of p300/CBP have *trans*activation function. The HAT activity maps to the central region of the protein. The regions in p300/CBP that binds to target proteins are indicated.

Transcription regulation by p300/CBP

p300/CBP are versatile transcriptional integrators which have at least three different modes of regulating transcription. Here, I describe each mode in detail.

i) A bridge to the transcriptional machinery.

The initiation of transcription by RNA polymerase II requires sequence-specific transcription factors to bind to the promoter and/or enhancer elements, and the basal transcription machinery which physically carry out the transcriptional function. Unless the transcription factors can directly interact with the basal transcription machinery, bridging proteins are required to establish the contact between these two sets of proteins. p300/CBP is known to bind to a variety of transcription factors (reviewed in 75, 147), and interact directly with various components of the transcriptional machinery, including TBP, TFIIB, TFIIE and TFIIF (48, 68, 140). Therefore, in the simplest view, p300/CBP functions as a 'bridge' to connect transcription factors with the basal transcription machinery. An important feature of this mechanism is how the 'bridge' can be regulated, thereby providing specificity to the transcriptional regulation. The analysis of $p300^{+/-}$ and $cbp^{+/-}$ mice suggested that there is a limiting amount of p300/CBP proteins under physiological conditions, as various levels of embryonic lethality were observed in the heterozygous mice (189). In keeping with these observations, over-expression experiments suggested that transcriptional repression among certain transcription factors can also be mediated through p300/CBP (reviewed in 147). Since the number of signal transduction pathways which require the activity of p300/CBP is so large, Rosenfeld and colleagues (76) proposed that a co-ordinated re-distribution of p300/CBP activity among different classes of factors in a signal-dependent manner would allow the specificity of transcriptional regulation (76, 163). For example, the

engagement of p300/CBP by certain hormone receptors was shown to inhibit AP-1 transcription (76). Over-expression of E2F-1 can block p53 *trans*activation in a p300/CBP dependent manner (92). Similarly, over-expression of p53 represses TRE-regulated promoters and such inhibition relies on the physical interaction between p53 and p300 (8). Furthermore, agonists of the Ras pathway can inactivate cAMP-responsive genes, specifically interfering with CREB-dependent transcription (125). A plausible mechanism for these results suggests that the activation of the Ras pathway promotes p300/CBP association with mitogen-regulated S6 kinase $pp90^{Rsk}$. The physical interaction of $pp90^{Rsk}$ with p300/CBP may be sufficient to compete off CREB-dependent transcription since a catalytically inactive $pp90^{Rsk}$ can still block CREB function (125). Last but not the least, binding of E1A to p300/CBP inactivates a number of cellular and viral promoters and enhancers (147, 155). For example, the expression of genes induced by muscle differentiation is inhibited by E1A (132, 133). E1A also antagonises SV40 and polyoma virus enhancers in a p300/CBP dependent manner (147, 155). Taken together, these findings suggest that p300/CBP is a focal point whereby different signal transduction pathways compete for a limited pool of these promiscuous activators, thereby targeting their specificity towards certain promoters. Viral oncoporteins, such as Ad E1A and SV40 LT, sequester p300/CBP activity, thereby suppressing differentiation in favour to retain cells in an active proliferative state.

ii) An assembly platform for multi-protein complex formation.

The p300/CBP proteins act to nucleate the assembly of diverse co-factor proteins into co-activator complexes (87, 178, 184). Moreover, p300/CBP has been frequently found in complex with other HATs, including pCAF (188), SRC-1 (154), and P/CIP/ACTR/AIB1 (26). By providing an assembly platform for transcription factors,

other HATs and components of the basal transcriptional machinery, they might increase the relative concentration of these factors in a local environment to facilitate protein-protein and protein-DNA interaction. Maniatis and colleagues (81) have proposed an enhanceosome concept which can explain the specificity of transcription regulation mediated by p300/CBP (23, 81). The current view is that in order for a cell to use a limited repertoire of activators or co-activators in response to diverse regulatory cues, co-operativity and transcriptional synergy are employed where a small combination of ubiquitous, signal- and tissue- specific activators can define an exponentially large number of regulatory decisions. Studies on the human interferon (IFNβ) enhancer suggested that the surface of p300/CBP provides an assembly platform for different components of the transcription apparatus (81, 121). The recruitment of p300/CBP, together with transcription factors, such as ATF2/c-Jun, p50/p65 of NF-κB, and interferon regulatory factor 1, and architectural proteins such as high mobility group proteins (HMG), would determine the exact three-dimensional positioning of these multiple binding sites, which is important for co-operativity. Subsequently, it was shown that both p300/CBP and the p300- associated factor pCAF are required for the activation of transcription from the IFNβ enhanceosome (121). Interestingly, p300/CBP and pCAF were found to acetylate the HMG protein in the enhanceosome, and the acetylation of HMG I by p300/CBP also leads to the destabilization and disassembly of the enhanceosome, which is important in turning off IFNβ gene expression (121).

Since many of these multi-protein complexes also contain multiple HATs, it is a surprise at first if they only acetylate nucleosomes. One possible scenario is that transcription factors need to recruit multiple HATs in order to achieve a critical mass of enzymatic activity to overcome deacetylation on promoters. Subsequently, many non-

chromosomal proteins were shown to be acetylated by p300/CBP and pCAF. This suggests that different HATs might have different substrate specificity, and the combination of active HATs is specific to each promoter in a context dependent manner. The three dimensional structure studies on the bromodomain, which is commonly found in many HATs identified so far, revealed its unique role in recognizing acetylated peptides (39, 74). The double bromodomain in $TAF_{II}250$ binds to acetylated peptide 70 times stronger than the pCAF bromodomain (74). The distance of the two binding pockets on $TAF_{II}250$ is about seven amino acid residues apart, which fit well with sites of acetylation observed on H4 *in vivo* (74). Therefore, another possibility exists that the multiple HATs provide a combination of bromodomains in recognizing unique patterns of acetylated nucleosome in promoter regions. Furthermore the bromodomain may also mediate protein-protein and protein-nucleosome interactions. From the studies of the IFNβ enhanceosome, it also indicated that different HATs might function at different stages during the transcription process. In this case, p300/CBP HAT activity is required to terminate IFNβ gene expression, suggesting a complex scenario where HAT activity plays roles in both transcriptional activation and post-induction turnoff (121).

iii) Acetylation-dependent transcription regulation

Acetylation of multiple sites in the core histone tails is known to be associated with transcriptional activity; hypo-acetylation is generally correlated with transcriptional repression and hyper-acetylation is correlated with transciptional activation (reviewed in 21, 54, 60, 170). It was thought that the neutralization of positive charge on histone tails by acetylation might weaken the histone/DNA interaction, thereby facilitating RNA polymerase II and its associated factor to access the DNA template. Crystallographic

studies on the nucleosome structure further indicated that the histone tail was involved in maintaining higher order chromatin structure, and acetylation might relieve chromatin structure to a more 'open' state for transcriptional activation (104). When p300/CBP was found to possess HAT activity, it was immediately assumed that the intrinsic HAT activity was important for its co-activation function. *In vitro*, p300/CBP can acetylate all four core histones, with preference toward H3 and H4 (12, 128). By tethering the p300/CBP HAT domain to the Gal4- DNA binding domain, it was shown that the HAT activity is required to stimulate transcription from the adenovirus major late (AdML) and E4 promoters, but not E1B or the SV40 promoters (109). This demonstrated the promoter selectivity of the HATs. Mutations which abolish HAT function also correlate with loss of stimulation from the AdML and E4 promoters (109). These studies provided evidence that the HAT activity is required for the transcriptional activation. However, it is yet to be established that if the HAT activity acts on nucleosomes, and participitates in chromatin remodelling-related mechanisms. Furthermore, acetylation of specific lysine residues on histone tail (such as K8 and K16 on H4, and K9 and K14 on H3) appear to be most important for transcriptional regulation (21, 54). It is not clear what is the substrate specificity of p300/CBP HAT *in vivo*.

Abundant evidence also highlights the importance of non-chromosomal protein acetylation in regulating gene expression. A growing list of transcription factors, such as p53 (56, 100, 139), E2F (110, 111), c-Myb (160), MyoD (141), GATA-1 (17), EKLF (195) and HNF-4 (153), were found to be acetylated by p300/CBP and/or pCAF. In all cases, acetylation of these transcription factors was reported to enhance their DNA binding activity in gel shift assays. Acetylation neutralises the positive charge on the ε-amino group of the lysine residues. This charge modification could cause conformational change, which seems to be a plausible explanation for the enhanced

DNA binding activity observed for p53 (56). Alternatively, acetylation may create a motif which enhances protein-DNA recognition. This mechanism seems to accommodate well with the observation that so many transcription factors have been independently reported to have enhanced DNA binding activity upon acetylation. HIV-Tat is another substrate acetylated by p300/CBP and pCAF (78). Acetylation of Tat synergies with its transcriptional activation of the HIV-1 LTR (78). In this case, acetylated Tat was also found to promote its dissociation from TAR RNA, and enhanced its binding to the Tat-associated kinase, CDK9/P-TEFb. One example exists whereby acetylation inhibits transcription. Acetylation of dCTF inhibited its binding to Armadillo, thereby leading to down-regulation of transcription (172). The basal transcription factors, TFIIE and TFIIF, were reported to be acetylated by p300/CBP (68). The consequence of this modification on basal transcription machinery function has not been explored. Overall, evidences suggested that nucleosome and protein acetylation are both relevant towards p300/CBP-dependent transcriptional regulation.

Regulation of cell cycle by p300/CBP.

The fact that p300/CBP are specific targets of E1A immediately suggested their potential importance in cell cycle regulation (182, 185). Detailed studies on the interaction between E1A and p300/CBP support such a role in inducing cellular DNA synthesis and S phase progression (155). Furthermore, E1A mutants deficient in binding to p300 are defective in transformation function (173). Direct evidence that p300/CBP are important in cell cycle regulation was demonstrated in the study of *p300* and *cbp* knockout mice (189). p300/CBP are required in both cellular proliferation and differentiation. *p300* nullizygous embryos start to die about E10.5, showing defects in neural tube closure and heart development. Specifically, defects

in cardiac myocyte differentiation were thought to contribute to the early lethality in these homozygous mice. Consistently, cell-based assays indicated that p300/CBP collaborate with the MyoD family of proteins in modulating the expression of downstream myogenic factors such as myogenin and MEF2, and promote cell cycle withdrawal in myocytes induced to differentiate (132, 133, 141, 192). Furthermore, the p300/CBP interacting protein pCAF is also required for this myogenic program since micro-injection of either anti-p300/CBP or anti-pCAF antibodies block MyoD-dependent transcription in muscle cells. Whilst the p300 HAT domain was dispensable for MyoD transcription, mutation in the HAT domain of pCAF impairs Myo-D-dependent *trans*activation (141). $cbp^{+/-}$ mice showed defects in haematopoietic differentiation, which subsequently led to haematologic malignancies at an advanced age (89). In this case, the differentiation promoting properties of CBP contributes a tumour suppression function. Consistently, p300/CBP has been implicated in the differentiation of haematopoietic tissues, such as B cells (46). Strikingly, inactivating p300/CBP homologue *cbp-1* gene in *Caenorhabditis elegans* was shown to block nearly all differentiation programs except neuronal differentiation, which is the default pathway (145); mesodermal, endodermal, and hypodermal cells were completely absent in most of the embryos. A plausible mechanism is that two *C. elegans* transcriptional activators SKN-1 and PAL-1 require p300/CBP to initiate differentiation. Interestingly, inactivation of components of the HDAC complexes, such as *hda-1*, *rba-1* and *rba-2*, can rescue certain *cbp-1* phenotypes (145). This provides indirect evidences that p300/CBP HAT activity is required for differentiation, at least in *C. elegans*, to counteract the repressive role on differentiation imposed by HDACs. These findings established a role for p300/CBP in cellular differentiation, which could partially contribute to its tumour suppressive function.

While it makes good sense for viral oncoproteins such as E1A to antagonise p300/CBP function, and thereby suppress cellular differentiation in favour of cellular proliferation, it is

interesting to note that p300/CBP may also be required for cellular proliferation. *p300*$^{-/-}$ embryos are significantly smaller than their wild-type littermates, showing defects in cellular proliferation (189). *p300*$^{-/-}$ MEFs (mouse embryonic fibroblasts) grow more slowly compared to wild-type cells, with phenotypes reminiscent of senescence. Since binding of E1A to p300 also correlates with E1A-induced DNA synthesis (155), it is possible that the E1A-p300 complex is an active complex in stimulating cellular growth and proliferation.

Analysis of the *cbp-1* deficient embryos of *C. elegans* suggested that apoptotic pathways were not affected (145). However, cell based assays indicated that p300/CBP can be involved in apoptosis. Cells deficient in p300, but not in CBP, have impaired IR (ionizing radiation) sensitivity (193). Moreover, p300/CBP deficient cells were shown to be resistant toward retinoic acid induced apoptosis (77). Functional sequestration of p300/CBP activity via E1A, or with a dominant negative fragment of p300, blocks p53-dependent apoptosis (8, 97), suggesting that p300 is involved in p53-dependent apoptosis. In this case, contradicting evidence also exists that co-expression of p300 with p53 induces p53-mediated G1 arrest, but prevents p53-dependent apoptosis in SAOS2 cells (92, 160). However, in those assays, p300 augmented E2F-1 dependent apoptosis (92). An additional level of complexity arises when p300 was demonstrated to regulate p53 turnover (53). The N-terminal region of p300 can mediate a ternary complex between p300-p53-MDM2, which in turn controls p53 stability. The proto-oncogene MDM2 is important in regulating p53 stability, via a ubiquitin-dependent protein degradation pathway (53, 101). MDM2 mutants capable of binding to p53, but deficient in binding to p300, fail to promote p53 degradation. Similarly, a short p300 fragment consisting of the p53 binding region in the N-terminus acts in a dominant negative fashion to enhance p53 stability (53). Therefore, p300 has a dual role in controlling p53 activity, both in augmenting p53 *trans*-activation function and promoting p53 degradation. This provides a possible explanation of the contradictory observations for p300 in regulating p53-mediated apoptosis. The exact experimental outcomes

may very much depending on the cellular status, whether in favour of p300 in activating p53 transcription, or in promoting p53 degradation. The p300/CBP-pCAF complex can arrest the cell cycle (188). This protein complex might regulate target gene expression which is crucial in controlling G1/S transition, such as p21/WAF1 (47). Over-expression of E1A, which antagonises pCAF binding to p300/CBP, drives cells into S phase (188). As discussed above, the p300/CBP-pCAF complex can co-operate with MyoD in myogenesis. Overall, p300/CBP proteins are involved in all cellular programmes, from differentiation, to proliferation and apoptosis. This leads on to the next question as to how p300/CBP are regulated in a timely manner to exert biological specificity.

Regulation of p300/CBP activities

p300/CBP are nuclear phosphoproteins (185). CBP, together with various factors known to be important regulators of the cell cycle and transcription, such as p53, pRb, Daxx, PML, BLM and SUMO-1, reside in a unique nuclear structure called the nuclear body (reviewed in 198). At present, the biological significance of the nuclear body is not well understood and it is proposed that it is a depot for storage and titration of nuclear proteins. The phosphorylation status of p300/CBP is cell cycle regulated, being hyperphosphorylated at mitosis (185). p300 isolated from undifferentiated F9 cells is unphosphorylated. It is then phosphorylated and becomes competent to activate transcription upon treatment with retinoic acid which induces differentiation (83). Viral oncoproteins, SV40 LT and E1A, bind to p300/CBP in the same region, but have a different effect on p300/CBP phosphorylation status (11, 45). T antigen co-immunoprecipitates with the hypophosphorylated p300, and suppresses p300/CBP phosphorylation (45). In contrast, E1A simulates p300/CBP phosphorylation, probably via cyclin/CDKs (45). Cyclin-dependent kinases, Cdk2 and Cdc2 (11) have been found to phosphorylate p300 *in vitro*. p300 is required for the induction of p53-independent p21 gene

expression, which is an inhibitor of CDK activity. Therefore differentiation can also be associated with hypo-phosphorylated p300 (114). The C-terminal region of p300/CBP interacts with cyclin E-Cdk2, as well as TFIIB and E1A. 12S E1A, which is an inhibitor of p300-dependent transcription, does not affect p300 association with cyclin E-Cdk2 (48). However, 13S E1A, which is also a pleiotropic transcription activator, enhances p300/cyclin E-cdk2 association (48).

p300/CBP can also be phosphorylated by other kinases in response to various upstream signals. For example, CBP is phosphorylated by MAPKs (mitogen-activated protein kinases) or PKA (protein kinase A), both of which enhance CBP *trans*activation potential (75). The bromodomain of GCN5 was reported to be phosphorylated by the DNA-PK family of kinases, which repressed GCN5 HAT activity (14). It is possible that the p300/CBP bromodomain might be similarly regulated by DNA-dependent protein kinases.

An important question regarding p300/CBP function is how its HAT activity is controlled during the cell cycle. Ait-Si-Ali *et al* (2) showed that HAT activity peaks at G1/S transition. Furthermore, phosporylation of p300/CBP by cyclin E-cdk2 complexes in the C-terminal region of the protein stimulated the HAT activity (2). In the same study, E1A was also demonstrated to activate the HAT activity (2). These observations suggest that the HAT activity drives cell cycle progression, and that viral oncoproteins might mimic proliferative signals to activate p300/CBP HAT activity. Similarly, E1A was shown to immunoprecipitate HAT active fractions of p300/CBP and pCAF (12). However, this scenario is challenged by several reports indicating that E1A inhibits p300/CBP and pCAF HAT activity *in vitro* (25, 58). Furthermore, p53 acetylation is also inhibited in the presence of E1A (25). Results from various laboratories have indicated that E1A's effect on histone acetylation is concentration dependent; at a low level, it enhances histone acetylation, whereas at a high level, it represses histone acetylation. The physiological relevance of these observations is not clear. However, one noticeable feature

is that E1A does not inhibit the intrinsic HAT activity of p300/CBP. E1A was acetylated in those *in vitro* assays, and p300/CBP was auto-acetylated. Given p300/CBP proteins are consistently found in complex with other HATs, it is possible that other HATs, such as pCAF or SRC-1, might acetylate p300/CBP as a substrate. How acetylation controls p300/CBP function awaits further investigation.

p300/CBP in human diseases.

Accumulating evidence has demonstrated that mutations in *p300* and *cbp* genes are linked to the onset of a number of human tumours, suggesting that p300/CBP may function as classical tumour suppressors (reviewed in 51). This is consistent with the early working model that viral oncoproteins, such as E1A and SV40 LT, target and antagonize p300/CBP tumour suppression functions. Mutations in *cbp* were first described in RTS patients, which is an autosomal-dominant disease characterized by mental retardation, skeletal abnormalities and high incidence of neoplasia (129). Most of the RTS patients are heterozygous for the mutation, suggesting a full complement of *cbp* gene dosage is crucial for normal development. Consistently, $cbp^{+/-}$ mice showed skeletal abnormalities reminiscent of RTS patients (158). As discussed above, $cbp^{+/-}$ mice have defects in haematopoietic differentiation, which subsequently leads to haematologic malignancies at advanced age, providing direct evidence that CBP functions as tumour suppressor (89). Furthermore, bi-allelic inactivating somatic mutations in the *p300* gene have been observed in gastric, colon and breast cancers (50, 122). Various *p300* mutations leading to truncated proteins were detected in primary tumours and tumour cell lines (50). In many cases, the somatic mutations are associated with loss of heterozygosity (LOH) of the second allele. These observations are consistent with Knudson' hypothesis that *p300* behaves as a classical tumour suppressor gene.

The *p300/cbp* genes are also found in various translocation events, and contribute to haematological malignancy probably through gain of function mutation. For example, MOZ-CBP fusion was reported in one AML (acute myeloid leukemia) patient (16). The *MOZ* gene encodes a putative HAT based on it homology to a yeast HAT, SAS2. The hybrid protein consists of 5'-MOZ/CBP-3' and retains the HAT domains from both proteins. Similarly, a second translocation of the *cbp* gene to the *MLL* gene (mixed lineage leukemia) was observed in chronic myeloid leukemia and myelodysplastic syndrome (152). Most of these translocations arise as a consequence of anti-cancer treatments. In another case, one patient suffering from therapy-related AML was identified as having an in-frame fusion of *MLL* with *p300* gene (67). Since both MOZ and MLL have been implicated in chromatin remodeling functions, these fused proteins may cause aberrant and deregulated gene expression to prevent proper differentiation and cell cycle control. Whilst the p300/CBP tumour suppression function is not well understood, the fact that they interact with both p53 (55, 92, 97) and pRb/E2F (92, 164) pathways may provide clues as to the underlying mechanism. As demonstrated by Lee *et. al.* (92), the ability of p300 to augment p53 mediated growth arrest, and E2F-1 dependent apoptosis may be one explanation to its tumor suppressive function.

Discussion

It has becoming clear that p300/CBP are versatile transcription co-activators, participating in many cellular processes. These inculde: 1) the formation of a physical bridge between transcription factors and the basal transcription machinery; 2) an intrinsic HAT activity, which regulates cellular and nucleosomal targets; 3) a scaffolding function for multi-protein complexes formation; 4) providing basic cellular functions, including DNA replication, cell growth, differentiation and apoptosis; 5) mediating cross-talk among different signal transduction pathways.

Through studies on p300/CBP, a lot has been learned about transcriptional regulation and cell cycle regulation. However, many questions relating to p300/CBP function remain to be answered. For example, there are uncharacterised members of the p300/CBP family, and it will be interesting to establish how their properties differ from each other. Secondly, the regulation of p300/CBP protein function is relatively unexplored at present. It is known that p300/CBP can be phosphorylated and acetylated. CBP co-localizes with PML proteins in nuclear bodies, which are discrete nuclear structures that contain other proteins known to carry key function in cell cycle regulation, transcription and translation control. Curiously, SUMO-1, which performs another kind of post-translational modification called SUMOylation by attaching a ubiquitin-like protein SUMO to the lysine residues, is also localised in the nuclear bodies (198). It is possible that p300/CBP can be SUMOylated, in common with various other proteins in the nuclear body. How the p300/CBP family protein activity is modulated at the post-translational level requires further investigation.

Although the intrinsic HAT activity of p300/CBP is undoubtedly involved in transcriptional control, its physiological substrates are unclear. Furthermore, it is not known whether p300/CBP HAT activity acts only on nucleosomes. The picture is much more complicated as many transcription factors, and non-chromosomal proteins, were found to be acetylated by p300/CBP *in vitro*. Protein acetylation could be a much more common event than previously thought, and contribute in many aspects of cellular function apart from transcription. In this case, evidence does exist that acetylation might also regulate nuclear transport (13). Genetic and biochemical analysis suggests that p300/CBP are classical tumour suppressors, although the underlying molecular mechanisms are still poorly understood. The HAT activity appears to contribute to their tumour suppressive function, as p53 acetylation was shown to be induced under certain DNA damaging conditions (139). Moreover, genetic analysis supports a

role for the HAT activity in promoting cellular differentiation, which may also indirectly contribute to the tumour suppressive effect.

An intriguing report indicates that p300 is involved in controlling p53 stability together with MDM2 (53). This observation will surely lead to many more investigations on the functional interaction between p300/CBP and the ubiquitin-dependent protein degradation system, which is important in cell cycle control. Further experiments will be required to substantiate this finding. Since both acetylation and ubiquintination occurr on lysine residues, it is possible that the HAT activity also plays a role in regulating protein stability.

Chapter 2: Materials and Methods

Plasmids

pGEX-Rb and Δ21&Δ22 mutants have been described in Bandara *et. al.*, 1991 (10). pGEX-Rb 763-928, pCDNA3-9E10Rb, pGEX-107, pGEX-p53 were kind gifts from Robert White and Sybille Mittnacht. pGEX-Rb 794-829, 844, 857, 864, 876, 884, 896, 910 (1) were kindly provided by Peter Adams. pGEX-Rb 830-884, 830-928, 881-928, 792-928, 702-766, and 641-775 were cloned by direct PCR (for primers, see table 2.1) from pCDNA3-9E10-Rb. pGEX-Rb 379-656 was cloned by restriction digest using NheI and EcoRI from pGEX-Rb 379-928. pGEX-E1A (12S), ΔN36, pm928, were cloned by PCR (for primers, see table 2.1) from pCMV-E1A or pCMV-E1A pm928 constructs (88). pGEX-E1AΔCR1 and E1AΔCR2 were cloned by PCR (for primers, see table 2.1) from Sp 53 and Sp CS (9). The PCR products were cloned directly into pGEX-KG vector (Pharmacia). His-p300$^{1195\text{-}1673}$, Flag-p300$^{1134\text{-}2414}$ bacterial expression vectors and Flag-300 (FL) baculovirus were kind gifts from Nakatani (128, 188). Mammalian expression vectors for MDM2, cyclin E, and CDK2 also have been described (101, 119). Flag-HDAC was a gift from Linda Smith. 3X-E2F (luc) and 3x-E2Fmut (luc) were gifts from Lan Bandara. pGEX-cyclin D was a gift from Rene Bernards. pGEX-E7 was a gift from Kate Moore. $TAF_{II}250$ baculovirus was a kind gift from Peter Verrijzer. pG4-p53, pG4-E2F-1, pHA-E2F-1, pCMV-βgal, pVP-16, pG4-p300$^{611\text{-}2284}$, and pG4-p300$^{1572\text{-}2284}$ plasmids have been described in Lee *et. al.* (92). pG4-AdML(luc) reporter was cloned from pG4-AdML(CAT) reporter into pGL13-luc backbone (France *et. al.*, personal communication). pGEX-p300$^{1\text{-}595}$ and pGEX-p300$^{1709\text{-}1913}$ were gifts from Richard Eckner (45). The pHA-NAP-2$^{1\text{-}123}$, $^{98\text{-}386}$, $^{1\text{-}279}$ and $^{234\text{-}386}$ were generated by direct PCR from pHA-NAP-2 (for primers, see table 2.1). The PCR products were cloned into the pCDNA3-HA vector using BamH1/XhoI restriction enzymes. pHA-NAP-2,

pHA-NAP-2$^{110-230}$, pHA-NAP-2$^{\Delta 110-230}$, His-NAP-2 and pVP16-NAP-2 plasmids were cloned using standard procedure (Shikama *et. al.*, in press). For the construction of various pGEX-p300 constructs, the appropriate fragments were re-cloned from pG4-p300 constucts which were described in Lee *et. al.* (92). In short, pG4-p300$^{1818-2080}$ was cloned by PvuII digestion from the pG4-p300$^{1572-2284}$ backone. The insert was cloned into the XhoI/BglII sites of the pG4 backbone. pG4-p300$^{1906-2284}$ was cloned using BstEII and BglII digestion from the pG4-p300$^{1572-2284}$ backbone. To generate pGEX-p300$^{1302-1737}$, pG4-p300$^{611-2284}$ was cut by XbaI and Eco47III, and the fragment was cloned into the XhoI(blunt)/XbaI sites of the pGEX-KG. To generate pGEX-p300$^{1195-1673}$, the fragment was cut out from pG4-p300$^{611-2284}$ using EcoRI and NotI (blunt), and cloned into the XhoI (blunt)/EcoRI sites of the pGEX-KG backbone. pGEX-p300$^{1572-1906}$ was cloned by cutting the fragment using XhoI (blunt)/SacI from pG4-p300$^{1572-1906}$, and cloned into the SmaI/SacI sites of pGEX-KG. pGEX-p300$^{1818-2080}$ was cloned by cutting the fragment from pG4-p300$^{1818-2080}$ using ClaI (blunt)/SacI, and cloned directly into the XbaI (blunt)/SacI sites of the pGEX-KG. pGEX-p300$^{1906-2284}$ was cloned by cutting the insert out from pG4-p300$^{1906-2284}$ using XhoI (blunt)/SacI, and cloned into the SmaI/SacI sites of the pGEX-KG.

Plasmids	5'-primer	3'-primer
GST-Rb (830-884)	5'-cgggatccagaatcttagtatcaattgg-3'	5'-cggaattctcattcatctgatccttcaatatc-3'
GST-Rb (830-928)	5'-cgggatccagaatcttagtatcaattgg-3'	5'-cggaattctcatttctcttccttgtttg-3'
GST-Rb (881-928)	5'-cgggatccggatcagatgaagcagatg-3'	5'-cggaattctcatttctcttccttgtttg-3'
GST-Rb (792-928)	5'-cgggatcccctagttcacccttacgg-3'	5'-cggaattctcatttctcttccttgtttg-3'
GST-Rb (641-775)	5'-ccgggattccattgaaatctacctctc-3'	5'-tcacctggtggaagcatactgc-3'
GST-Rb (702-766)	5'-cgggatcccaaattatgatgtgttccatg-3'	5'-ccctcgagtcaaatatttgttttcagtctctg-3'
GST-E1A12S	5'-cgggatccatgagacatattatctgccac-3'	5'-ccctcgagttatggcctggggcgtttac-3'
GST-E1A(pm928)	5'-cgggatccatgagacatattatctgccac-3'	5'-ccctcgagttatggcctggggcgtttac-3'
GST-E1A(ΔN36)	5'-cgggatcccattttgaaccacctacc-3'	5'-ccctcgagttatggcctggggcgtttac-3'
GST-E1A(ΔCR1)	5'-cgggatccatgagacatattatctgccac-3'	5'-ccctcgagttatggcctggggcgtttac-3'
GST-E1A(ΔCR2)	5'-cgggatccatgagacatattatctgccac-3'	5'-ccctcgagttatggcctggggcgtttac-3'
NAP2(1-123)	5'-cgggatccatggcagaaaacagtctt-3'	5'-ccactcgagctaccacgctgactctgc-3'
NAP2(98-386)	5'-cgggatccttgtaccagcctcta-3'	5'-ccgctcgagtctcactgctgcttgc-3'
NAP2(1-279)	5'-cgggatccatggcagaaaacagtctt-3'	5'-ccactcgagctaggttcgtacggtgcc-3'
NAP2(234-386)	5'-cgggatccgacccgttttcttttg-3'	5'-ccgctcgagtctcactgctgcttgc-3'

Table 2.1 Primers used for PCR cloning.

Site-Directed Mutagenesis

pDNA3-9E10Rb point mutants were generated using the Quickchange site-directed mutagenesis kit (Stratagene). In short, primer pairs (one for the sense strand, and the other for the anti-sense strand) for specific mutants were synthesized (Genosys), see table 2.2 for the primers for each mutant. The mutation sequences were introduced into the middle of the primers, and the primers were designed to have a T_m approximately 78°C (T_m=81.5 + 0.41(%GC) – 675/(number of base pair of the primer) - % mismatch). PCR reactions were carried out to generate the mutant DNA sequences. Since some of the primers have strong secondary structure, some PCR reactions were carried out with modification. For example, in some reactions, 10% glycerol was added to the reaction mixture to relax the primer secondary structure. In other cases, first the sense and the anti-sense primers were used in independent PCR reactions for 3 cycles, then the two halves of the PCR reaction mixtures were mixed for another 16 cycles. Using this method gave better product yield. The parental DNA, which was isolated from *E.coli* and contained methylation modification, was digested away using DpnI restriction enzyme. The restriction digest mixtures were used to transform XL1-Blue supercompetent cells. The cloning strategy was such that by introducing the specific mutations, it would either create new restriction site(s), or destroy certain existing restriction site(s). The first screening was therefore carried out using restriction digest. 2-4 of the positive clones were then selected for DNA sequencing to confirm the correct mutants were made. All DNA sequencing was carried out by MWG Biotech. The DNA sequencing covered approximately the last 350 amino acids of the pRb sequences. In order to eliminate possible mutations elsewhere due to PCR, the mutant sequences were re-cloned into the wild-type backbone of pCDN-3-9E10Rb using NheI/XhoI digest. Generation of pGEX-Rb point mutants was done using Nhe1/EcoRI digests from the

respective pCDNA3-9E10-Rb mutant constructs. To generate the pG4-Rb and mutants, the EcoRI fragment from the pCDNA3-9E10-Rb or mutants were sub-cloned into the XhoI (blunt) of pG4m backbone(177). All pG4-Rb constructs were from amino acid 300-928.

Plasmids	Primer pair
713KA	5'-gtgttccatgtatggcatatgcgcagtgaagaatatagacc-3' 5'-ggtctatattcttcactgcgcatatgccatacatggaacac-3'
713KR	5'-gtgttccatgtatggcatatgcagagtgaagaatatagacc-3' 5'-ggtctatattcttcactctgcatatgccatacatggaacac-3'
765KA	5'-cggtcttcatgcagagactggcaacaaatattttgcagtatgc-3' 5'-gcatactgcaaaatatttgttgccagtctctgcatgaagaccg-3'
765KR	5'-cggtcttcatgcagagactgcgcacaaatattttgcagtatgc-3' 5'-gcatactgcaaaatatttgtgcgcagtctctgcatgaagaccg-3'
740KA	5'-gctgttcaggagacattcgcacgtgttttgatcaaagaagag-3' 5'-ctcttctttgatcaaaacacgtgcgaatgtctcctgaacagc-3'
720,722KA	5'-gcagtgaagaatatagaccttgcattcgcaatcattgtaacagcatacaagg-3' 5'-ccttgtatgctgttacaatgattgcgaatgcaaggtctatattcttcactgc-3'
729KA	5'-cattgtaacagcatacgcggatcttcctcatgctgttcag-3' 5'-ctgaacagcatgaggaagatccgcgtatgctgttacaatg-3'
873,874RR	5'-ccctcctaaaccactgagaagactacgctttgatattgaag-3' 5'-cttcaatatcaaagcgtagtcttctcagtggtttaggaggg-3'
873,874QQ	5'-caaccctcctaaaccactgcagcaactacgctttgatattgaag-3' 5'-cttcaatatcaaagcgtagttgctgcagtggtttaggagggttg-3'

Table 2.2 Primers used for site-directed mutagenesis.

In vitro protein acetylation assay

PAGE analysis of protein acetylation was performed as described (128), with slight modification. Indicated amounts of input proteins were incubated at 30°C for 45-60 minutes in 30μl volumes with 5X HAT assay buffer (250mM Tris pH 8.0, 25% glycerol, 0.5mM EDTA, 250mM KCl, and 10mM sodium butyrate), 90pmol [^{14}C]acetylate CoA (55 mCi/mol, Amersham Life Science Inc.) and appropriate amount of water. After incubation, reactions were stopped by adding 15μl of 3X SDS loading buffer (150mM Tris-HCl (pH6.8), 6% SDS, 30% glycerol, 0.3%

bromophenol blue, 3% mercaptoethanl), and analysed on SDS-PAGE. The gels were dried and exposed for autoradiography for 48-96 hours. Quantiation of protein acetylation level was done by phosphoimaging analysis typically after 16-24 hours of exposure.

For the filter scintillation assay, samples were prepared as described for in gel assay. Chicken histones used in the assay were purified and kindly provided by Linda Smith and William Cairns. After incubation, the reaction mixture was spotted onto Whatman P-81 phosphocellulose filter paper and washed with 0.2M sodium carbonate buffer (pH 9.2) at room temperature 2-3 times, and once with acetone. The dried filters were counted in a liquid scintillation counter. The synthetic NAP peptides 141 (GDEEGEDEDDADVNPKK) and 142 (NSLSDGGPADSVEAAKN), and the Rb peptide (see chapter 3) and the histone H4 peptide (SGRKGGKGLGKGGAKRHRK) were provided by Newcastle University.

Expression and Purification of Glutathione S-transferase Fusion Proteins

Glutathione S-transferase fusion protein expression and purifications were performed as described in the company protocol (Pharmacia). Fresh overnight cultures of BL21(DE3) pLys (Invitrogen) transformed with the appropriate pGEX-recombinants were diluted 1:10 in Luria-Bertani medium (LB) containing ampicillin (100μg/ml) and incubated at 37°C with shaking. After 2 hours of growth at 37°C, isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) was added to final concentration of 0.5mM. The cultures were subsequently incubated at 30°C for 4-5 hours for protein expression before harvesting.

For fusion protein purification using glutathione-Sepharose (Pharmacia), bacterial cultures were pelleted by centrifugation at 6,000rpm for 10 minutes at 4°C. The pellets were resuspended in cold PBS (Sigma, 5ml PBS/100ml of bacterial culture, PBS supplemented with protease inhibitors cocktail (Calibiochem)). The bacteria were then lysed on ice by mild sonication, and Triton-X-100 (Sigma) was added to the lysate to a final concentration of 1%.

The lysate was incubated at 4°C for 30 minutes, then centrifuged at 13,000rpm, 4°C for 30 minutes. The bacterial supernatants were rocked for 30-45 minutes at 4°C with glutathione-Sepharose (200µl of beads/100ml culture). The glutathione-Sepharose beads were washed three times with 20ml of cold PBS supplemented with 1% Triton, and once with 20ml of cold PBS. For analysis of bound proteins, appropriate amounts of beads were boiled in 1X sample buffer (50mM Tris-HCl (pH6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue, 1% mercaptoethanl), and loaded onto a SDS-polyacrylamide gel. Proteins were visualized by Coomassie blue staining. GST-fusion proteins were eluted in elution buffer (50mM Tris-HCl (pH8.0), 10mM reduced glutathioine, 120mM NaCl). All fusion proteins were subsequently dialysed into BC100 buffer (20mM Tris-HCl (pH8.0), 0.5mM EDTA, 100mM KCl, 20% glycerol, 0.5mM DTT, 0.5mM PMSF).

Expression and Purification of His-tagged fusion proteins

The procedure for His-tagged fusion protein expression is similar to that described for GST-fusion protein expression. To purify His-tagged fusion protein, the procedure was adopted from the company instruction manual (Pharmacia). In short, 5ml of PBS (supplemented to final 0.5M NaCl, 10mM imidazole pH7.4 and protease inhibitor cocktail (Calibiochem)) was used to resuspend bacterial pellets from 100ml cultures. The bacteria were then lysed on ice by mild sonication and pelleted at 13,000rpm for 30 minutes, at 4°C. The supernatant was collected and incubated with Ni-NBT agarose (QIAGEN) at 4°C for 1 hour. After incubation, the beads were washed 5 times in PBS (supplemented to final 0.5M NaCl, 40mM imidazole pH7.4). Elution was carried out using BC100 buffer supplemented with 200mM imidazole. His-E1A (13S) protein was a kind gift from Laurent Delavaine.

Production of Flag-p300 $^{1135-2414}$ baculovirus

Flag-p300$^{1134-2414}$ bacterial expression vector was a kind gift from Y. Nakatani. To generate the baculovirus of Flag-p300$^{1135-2414}$, the BAC-To-BAC Baculovirus Expression system from Gibco Life Technologies was used. In short, Flag-p300$^{1134-2414}$ sequence was taken out from the bacterial expression vector by RsrII and NotI digestion. The fragment isolated was cloned directly into the RsrII and NotI sites of pFastBacHTa vector. After selecting the correct pFastBacHTa-Flag-p300$^{1134-2414}$ clones, the plasmid was transformed into Max Efficiency DH10Bac Cells, which contained Bacmid and helper plasmid. The colonies containing the recombinant bacmid showed white in colour, and recombinant bacmid DNA was isolated and used to transfect insect cells using CellFectin Reagent. Recombinant baculovirus containing Flag-p300$^{1135-2414}$ was harvested 48 hour after transfection. The virus was amplified to a titre of 1 x 10^8 pfu/ml and used for infection for protein purification.

Expression and Purification of Flag-p300 from sf9 cells

To express Flag-p300 proteins in sf9 cells, 1.5 x 10^7 sf9 cells were infected with the appropriate baculovirus at a multiplicity of infection (M.O.I.) of 10. The sf9 cells were harvested for protein purification after 48 hours of incubation at 25°C. The method of purification was essentially described in Chen and Tjian, 1996 (29). The sf9 cells were pelleted by mild centrifugation at 700rpm, washed twice with cold PBS and lysed in 2ml of HEMG buffer (25mM Hepes-KOH (pH7.6), 0.1mM EDTA, 12.5mM $MgCl_2$, 10% glycerol) supplemented with 400mM KCl, 0.1% NP-40 and protease inhibitor cocktail (Calibiochem). The samples were quickly freeze-thawed three times and centrifuged at 13,000rpm, 4°C for 30 minutes. The supernatant was collected and incubated with anti-flag M2 affinity gel (Sigma, 200ul of beads/ml of supernatant) in HEMG buffer containing 200mM KCl. After 2 hours of incubation

at 4°C, the beads were washed 5 times with HEMG buffer supplemented with 200mM KCl. Proteins bound on the beads were eluted in elution buffer which was composed of basically HEMG buffer supplemented with 160mM KCl and 1mg/ml Flag-peptide (Sigma). Elution was carried out at 4°C for 2 hours. Proteins eluted were analysed by SDS-PAGE and visualized by Coomassie blue staining

Tissue Culture and Transfection

The C33A, U2OS, SAOS2, T98G, HEK293, HeLa, and A31 cells were all cultured in Dulbecco modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. Sf9 cells for baculovirus production and protein expression were maintained in TC100 medium (Gibco) supplemented with 5% fetal bovine serum at 25°C. The U2OS cell line with the integrated reporter described in chapter 4 was a kind gift from Rene Bernards. HELA nuclear extracts were purchased from Computer Cell Culture. TSA were purchased from Sigma. Transfections were carried out using the calcium phosphate method as described in the Promega manual. For the mammalian two-hybrid assay, 1μg of the indicated pG4- plasmids were transfected with 0.5μg of pVP16 or pVP16-NAP2 into U2OS cells. Experiments were repeated in C33A cells. For the luciferase assay, cell extracts were prepared in reporter lysis buffer (25mM Tris-H_3PO_4 pH7.8, 2mM 1,2-diaminocyclohexane tetra acetic acid, 2mM DTT, 10% glycerol and 1% Triton X-100), and centrifuged at 13,000rpm at 4°C, 10 minutes. The supernatant was assayed for luciferase activity by mixing 300μl luciferase assay reagent (1:4, Promega) with 60μl cell extract and measuring the activity with a luminometer (Berthold Lumat). To measure β-galactosidase activity, 100μl cell extract was mixed in 100μl of 2X assay buffer (200mM Na phosphate buffer, pH7.3, 2mM $MgCl_2$, 100mM β-mercaptoethanol, 1.33mg/ml ONPG (Sigma)). The reaction mixtures were incubated at 37°C until a reasonable

yellow colour developed. The reaction was stopped by adding 500µl of 0.5M Na_2CO_3 to each sample, and the absorbance was measured at 420nm. The transcriptional activity of the reporter assay was normalised by luciferase activity/β-galactosidase activity. Immunoblotting was also carried out to confirm the expression of the transfected constructs. All reporter gene assays presented have been repeated at least 2-3 times to ensure the reproducibility of the result.

Western Blot and Immunoprecipitation

Anti-Ac-K antibody was purchased from New England Biolab. Anti-Ac-H4 antibody was purchased from Serotec. Anti-Rb antibody IF-8 was kind a gift from Julian Gannon. G3-245 anti-Rb monoclonal antibody was purchased from Pharmingen and C-15 anti-Rb polyclonal antibody was purchased from Santa Cruz. E2F-1 antibody KH95 was purchased from Santa Cruz. Anti-MDM2 antibody H221 was purchased from Santa Cruz and Ab-1 monoclonal antibody was purchased from Calibiochem. Anti-Flag antibody (M2) was purchased from Sigma. Anti-p300 antibodies, C-20 and N-15 were purchased from Santa Cruz. Monoclonal anti-p300 antibody Ab-1 was purchased from Calibiochem. Anti-myc antibody 9E10 (Santa Cruz); anti-G4 antibody (Santa Cruz); anti-HDAC2 antibody (Santa Cruz); anti-HA antibody (Roche) were used for immunoblotting for the respective proteins.

To immunoprecipitate the endogenous acetylated pRb, cell pellets of SAOS2, U2OS, T98G, A31, C33A and HEK293 were lysed in IPH buffer (50mM Tris pH 8.0, 150mM NaCl, 5mM EDTA, protease inhibitor cocktail (Calibiochem), 5µM TSA (Sigma)), supplemented with 0.5% NP-40. Protein concentrations of the extracts were determined using Bradford assay (BioRad). Protein-A-agarose was first incubated with anti-Rb antibodies IF-8 and C15 for 1-2 hours at 4°C. The beads were washed 3 times with IPH buffer (with 0.5% NP-40), and incubated with cell extracts overnight at 4°C. After overnight incubation, the beads were washed three

times with IPH buffer. Proteins bound to the beads were eluted with 3X SDS loading buffer and analysed by Western blotting using the appropriate antibodies.

All other immunoprecipitations described were carried out in the same way as above, with slight modification of the NP-40 concentration. To immunoprecipitate pRb with MDM2, the NP-40 in the lysis buffer was diluted to final of 0.1%. Subsequent washes were carried out in IPH buffer with 0.1% NP-40.

In vitro-pulldown Assays

For the pRb/MDM2 experiments, 2μg of GST-pRb was actylated by Flag-p300 (FL) in a standard 30μl reaction as described above, but using 1μl of 10mM non-radiolabelled acetyl-CoA instead. For unacetylated pRb samples, acetylation reactions were carried out without acetyl-CoA. After the acetylation reaction, the reaction mixture was incubated with Flag beads (Sigma) in 400μl of IPH buffer (0.5% NP-40) to remove Flag-p300. The supernatant was collected and immunoprecipitated with either anti-Ac-K antibody or anti-Rb antibody to purify the acetylated and unacetylated pRb respectively. *In vitro* transcribed and translated MDM2 (Promega IVT manual) was incubated either with acetylated or unacetylated pRb to assess binding efficiency. After incubation, the beads were washed three times with IPH buffer (0.25% NP-40), and samples analysed on SDS-PAGE followed by Western blot analysis. Anti-MDM2 antibody H221 (Santa Cruz) was used to detect MDM2 binding to pRb.

To map the p300/NAP-2 binding domain(s), the indicated NAP-2 derivatives were *in vitro* transcribed and translated, and ^{35}S-labelled using TNT-coupled reticulocyte lysate system (Promega) and incubated with GST-beads loaded with the indicated fusion proteins in IPH buffer (0.5% NP-40). After incubation at 4°C for 2 hours, the beads were washed three times in 1ml of IPH buffer. Proteins bound to the beads were eluted with 3X SDS loading buffer and analysed by SDS-PAGE, followed by autoradiography. Similar experimental procedures were

used for identifying NAP-NAP interaction domains. All mapping experiments were repeated at least twice to ensure the reproducibility of the results. All *in vitro* pulldown assays described in chapter 5 were carried out in the same way as described above.

Cell cycle analysis

For flow cytometry analysis, 10μg of an expression vector for the cell surface protein CD20 was co-transfected into cells (grown in 10% FCS) together with 10μg of the indicated pCDNA3-9E10-pRb plasmids. Cells were washed and refed after 16 hour of transfection, and harvested 40-48 hour later by washing in PBS and thereafter treating with cell dissociation medium (Sigma) for 15 minutes. Cells were washed in DMEM by centrifuging at 700rpm and resuspended in a small volume of DMEM containing the anti-CD20 antibody leu16 (Becton Dickinson) coupled to fluorescein isothiocyanate (FITC). Cells were incubated upon ice for 45 minutes, further washed twice in PBS and then resuspended in 50% PBS in ethanol at 4°C overnight. Cells were collected by centrifugation and treated with RNase (125U/ml) for 30 minutes, harvested by centrifugation and suspended in propidium iodide (20μg/ml) in PBS at 4°C 30 minutes. Flow cytometry was performed on a Becton Dickinson fluorescence activated cell sorter. The intensity of propidium iodine staining was analysed in cell populations that were positive for FITC staining to determine the cell cycle profile of the transfected population using the Consort 30 software. The data presented shows a representative example from multiple assays.

Mass spectroscopy and peptide sequencing

Acetylation of the synthetic peptide was carried out in a standard procedure described in the section of *in vitro* acetylation, with un-radiolabelled acetyl-CoA (1μl of 10mM stock). The peptide sequencing was carried out at Welmet, Edinburgh University. All mass spectroscopy

analysis was performed in Micromass Ltd, Wythenshawe, UK (please see appendix for details). Experts in Micromass UK Ltd (Wythenshawe) carried out sample preparation prior to mass spectroscopy analysis. In short, the samples were first desalted by means of a C18 Ziptip, and diluted to approximately 2pm/μl in 50%MeOH plus 0.1% HCOOH prior to ESI-MS analysis. Electrospray MS and MS/MS data were acquired on a micromass Q-Tof mass spectrometer (Wythenshawe, UK.), fitted with a Z-spray nanoflow electrospray ion source. For details of the procedure, please see appendix for report directly submitted from Micromass.

Chapter 3: Acetylation Control of the retinoblastoma tumour suppressor protein

Introduction

pRb and p300 are targets of the viral oncoprotein E1A (182, reviewed in 42, 118 and references therein). E1A mediates a trimeric complex between pRb/E1A/p300, and this complex is important for E1A-mediated transformation (155, 173). While E1A mutants deficient in binding to pRb or p300 fail to transform cells, the transformation ability can be restored when the mutants are co-infected into the cells (173). However, the restored transformation is at least ten times less efficient when compared to the wild type, indicating the significance of the complex.

After p300 was cloned (44), it was recognised as a versatile transcriptional co-activator (5, reviewed in 52, 75, 147). Subsequently, the p300/CBP family of proteins was found to possess HAT activity (12, 128), which transfers an acetyl- group to the ε-side chain of the lysine residue. In general, acetylation correlates with transcriptional activation and deacetylation correlates with transcriptional repression (reviewed in 20, 60). In the early 90s, only chromosomal proteins such as histones and HMG proteins were known to be acetylated. Studies during the last decade suggest that acetylation might alter chromosomal structure, facilitate chromatin remodelling, and regulate transcription. Recently, a growing list of transcription factors such as p53 (56), HIV-1 Tat (78), GATA-1 (17), myo-D (141), EKLF (195), c-Myb (161) and E2F (110, 111), were reported to be acetylated by p300/CBP and/or pCAF. Acetylation of most of these transcription factors resulted in enhanced DNA binding activity. Acetylation of p53 was shown to be stress-inducible, and correlates with certain phosphorylation events in p53 (100, 139). Acetylation of E2F-1 enhances its stability (110). Interestingly, one

example also exists whereby acetylation can inhibit transcriptional activation. Acetylated dCTF inhibits its binding to Armadillo, thereby down-regulating transcription (172). An emerging theme suggests that acetylation might mimic phosphorylation in controlling protein activity (15).

pRb is an intensively studied tumour suppressor protein (reviewed in 43, 62). Phosphorylation control of pRb by G1 cyclin-dependent kinases plays an important role in regulating pRb function (reviewed in 115, 149). Most of the pRb's growth suppressive properties are ascribed to its ability to sequester the E2F family of transcription factors (reviewed in 43, 62 and references therein). During G0/G1 hypophosphorylated pRb binds to E2F, inactivating E2F-dependent transcription and thereby preventing cell cycle progression. Cyclin D/cdk4/6 and cyclin E/cdk2 progressively phosphorylate pRb at late G1 to early S phase. In S phase, pRb phosphorylation is maintained by cyclin A/cdk2.

pRb binds to the *trans*activation domain of E2F, providing a simple model of how pRb inhibits E2F-dependent transcription. In addition, pRb possesses intrinsic transcription repression activity, which is independent of its association with E2F (177). The identification of HDACs (18, 103, 106) as pRb binding proteins suggests a second mechanism in which transcriptional repression is achieved by recruiting HDACs to promoters to deacetylate histones. Furthermore, hSWI/SNF complexes, which are ATPase-dependent chromatin remodelling factors, exist in a ternary complex with pRb and HDACs, further substantiating pRb's role in regulating transcription via chromatin remodelling related mechanisms (196). Both E1A and E7 block pRb binding to HDACs, providing an additional mechanism for their oncogenic activity. Interestingly, pRb also binds to a HAT, $TAF_{II}250$, which is the largest subunit of TFIID (116, 143, 144). Whilst pRb was shown to inhibit the intrinsic kinase activity of $TAF_{II}250$ (150), it is unclear if pRb also regulates $TAF_{II}250$ HAT activity.

Here, evidence is presented to indicate that pRb is acetylated both *in vitro* and *in vivo*. *In vitro* acetylation assays were carried out which identified that the pRb family member p107, and

the pRb target E2F-1, were also acetylated by p300. While E2F-1 was acetylated by both p300 and pCAF, pRb was only acetylated by p300. Domain mapping experiments suggested that pRb is acetylated in the pocket and in the C-terminal region. Furthermore, Ad E1A enhanced pRb acetylation via a physical interaction mediated by its N-terminal transformation-sensitive domain. The MDM2 oncoprotein was found to interact more efficiently with the acetylated form of pRb. Site-directed mutagenesis studies identified residue K873, 874 as two possible lysines directly acetylated by p300 *in vitro*. When these lysines were mutated to glutamine, which might mimic the acetylated status of the lysine, the mutants showed defects in phosphorylation by cyclin/CDK complexes *in vivo*. Overall, the study suggests that pRb acetylation is an additional mechanism in regulation of pRb function.

Results

In order to investigate if components of the pRb/E2F pathway are subject to acetylation control, recombinant pRb, E2F-1, cyclin D, p107 and various other substrates were screened in an *in vitro* acetylation assay. The enzymes used in the *in vitro* acetylation assay included recombinant Flag-p300 (FL), Flag-p300$^{1135-2414}$ and Flag-pCAF purified from sf9 insect cells infected with the respective baculovirus, or His-p300$^{1195-1673}$ (which contains the minimal HAT domain of p300) purified from bacteria (Fig 3.1a and data not shown). From this initial screening, pRb, p107, E2F-1, p53, histones and E1A were shown to be acetylated (Fig 3.1b). Furthermore, both p300 and pCAF were found to be auto-acetylated (Fig 3.3b, 3.7b and data not shown).

pRb, p107 and E2F-1 are acetylated in vitro

Using *in vitro* acetylation assays, pRb and its family member p107 were shown to be acetylated by p300, but not by pCAF (Fig 3.2c). Furthermore, pRb and p107 can be acetylated with the minimal HAT domain of p300 (Fig 3.2c, left panel). Two pRb pocket mutants, Δ21 and Δ22, show much reduced levels of acetylation (Fig 3.2c), suggesting the integrity of the pocket is important for efficient acetylation. In the same experiments, pRb C-terminal region was found to be efficiently acetylated (Fig 3.2c). Interestingly, in these assays, E2F-1 was found to be acetylated by both pCAF and p300 (Fig 3.2c, middle and right panel). While E2F-1 was poorly acetylated by the minimal HAT domain of p300, Flag-p300$^{1135-2414}$ efficiently acetylated E2F-1. Previously, the C-terminal region of p300 was shown to interact with E2F-1 (165), indicating efficient acetylation of E2F-1 by p300 requires the E2F-1 binding domain in p300. In this study, efforts were concentrated on understanding the role of pRb acetylation.

pRb is acetylated in vivo

Whilst pRb was found to be acetylated *in vitro*, it was important to substantiate these findings *in vivo*. Two commercially available antibodies against anti-Ac-K were characterised to specifically recognise *in vitro* acetylated proteins, including *in vitro* acetylated pRb, E2F-1 and p300 (Fig 3.3a and b and data not shown). These antibodies were subsequently used to investigate whether pRb is acetylated *in vivo*. Since the antibody against Ac-K (New England Biolab) had much reduced background in immunoblotting (compare Fig 3.3b to Fig 3.3a), and showed a clear specificity in the immunoprecipitation assay (Fig 3.3c, compare track 2 with 6), all work presented in this thesis used this particular antibody to detect and immunoprecipitate acetylated forms of pRb. To investigate if pRb is acetylated *in vivo*, SAOS2, U2OS and C33A cells were over-expressed with pRb, and treated with TSA prior to harvesting. Immunoprecipitations were performed using anti-Rb antibodies and control antibodies, followed by immunoblotting with the anti-Ac-K antibody. The anti-Ac-K antibody recognised a specific polypeptide from the sample immunoprecipitated with the anti-Rb antibody with a molecular weight of 105 KDa, but not in the control sample (Kristic-Demonacos *et. al.*, personal communication). Using the same experimental procedure, further evidence that pRb is acetylated under physiological conditions was demonstrated when endogenous pRb was found to be acetylated in 293 HEK cells (Fig 3.3d, middle panel, track 2). Althought similar amount of pRb was immunoprecipitated from 293 HEK, U2OS and T98G cells (Fig 3.3d, top panel, track 2, 3 and 4), no significant amount of endogenous pRb was found to be acetylated in U2OS or T98G cells (Fig 3.3d, middle panel, track 3, 4). A31 (Fig 3.3d, track 5) is a murine cell line, and since the pRb antibodies did not immunoprecipitate murine pRb (Fig 3.3d, top panel, track 5), this cell line was used as a negative control in the experiment. Furthermore, SAOS2 cell, which is $Rb^{-/-}$,

was also used as negative control for the immunoprecipitation and immunoblotting of pRb (Fig 3.3d, top panel, track 6).

Domain Mapping of Acetylation sites in pRb

Since the pRb pocket mutant Δ21 and Δ22 have diminished levels of acetylation, investigation was undertaken to determine if the regions deleted in these mutants contain site(s) for acetylation. From the three dimensional structure of the pRb A/B pocket in complex with the E7 peptide (94), a group of conserved and functionally important lysine residues (K713, 720, 722, 729) were implicated in exons 21 and 22 (also see chapter 5, Fig 5.1c and d). A peptide (Fig 3.4a) covering this region was synthesized and tested in the *in vitro* acetylation assay. The peptide was acetylated *in vitro* by p300, and mass spectroscopy analysis suggested that the peptide was singly acetylated (Fig 3.4c). Using both mass spectroscopy analysis and peptide sequencing, it was found that the first lysine in the peptide (K713 in pRb) was predominantly acetylated (Fig 3.4b, d and e). From peptide sequencing, the majority of the K713 was not modified (Fig 3.4b), as indicated by K in the spectrum. Approximately 10% of the K713 was acetylated, as indicated by Ac-K in the spectrum. This peak co-migrates at the same position as the acetylated-lysine amino acid standard. Also in the spectrum indicates the 5^{th} amino acid in the peptide, Valine (V), of the peptide. This is a secondary peak due to the heterogenity of the peptide population.

In order to substantiate the result that K713 is the site of modification, site directed mutagenesis was carried out to mutate K713 to A or R (also see chapter 5, Fig 5.1). GST-pRb 713KA and 713KR mutants, which had the K713 mutated to A and R respectively, exhibited a slight reduction in the level of acetylation when compared to the wild type protein (Fig 3.5b and c). Therefore, there are other lysine residue(s) apart from K713 that can be acetylated by p300 *in vitro*.

In order to define the general domain(s) of acetylation in pRb, various GST-pRB fusion proteins were purified and tested in the *in vitro* acetylation assays. GST-pRb (379-656), which contains the A pocket and the linker region, showed a low, but detectable level of acetylation (Fig 3.6b). GST-pRb (641-775), which contains the B pocket, had an undetectable level of acetylation (Fig 3.6b). However, one particular problem with GST-pRb (641-775) was that it generated much less recombinant protein from the purification. Two more GST-pRb fusion constructs containing the B pocket were made to try to overcome this problem, namely GST-pRb (702-766) and GST-pRb (702-792). However, neither of these constructs yielded significant amounts of recombinant protein (data not shown). Therefore, it is not clear whether or not the B pocket can be acetylated *in vitro*. It is very likely that under these conditions, truncation within the pRb small pocket will not yield properly folded proteins, which might therefore complicate the interpretation of the data, and no further detailed domain mapping was carried out within the small pocket. The C terminal region of pRb however was strongly acetylated by p300 *in vitro* (Fig 3.2 and 3.6). By studying the acetylation of an extensive panel of mutant derivatives taken from the C-terminus, the major regions of acetylation were mapped to residues between 794-829 and 830 to 884 (Fig 3.6d and f). GST-pRb (881-928), which contains seven lysine residues, failed to be acetylated. In conclusion, multiple domains on pRb can be acetylated. In the C-terminus, the major sites of acetylation occur on lysine residues within region of 794-881, which contains 8 lysines altogether.

pRb acetylation by p300 is enhanced by E1A

Both pRb and p300 are targets of viral oncoprotein E1A, and a ternary complex can be formed of pRb, p300, and E1A (45, 173). 293 HEK is a cell line which contains endogenous E1A. Using anti-Rb antibodies for immunoprecipitation, p300 was indeed detected in immunoprecipitates from 293 HEK cells (Fig 3.3d, bottom panel, track 2). Anti-Rb antibodies

did not immunoprecipitate a detectable level of p300 from cell lines (U2OS, T98G, C33A) lacking E1A expression (Fig 3.3d and data not shown). Furthermore, an *in vitro* binding assay using recombinant E1A, pRb and p300 also indicated that E1A could mediate the formation of the trimeric complex (data not shown).

There are contradicting reports regarding E1A's ability to regulate p300 HAT activity (2, 25, and 58). Given the ability of E1A to mediate the formation of pRb/E1A/p300 ternary complex, experiments were carried out to determine if E1A affects p300's ability to acetylate pRb. By titrating His-E1A (13S), E1A enhanced pRb acetylation (Fig 3.7b). This effect is specific to pRb as in the same experiment, His-E1A inhibited E2F-1 acetylation, and caused a moderate change in the level of histone acetylation (a slight stimulation of 1.8 fold with 0.3μg of E1A, Fig 3.7b). Furthermore, E1A did not significantly alter p300 autoacetylation. To further verify that E1A enhances pRb acetylation by p300, a GST-E1A (12S) fusion protein was purified, and similar experiments were performed. Consistent with the result observed with His-E1A, GST-E1A also enhanced pRb acetylation at range where there was less E1A protein compared to pRb protein (molar ratio of E1A to pRb is approximately 1:1 in Fig 3.7c, track 4). When the molar ratio of E1A to pRb was about 5:1 (Fig 3.7c, top panel, track 5), the enhancement of pRb acetylation was lost. However, it is clear that under those conditions, E1A did not repress pRb acetylation by p300. GST-E1A specifically enhanced pRb acetylation as p53 acetylation was reduced by GST-E1A at all concentrations in the same experiment (Fig 7c, bottom panel).

Next, experiments were performed to identify the domains of E1A which are required to enhance pRb acetylation. A panel of GST-E1A mutants were created to answer the question. The N-terminus deletion mutant Δ36 is defective in binding to p300 (173). The pm928 mutant has been previously reported to be defective in binding to pRb (173). The ΔCR1 and ΔCR2 mutants

contain deletions in the conserved region 1 and 2 of E1A respectively, and invariably they were reported to be defective in binding to pRb (9). *In vitro* acetylation experiments with various E1A mutants suggested that all E1A mutants were defective in enhancing pRb acetylation when compared to the wild type (Fig 3.8b, d, and e). The E1A Δ36 mutant was completely inactive in enhancing pRb acetylation by p300. Therefore, E1A requires both the p300 and pRb binding domains to enhance pRb acetylation. These observations are consistent with the idea that E1A, by mediating a ternary complex among the three proteins, actively directs the p300 HAT activity to acetylate pRb.

Acetylation of pRb enhances pRb/MDM2 interaction

The C-terminal region of pRb is known to contribute to pRb tumour suppressor function, and was reported to bind to the oncoprotein MDM2 (64, 135, 183). Using a panel of GST-pRb C-terminus derivatives (Fig 3.9a), the minimal domain in pRb for binding to MDM2 was mapped to residues 792-928, which agrees with previous reports (183). Experiments were performed to investigate if pRb acetylation affects its interaction with MDM2. In the *in vitro* pulldown assay, MDM2 preferentially interacted with the acetylated form of pRb (Fig 3.9b). Furthermore, immunoprecipitation experiments using anti-Rb antibodies demonstrated the presence of an easily detectable complex between pRb and MDM2 in 293 HEK cells, but not in U2OS or T98G cells (Fig 3.9c). As shown in previous section, 293 HEK cells contain significant amounts of acetylated pRb (Fig 3.3d). When T98G cells were treated with Trichostatin A (TSA), which is a deacetylase inhibitor (103, 127) and therefore presumably enhances pRb acetylation level, it was also found to have an increased amount of MDM2 in the immunocomplex with pRb (Fig 3.9d). TSA treatment did not change MDM2 and p53 protein level (Fig 3.9d and data not shown), though it was noted that pRb was shifted to a hypophosphorylated status (Fig 3.9d and data not shown). These findings are consistent with previous reports (127). The *in vivo*

immunoprecipitation results are consistent with the findings of the *in vitro* binding assay, suggesting that MDM2 preferentially binds to the acetylated pRb.

Lys873/874 are important in regulating pRb phsophorylation by CDKs

One particular domain in the pRb C-terminal region (residues 830-884) was shown to be efficiently acetylated by p300 *in vitro* and was reported to contain sequences that are important in regulating pRb phosphorylation by cyclin/CDK complexes (1). Careful analysis of the sequence identified a lysine-rich stretch (KXXXK), which also showed some homology in other proteins which were found to be acetylated (Fig 3.10b). To investigated if these lysines might be regulated by acetylation, site-directed mutagenesis was carried out to mutate K873, 874 to arginine (RR), which will maintain the overall basic charge but cannot be acetylated, and therefore to mimic the unacetylated state; or to glutamine (QQ), which is expected to mimic the acetylated state of lysine (194). The K to Q mutation was widely used in studying how acetylation of the histone tails affects transcriptional regulation (194). When the GST-pRb (830-884)RR mutant was tested in the *in vitro* acetylation assay, no detectable acetylation was observed (Fig 3.10c). This suggests that K873/874 are residues modified by p300 *in vitro*. Overall, two critical lysine residues were identified; the mutation of which abolishes pRb acetylation in the region from amino acid 830-884.

Next, experiments were carried out to understand the functional significance of K873/874, in particular the role in regulating pRb phosphorylation by CDKs. First, the RR and QQ mutations were introduced into the full-length pRb. When the 873/874RR mutant was transfected into SAOS2 or U2OS cells, it behaved similarly as the wild type pRb to be phosphorylated to equivalent level (Fig 3.10d and e). However, the 873/874QQ mutant, which might mimic the acetylated state of the protein, showed a defect of phophorylation in both SAOS2 and U2OS cells (Fig 3.10d and e). In SAOS2 cells, the 873/874QQ mutants showed

incomplete phosphorylation upon expression of exogenous cyclin E/CDK2 when compared to the wild-type pRb or the 873/874RR mutant. U2OS cells were known to contain hyperactive endogenous CDK activity. Overexpression of pRb in U2OS cells showed accumulation of both the hypo- and hyperphosphorylated pRb (Fig 3.10e, track 2). While the 873/874RR mutant behaved similarly to the wild type pRb (Fig 3.10e, track 3), the 873/874QQ mutant showed defects in phosphorylation by endogenous CDKs, and accumulated in the hypophosphorylated form (Fig 3.10e, track 4). In conclusion, K873/874 are important in regulating pRb phosphorylation by cyclin/CDK complexes.

Discussion

The acetylation of pRb described here suggests an additional level of regulation of its activity. Although viral oncoprotein E1A recruits p300 and pRb into a ternary complex (42, 118), the functional significance of this complex is not well understood. E1A requires binding to both pRb and p300 for its transformation function (118, 173). With the demonstration that E1A enhances pRb acetylation by p300 comes the possibility that E1A acts to re-direct p300 HAT activity to modify pRb function. Furthermore, both E2F-1 (110, 111) and p53 (56, 100, 139) are known to be acetylated. It is very interesting to note that the two most studied tumour suppressor pathways, namely, the E2F/pRb and the p53 pathways, all subject to acetylation control. It was demonstrated that the acetylation of E2F-1 and p53 enhance their DNA binding activity. Detailed investigation of p53 acetylation demonstrates that p53 acetylation at the C-terminal region is stress-inducible, and probably directed by phosphorylation of S-33 and –37 in the N-terminal region (100, 139). Taken together, the data suggest that acetylation plays a role in regulating the p53 and E2F/pRb pathways. E1A also directly targets the two HATs, p300 and pCAF, used in the study (188), arguing that the disruption of the normal acetylation control of the p53 and E2F/pRb pathways by p300/pCAF could be an important feature of E1A oncogenic activity. Since E1A was found to enhance pRb acetylation, but reduce E2F-1 and p53 acetylation, the acetylation of pRb may inactivate its growth suppressive functions, whilst the acetylation of E2F-1 and p53 may enhance their tumour suppression functions. Further studies will be required to address these issues.

Post-translational phosphorylation control of pRb by G1 CDKs plays a major role in regulating pRb function (reviewed in 115). There are sixteen potential consensus phosphorylation sites in pRb. Phosphorylation at S-807 and -811 was demonstrated to release

the interaction of c-Abl from pRb (84). In contrast, the pRb/E2F interaction appears to be regulated by a number of phosphorylation sites in the C-terminal region, and possibly involving two serines in the spacer domain (85, 168). Phosphorylation at the pRb C-terminus by CDK4/6 initiates successive intramolecular interactions between the C-terminal region and the pocket domain which progressively inactivate pRb function (59). In parallel to pRb phosphorylation, this study shows that multiple domains on pRb can be acetylated. They occur both in the pocket and in the C-terminal region. It has so far not been tested whether acetylation occurs in the N-terminal region of the protein. In this respect, it will be crucial to further characterise the precise acetylation sites on pRb *in vitro* and *in vivo*. It is possible that the acetylation of pRb will have multiple effects on its functions, depending upon which residue(s) or combinations of residues are acetylated. While E1A enhances pRb acetylation, it will be interesting to determine whether E1A also alters the pattern of acetylation on pRb by preferentially enhancing acetylation at certain specific residues. Unlike the case of phosphorylation, there are no good consensus sequences for acetylation. Therefore, in the current study, it was difficult to identify the specific lysine residues modified by p300. It will be important to characterize all lysine residues which are modified by acetylation *in vitro* and *in vivo*. With the ever increasing power and resolution of the biophysical techniques using 2D gel analysis coupled to mass spectroscopy, this will be one way to identify the lysine residues acetylated in pRb. As demonstrated in this study, mass spectroscopy analysis successfully identified one lysine acetylated in a synthetic peptide. With the knowledge of the precise acetylation sites in pRb, mutagenesis studies could then be used to generate interesting information on how acetylation affects pRb function.

Since the C-terminal region of pRb was found to be the major domain to be acetylated, this leads to the question of how the acetylation in the pRb C-terminus affects its function. MDM2 is an oncoprotein known to bind to the C-terminal region (183). *In vitro* biochemical binding data suggest that acetylated pRb preferentially binds to MDM2. MDM2 was also found

to efficiently complex with pRb in 293 HEK cells which contain a relatively high level of acetylated pRb when compared to the other cell lines used in the study. Treatment of T98G cells with TSA, which is a deacetylase inhibitor and therefore is likely to enhance pRb acetylation, also enhanced MDM2 binding to pRb. Whilst these *in vivo* data are not exclusive proof that pRb acetylation enhances its interaction with MDM2, the findings are consistent with the *in vitro* data and suggest that acetylation regulates pRb-MDM2 interaction.

MDM2 was reported to inhibit pRb growth regulatory functions (183), possibly by relieving pRb from E2F. Furthermore, MDM2 can confer TGF-β resistance, possibly by interacting with the E2F/pRb pathway (156). Both reports provide evidence that MDM2 binding to pRb abrogates pRb growth suppression functions. These studies also provide a logic for E1A to enhance pRb acetylation, and therefore contribute to E1A's oncogenic activity. It is also to be noted that E1A can induce p53 and promotes apoptosis in primary cells, which is reflected in E1A's ability to enhance radio- and chemosensitivity (37). Interestingly, pRb binding to MDM2 was found to overcome both the anti-apoptotic function of MDM2 and the MDM2-dependent degradation of p53 (64). Therefore, the observation of enhanced MDM2 binding to acetylated pRb could be one explanation towards E1A's apoptotic function.

Although abundant evidence suggests that pRb phosphorylation is essential for cellular growth and proliferation, counter evidence also exists to suggest that there are other mechanisms which might bypass the need for pRb phosphorylation in driving cellular progression (reviewed in 115, and references therein). For example, it has been shown that the combined expression of c-Myc and oncogenic Ras allows entry of growth-factor-deprived REF 52 cells into S phase and does so without detectable pRb phosphorylation. Furthermore, overexpression of cyclin E also triggers S phase entry in cells expressing pRb lacking 10 out of the 16 CDK phosphorylation sites. It will be interesting to correlates the acetylation status of pRb in different phases of the cell cycle and determine if pRb acetylation is essential for cellular growth.

Mutational analysis of K873 and 874 identified these residues as potential targets of acetylation by p300 *in vitro*, though it has yet to be verified that they are targets of acetylation *in vivo*. An alternative explanation is that these lysines are part of a subtle consensus sequence important for acetylation. Therefore, mutations in these lysine residues may render other lysines un-acetylatable. Interestingly, these two residues are within the KPLKKL motif which was found to be important for pRb phosphorylation by cyclin-CDK2 complexes (1). Indeed, when the K873/874 was mutated to QQ, which might mimic the acetylated form of the protein, the mutant protein was defective in phosphorylation by cyclin/CDK complexes in both SAOS2 and U2OS cells. This suggests that there might be cross regulation between phosphorylation and acetylation. These findings are consistent with the model that acetylation of pRb has multiple functions. Since E2F is the most studied target of pRb, an investigation was carried out to determine if pRb acetylation affects its binding to E2F (data not shown). *In vitro* binding assays suggested that E2F-1 binds to the acetylated pRb, though these assays were not sensitive enough to determine if the affinity of the binding was affected upon acetylation. Given that the pocket domain was found to be acetylated *in vitro*, it will be important in future studies to investigate how acetylation changes the pocket function.

In the current study, pRb was found to be acetylated only by p300, but not by pCAF. This suggests a level of specificity in HATs. It is interesting to note that pRb also interacts with another HAT, $TAF_{II}250$, which is the largest subunits of the TFIID complex (143, 144). Previous studies suggested that pRb can inhibit the intrinsic kinase activity of $TAF_{II}250$, though pRb was not acetylated by $TAF_{II}250$ (150). $TAF_{II}250$ HAT activity is required for cell cycle progression (41). It will be worthwhile to investigate if pRb affects $TAF_{II}250$ HAT activity. Immunoprecipitation of pRb from U2OS, T98G and C33A cells, followed by immunoblotting with anti-Ac-K antibody indicated that there was an undetectable level of acetylated pRb under normal conditions. Similar to the case of p53 acetylation, only a low level of pRb protein seems

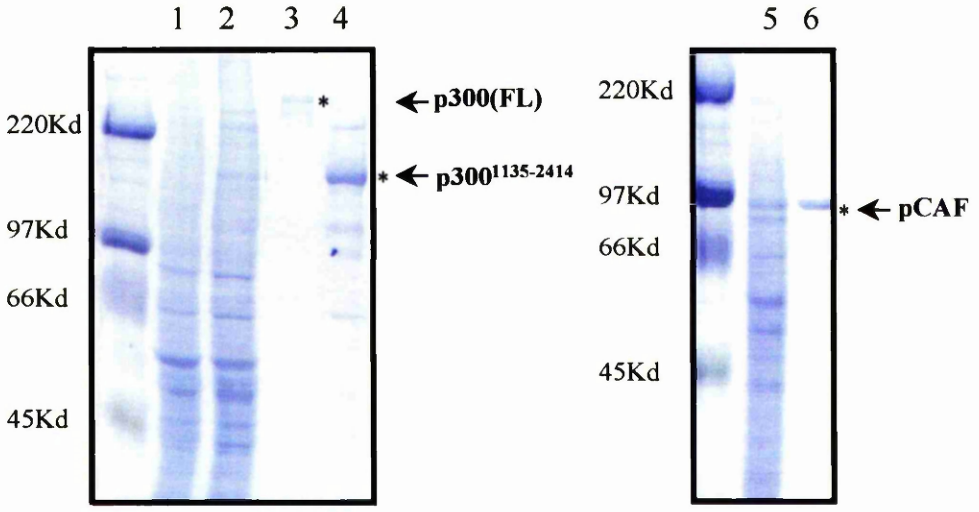
to be acetylated in the cells. In 293 HEK cells, which are transformed with E1A, a significant amount of acetylated pRb was observed. It will be important to understand how pRb acetylation is regulated in the cell. Since p53 acetylation was stimulated after treatment with DNA damaging agents such as UV and ionizing radiation (100, 139), experiments were carried out to determine if pRb acetylation might be similarly regulated. However, no detectable pRb acetylation was observed when U2OS cells were treated with actinomycin D (data not shown). Since pRb is hyperphosphorylated in U2OS cells, and therefore functionally inactive, this cell line might not be most suitable for that particular purpose. Similar experiments need to be repeated in other cell lines with an intact pRb/E2F pathway.

While E1A can bridge together a trimeric complex of pRb and p300, a cellular protein with similar activity has not been reported. MDM2 can bind to both p300 and pRb. Furthermore, p300 and pRb binds to non-overlapping domains on MDM2 (53, 64). *In vitro* binding experiments were performed using purified pRb and p300 proteins, together with *in vitro* translated MDM2 to determine if pRb/p300/MDM2 can be in a trimeric complex (data not shown). No trimeric complex was detected. It is very intriguing to speculate how pRb is recruited to p300 under normal conditions. Although p300 specifically acetylates pRb *in vitro*, evidence is still lacking that it is the HAT which acetylates pRb *in vivo*. It will be essential to identify the HAT(s) which acetylate pRb in the cell.

In conclusion, this report identifies an additional post-translational mechanism involved in regulating pRb function, namely acetylation. Given that the acetylation pathway is targeted by viral oncoprotein E1A, this indicates the potential significance of acetylation control on pRb. Future tasks remain to identify the precise acetylation sites on pRb, the HATs acetylating pRb under physiological conditions, and the functional consequences of pRb acetylation.

Figure 3.1

a)



b)

Substrates	p300 $^{1195-1673}$	p300 $^{1135-2414}$	p300 (FL)	pCAF
Histones	+	+	+	+
pRb	+	+	+	-
p107	+	+	+	-
E2F-1	-	+	+	+
p53	NA	+	+	+
E1A	NA	+	+	NA
JMY	-	-	-	-
NAP-2	-	-	-	-
DP-1	-	NA	NA	NA
Cyclin D	-	-	NA	NA
GST	-	-	-	-
BSA	-	-	-	-

Figure 3.1 Purification of Flag-p300/pCAF and table of summary of the *in vitro* acetylation results

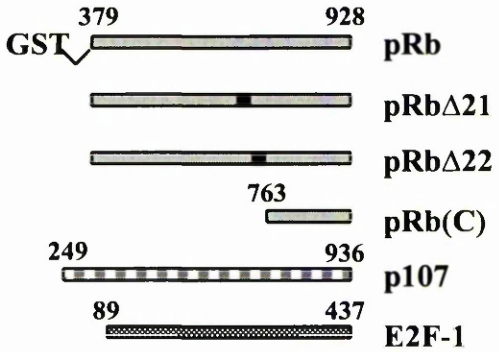
a) Purification of Flag-p300 and Flag-pCAF

Flag-p300, pCAF and $p300^{1135-2414}$ were purified from sf9 cell extracts infected with the respective baculovirus (sf9 extracts in lane 1, 2 and 5). The purified proteins (lane 3, 4 and 6) are indicated by *. The gels were stained with coomassie blue.

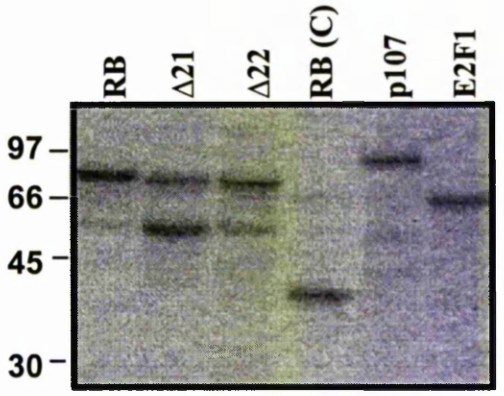
b) Summary of various substrates tested for acetylation *in vitro* using the indicated HAT. Substrates which were acetylated (+) and not acetylated (-) are indicated. NA (not applicable) means the tests were not performed.

Figure 3.2

a)



b) Coomassie Staining



c) Autoradiograph

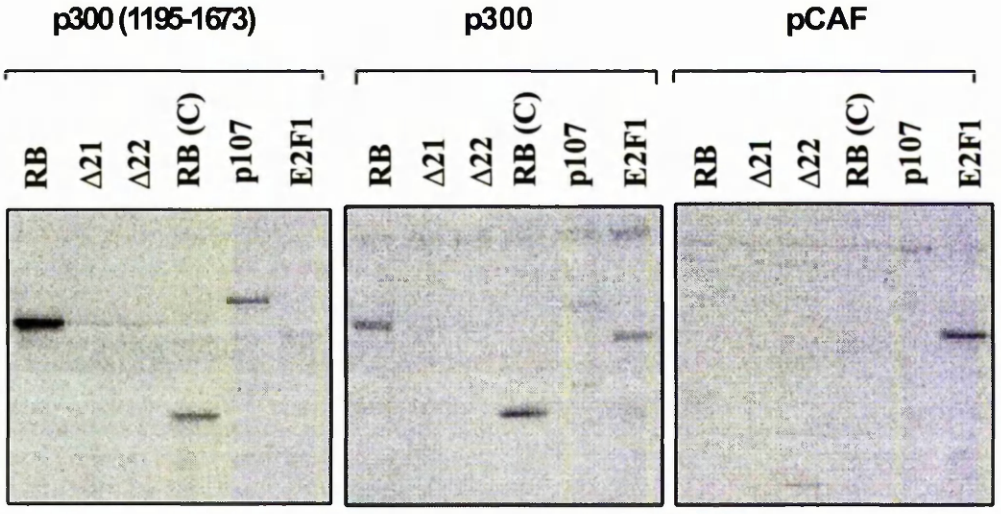


Figure 3.2

The pRb, p107 and E2F-1 are acetylated *in vitro*.

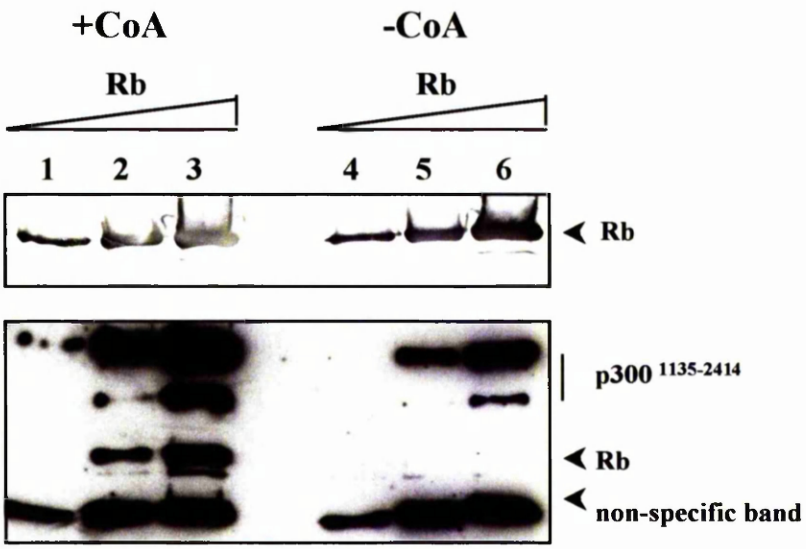
a) Schematic representation of various GST-Rb fusion proteins, GST-E2F-1 and GST-p107 used in the acetylation assays.

b) The indicated GST-Rb, E2F-1 and p107 fusion proteins were expressed, purified and analysed on an SDS gel. The gel was stained with coomassie blue.

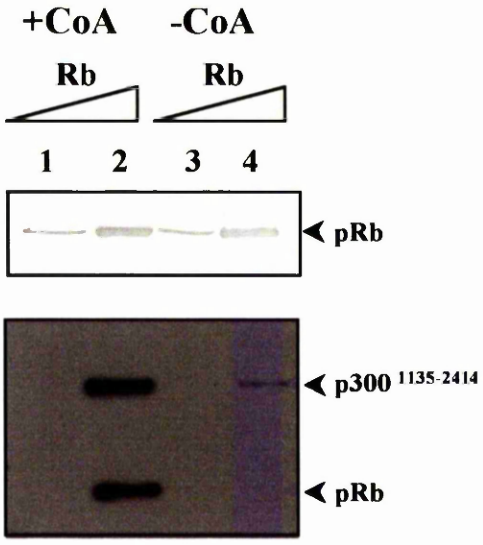
c) Autoradiograph of GST-fusion proteins acetylated by p300 and pCAF. The indicated GST-fusion proteins (2μg) were incubated with the indicated HATs (about 0.3μg) and ^{14}C-acetyl-CoA. The left panel shows the acetylation assay using His-p300$^{1195-1673}$, which contains the minimal HAT domain of p300. The middle panel shows the acetylation assay using Flag-p300 (FL) and the right panel shows the acetylation assay using Flag-pCAF.

Figure 3.3

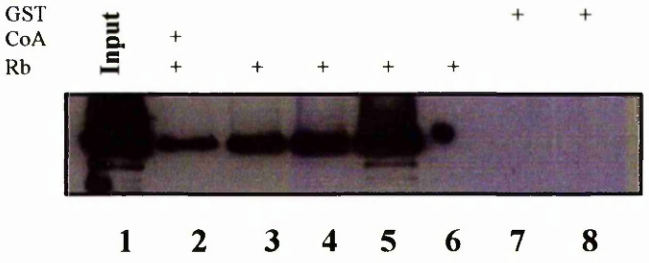
a)


+CoA
-CoA
Rb
Rb
1 2 3 4 5 6
Rb
p300 1135-2414
Rb
non-specific band

b)


+CoA
-CoA
Rb
Rb
1 2 3 4
pRb
p300 1135-2414
pRb

c)



d)

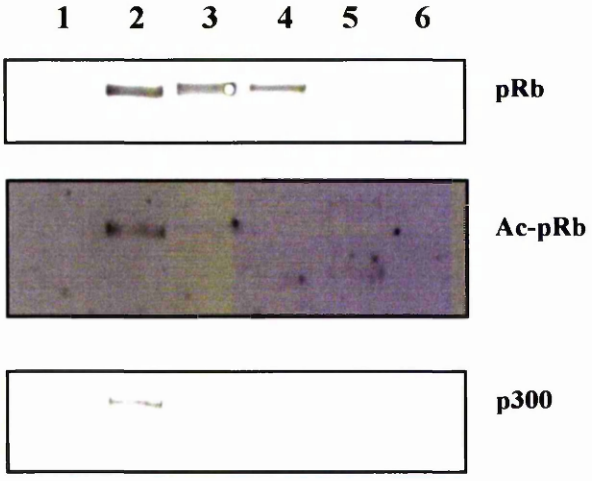


Figure 3.3

The retinoblastoma protein is acetylated *in vivo*.

a) Anti-Ac-H4 antibody (Serotec) recognises *in vitro* acetylated GST-Rb. 2μg of the GST-Rb was incubated with 0.3μg of flag-p300$^{1135-2414}$, in a typical 30μl acetylation reaction either with or without 1μl of 10mM acetyl-CoA. A titration of the reactions 1:3:6 was loaded into lane 1-3 with 4-6. The upper panel shows a western blot using anti-Rb antibody C15, which indicates the input amount of GST-Rb. The lower panel shows a western blot using anti-Ac-H4 antibody which specifically recognises acetylated GST-Rb (compare lanes 1-3 with 4-6). The antibody also recognises acetylated p300.

b) Anti-Ac-K antibody (New England Biolab) specifically recognises *in vitro* acetylated GST-Rb. The reactions were titrated 1:9 into lane 1, 2 and 3, 4. The upper panel shows a western blot using anti-Rb antibody C15, which indicates the input amount of GST-Rb. The lower panel shows a western blot using anti-Ac-K antibody which recognises acetylated GST-Rb (compare lane 2 with 4). The antibody also recognises acetylated p300.

c) Anti-Ac-K antibody immunoprecipitates *in vitro* acetylated GST-Rb. Acetylated GST-Rb (lane 2) and unacetylated GST-Rb (lanes 3-6) samples were prepared by performing an *in vitro* acetylation assay in the presence (lane 2) or in the absence of acetyl-CoA (lanes 3-6). GST-Rb was immunoprecipitated using anti-Ac-K antibody (lanes 2 and 6) or anti-Rb antibody C15 (lane 3), anti-Rb antibody IF-8 (lane 4) or direct pull-down using GST-beads (lane 5). Lane 7 and 8 were controls for C15 and IF-8 immunoprecipitation using purified GST protein. The immunoprecipitated samples were analysed by SDS-PAGE, followed by western blot using anti-Rb antibody C15. Lane 1 shows the input of purified GST-Rb. Anti-Ac-K antibody can specifically immunoprecipitate *in vitro* acetylated GST-pRb (compare lane 2 to 6). C15 and IF-8 also immunoprecipitate GST-Rb (compare lanes 3, 4 with 7, 8).

d) Endogenous pRb is acetylated.

Extract from 293 HEK, U2OS, T98G, A31 and SAOS2 cells (lanes 2-6) were immmunoprecipitated with anti-pRb antibodies (C15 and IF-8), and thereafter immunoblotted

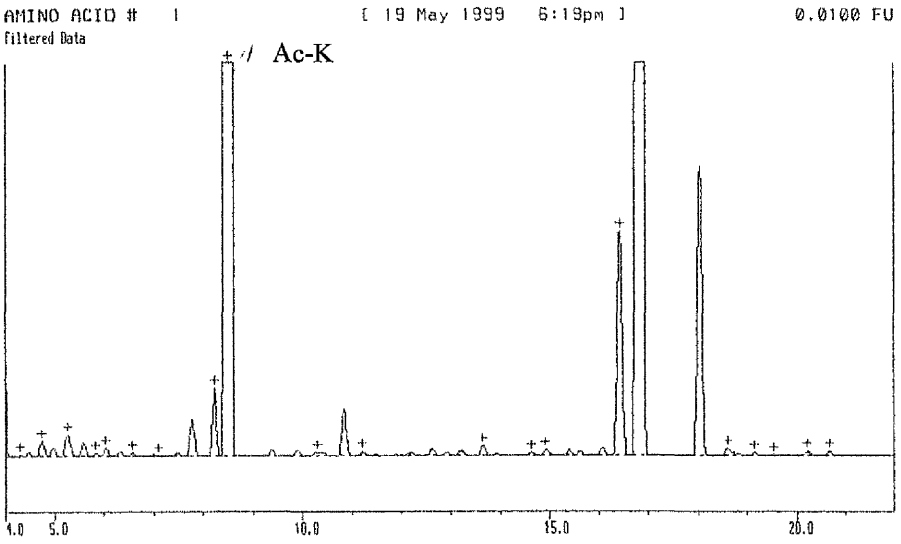
with anti-pRb antibody (G3-245, top panel), anti-Ac-K antibody (middle), or anti-p300 antibody (N15, bottome panel). Lane 1 is the control for immunoprecipitation from 293 cells using an irrelevant control antibody. Note that although a similar level of pRb is immunoprecipitated from 293, U2OS and T98G cells (lanes 2 to 4), only 293 HEK cells (which express E1A) contain high levels of p300 and acetylated pRb in the immunocomplex. A31 is a mouse cell line, which contains an abundant level of pRb (lane 5). This experiment indicates that the anti-pRb antibodies used do not efficiently immunoprecipitate murine pRb. SAOS2 cells is a $Rb^{-/-}$ cell line, which is used as a control for the immunoprecipitation and immunoblotting (lane 6).

Figure 3.4

a)


710
GICKVKNIDLKFKIIVTAYKDL
732

b)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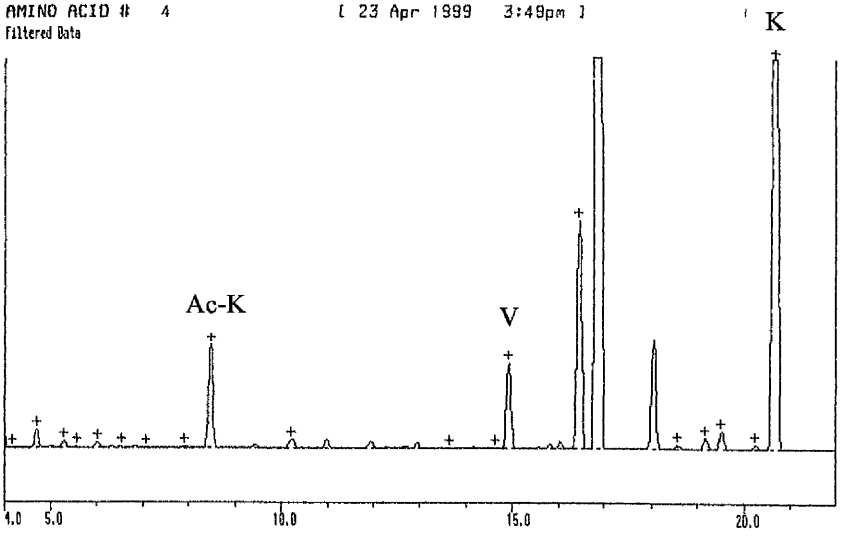
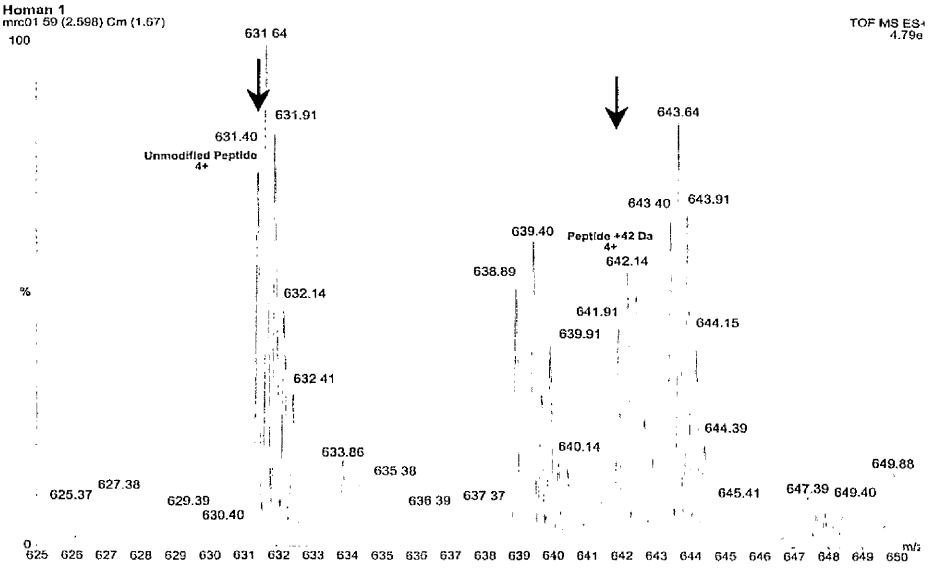
[19 May 1999 6:19pm]
0.0100 FU
Filtered Data
Ac-K
4.0
5.0
10.0
15.0
20.0


AMINO ACID # 4
[23 Apr 1999 3:49pm]
Filtered Data
K
Ac-K
V
4.0
5.0
10.0
15.0
20.0

c)



d)

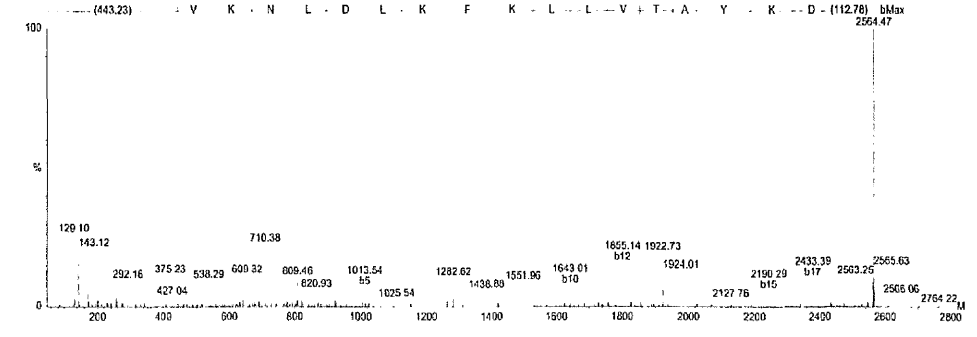
BioLynx - Peptide Sequencing Report
PepSeq file C:\PepSeq_Files\glasgow04.psq
Printed, Mon Aug 16 11:43:50 1999

Observed MW. 2563.1687 Precursor ion charge state. 1
M/z tolerance. 0.15 Intensity threshold 94 (0.500%)

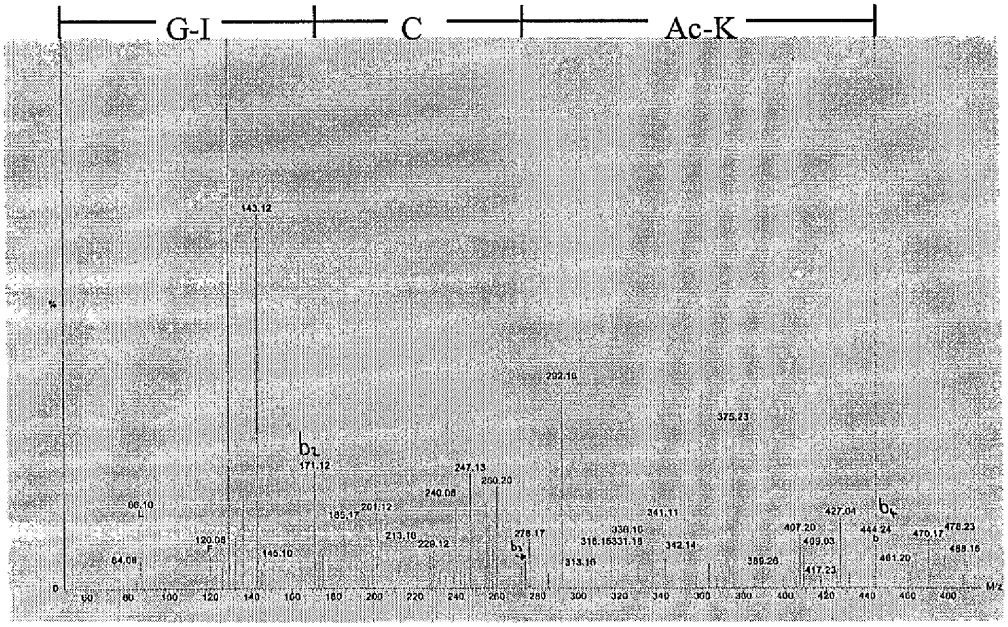
a	416.25	515.31	643.41	757.45	870.54	985.56	1098.65	1226.74	1373.81	1501.90	1614.99	1728.07	1827.14	1928.19	1999.23	2162.29	2290.38
	---	-0.00	---	---	-0.02	-0.01	0.02	---	---	---	-0.01	-0.00	-0.12	---	---	---	---
b	444.24	543.31	671.40	785.45	898.53	1013.56	1126.64	1254.74	1401.80	1529.90	1642.98	1756.07	1855.14	1956.18	2027.22	2190.28	2318.38
	0.00	0.01	0.02	0.03	-0.01	0.02	-0.02	0.00	0.01	-0.02	-0.02	0.04	-0.01	-0.01	-0.01	-0.01	0.05
	443.23	Val	Lys	Asn	Leu	Asp	Leu	Lys	Phe	Lys	Leu	Leu	Val	Thr	Ala	Tyr	Lys
y	2564.18	2120.94	2021.88	1893.78	1779.74	1666.65	1551.63	1438.54	1310.45	1163.38	1035.28	922.20	809.12	710.05	609.00	537.96	374.90
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
z	2547.15	2103.91	2004.85	1876.75	1762.71	1649.62	1534.60	1421.51	1293.42	1146.35	1018.25	905.17	792.09	693.02	591.97	520.93	357.87
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

a	2405.41	2518.17
	---	---
b	2433.41	2546.17
	0.02	---
	Asp	112.78
y	246.80	131.78
	---	---
z	229.77	116.75
	---	---

Acetylated peptide post ziptip clean up
glasgow04 MaxEnt 3 2 [Ev-178263,It50,En1] (0.029,120.08,0.191,1112.47,4



e)



Fragment ions (m/Z=1)	Peptide Seq	Fragment ions (m/Z=1)	Peptide Seq
58.03	G	58.03	G
171.11	G-I	171.11	G-I
274.12	G-I-C	274.12	G-I-C
402.22	G-I-C-K	444.23	G-I-C-Ac-K

Figure 3.4

Analysis of an *in vitro* acetylated peptide derived from pRb B pocket.

a) The sequence of the synthetic peptide from the pRb B pocket. The peptide covers exon 21, from amino acid 710 to 732. Conversed lysine residues among pRb from different species are indicated.

b) 5μg of the synthetic peptide was acetylated *in vitro* using 0.5μg of Flag-p300$^{1135\text{-}2414}$. The acetylated peptide was sequenced by Edman degradation (Welmet) to identify the acetylated lysine. The top panel indicates the position of the acetylated lysine amino acid standard in the spectrum. The bottom spectrum shows the 4th amino acid, which is K713 in pRb sequence.

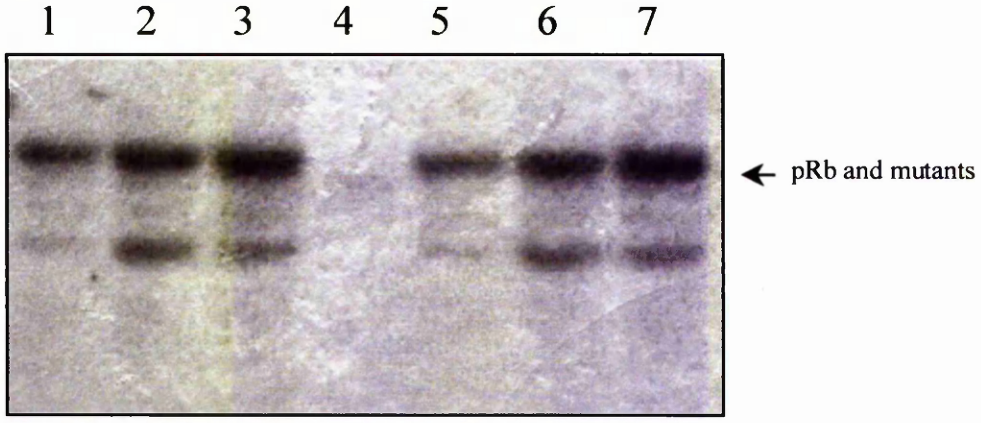
c) The acetylated peptide was subjected to mass-spectroscopy analysis. The peptide has a relative molecular mass of 2521.50. The expanded view of the spectrum shows a quadruply charged ion of low intensity at m/z 641.86 corresponding to the acetylated form of the peptide. The unacetylated peptide gives a signal of m/z 631.38 (see appendix).

d) Analysis of the MS/MS spectrum of the ion at m/z 631.40 with MaxEnt III software to produce a singly charged and de-isotoped MS/MS spectrum. Using the PepSeq software, the C-terminus of the peptide was determined, and indicated no modification. The modification was indicated to be present on one of the first four amino acids in the sequence (see appendix).

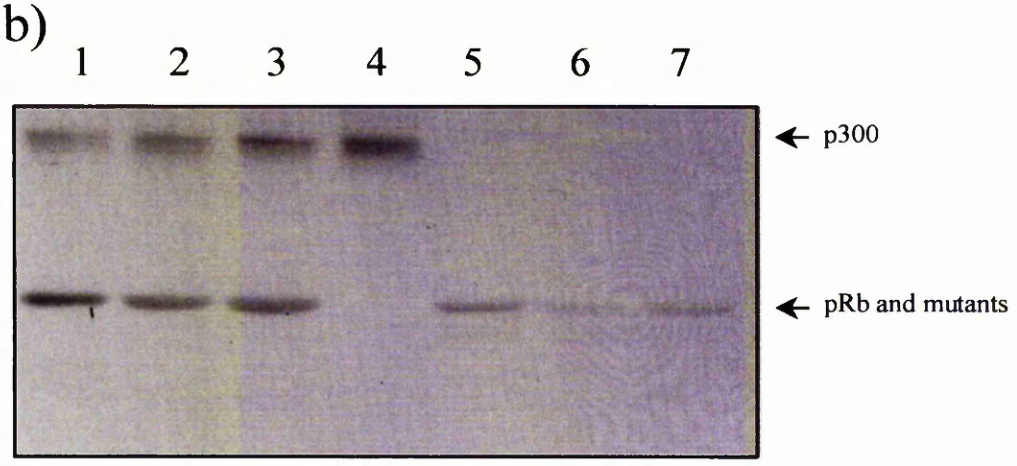
e) An expanded view of the region in m/z 50 to 500 indicated the expected mass spectral fragments of the first four amino acids of the pepide. The table indicates the expected spectral signal of the peptide sequence. In the spectrum, the presence of a peak corresponding to the acetylated lysine at 444.24, and the absence of a peak corresponding to the b4 fragment ion of the unmodified lysine at m/x402.22, strongly suggests the 4th lysine (or K713 in pRb) is acetylated in the peptide (see appendix).

Figure 3.5

a)



b)



c)

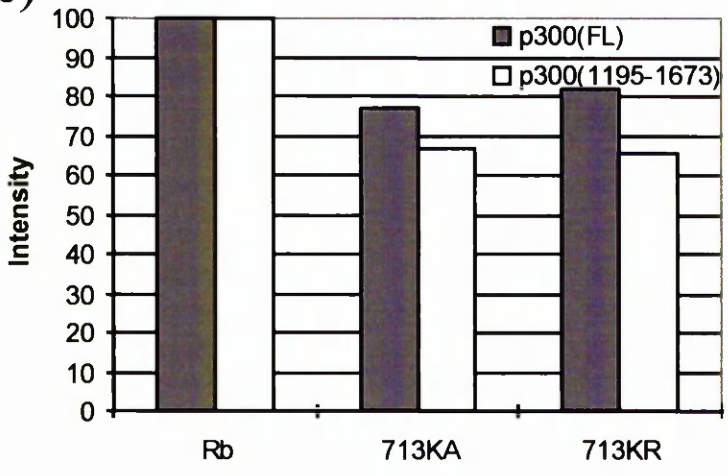


Figure 3.5

Characterisation of the pRb 713KA and 713KR mutants.

a) Coomassie stain of the GST-Rb (lane 1 and 5), and GST-Rb mutants (713KA and 713KR lane 2, 3 and 6, 7 respectively) used in the *in vitro* acetylation assay by Flag-p300$^{1135\text{-}2414}$ (lane 1 to 3) and His-p300$^{1195\text{-}1673}$ (lane 5-7). Lane 4 is a control using Flag-300$^{1135\text{-}2414}$, but without any GST-Rb fusion proteins. Approximately, 2μg of the GST-fusion proteins and 0.3μg of the p300 fusion proteins were used in the experiment. Acetylated protein is labeled with ^{14}C-acetyl-coA.

b) Autoradiograph of the *in vitro* acetylation of GST-Rb and GST-Rb mutants. Note: Rb mutants, 713KA and 713KR have reduced level of acetylation compared to the wild type (compare lanes 2, 3 with lane 1 and compare lanes 6, 7 with lane 5).

c) Quantitation of the level of acetylation of GST-Rb and pRb mutants in panel B. The intensity was normalised as intensity of the acetylated signal/intensity of input pRb protein. Wild type pRb was assigned with 100% of intensity. Both mutants have 65-80% of the wild type GST-Rb acetylation level.

Figure 3.6

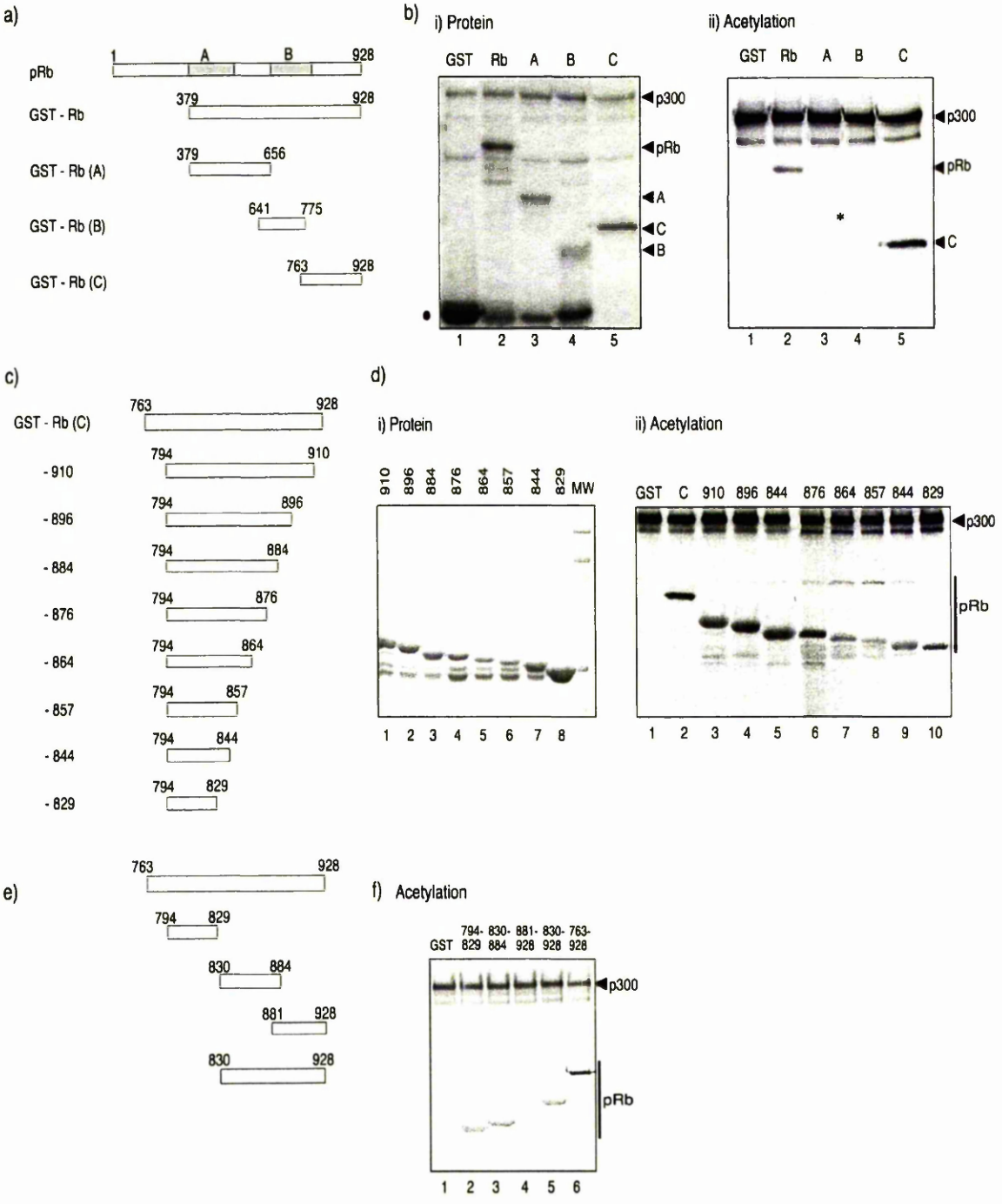
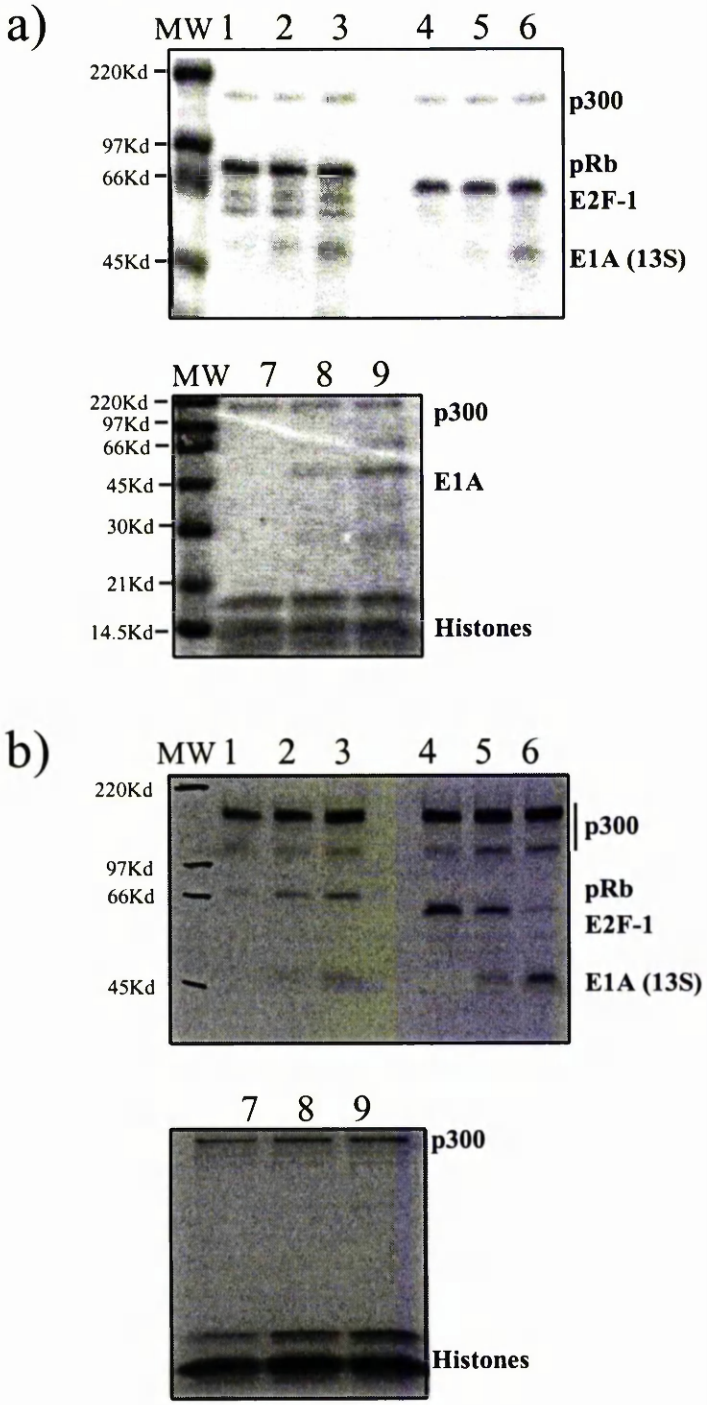

a)
pRb
1
A
B
928
GST - Rb
379
928
GST - Rb (A)
379
656
GST - Rb (B)
641
775
GST - Rb (C)
763
928
b)
i) Protein
GST Rb A B C
p300
pRb
A
C
B
1 2 3 4 5
ii) Acetylation
GST Rb A B C
p300
pRb
C
1 2 3 4 5
c)
GST - Rb (C)
763
928
- 910
794
910
- 896
794
896
- 884
794
884
- 876
794
876
- 864
794
864
- 857
794
857
- 844
794
844
- 829
794
829
d)
i) Protein
910 896 884 876 864 857 844 829 MW
1 2 3 4 5 6 7 8
ii) Acetylation
GST C 910 896 844 876 864 857 844 829
p300
pRb
1 2 3 4 5 6 7 8 9 10
e)
763
928
794
829
830
884
881
928
830
928
f) Acetylation
GST
794-829
830-884
881-928
830-928
763-928
p300
pRb
1 2 3 4 5 6

Figure 3.6

The C-terminal region of pRb is the major site of acetylation.

The indicated derivatives of pRb (a, c and e) were expressed and purified as GST-fusion proteins and analysed on a SDS gel by coomassie staining (bi and di). Each GST-pRb derivatives (about 2μg in b, and 5μg in d, f) was assesed for *in vitro* acetylation by Flag-p300$^{1135\text{-}2414}$ (0.3μg) in the presence of ^{14}C-acetyl-CoA. Acetylated pRb and autoacetylated p300 are indicated (bii, cii and f), and GST alone input is shown (bii, cii, and f, all in track 1). The results indicated two discrete domains in the pRb C-terminal region, from amino acid 794-829, and 830-884 contains possible acetylated lysines. The extreme C-terminal region from 881-928, although contain seven lysines, was not acetylated by p300 *in vitro*. The A pocket fusion (379-656) (indicated by *) showed a very low, but detectable level of acetylation by p300.

Figure 3.7


a)
MW 1 2 3 4 5 6
220Kd
97Kd
66Kd
45Kd
p300
pRb
E2F-1
E1A (13S)
MW 7 8 9
220Kd
97Kd
66Kd
45Kd
30Kd
21Kd
14.5Kd
p300
E1A
Histones
b)
MW 1 2 3 4 5 6
220Kd
97Kd
66Kd
45Kd
p300
pRb
E2F-1
E1A (13S)
7 8 9
p300
Histones

c)

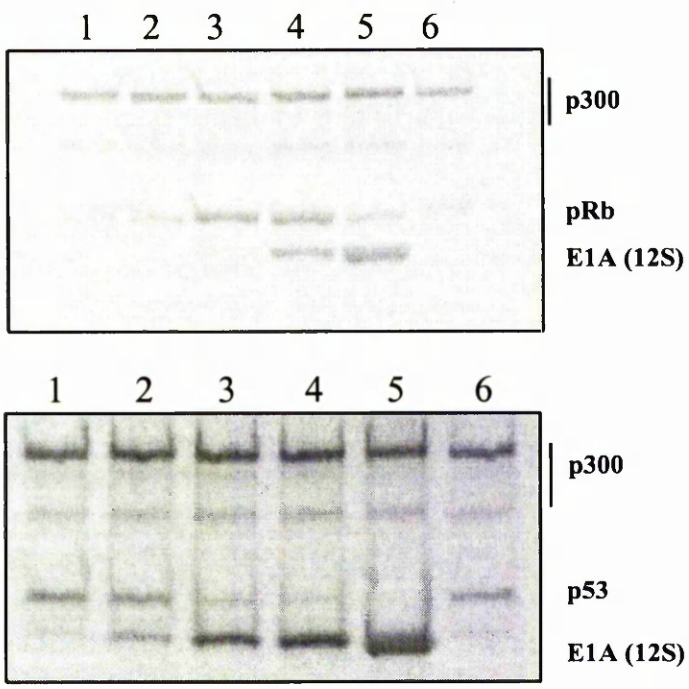


Figure 3.7

E1A enhances pRb acetylation, and inhibits E2F-1 and p53 acetylation by p300 *in vitro*.

a) Coomassie stain of GST-pRb (2μg), GST-E2F1 (2μg), chicken histones (1μg) used in the acetylation assay by Flag-p300$^{1135\text{-}2414}$ (0.3μg). His-E1A13S was titrated into the reaction from 0, 0.3, and 1μg into lanes 1-3, 4-6 and 1-9 respectively. The acetylated proteins were labeled with ^{14}C-acetyl-CoA.

b) Autodiograph of acetylated GST-Rb, GST-E2F1 and histones. E1A enhances GST-Rb acetylation (compare lanes 2, 3 to 1), represses E2F-1 acetylation (compare lanes 5, 6 to lane 4) and does not significantly alter histone acetylation (compare lanes 7, 8 and 9). In this experiment, p300 autoacetylation is not significantly altered by the presence of E1A. His-E1A was also acetylated by p300 *in vitro*.

c) Autoradiograph of GST-Rb and GST-p53 acetylation by p300$^{1135\text{-}2414}$ *in vitro*. Essentially the same experimental procedure was used as above. About 1μg of GST-Rb and 0.5μg of GST-p53 was acetylated by Flag-p300$^{1135\text{-}2414}$ *in vitro*. GST-E1A12S was titrated into the acetylation reaction from 0, 0.04, 0.2, 1, and 5μg (lanes 1-5). Lane 6 shows a control reaction using 1μg of GST alone. The top panel shows that GST-E1A enhances GST-Rb acetylation (compare lanes 3, 4 to lane 1). At a high level of GST-E1A (lane 5), the enhancement of GST-Rb acetylation by E1A was lost. The lower panel indicates GST-E1A represses GST-p53 acetylation (compare lanes 2-5 with lane 1). GST has no effect on pRb or p53 acetylation (compare lane 6 to lane 1).

Figure 3.8

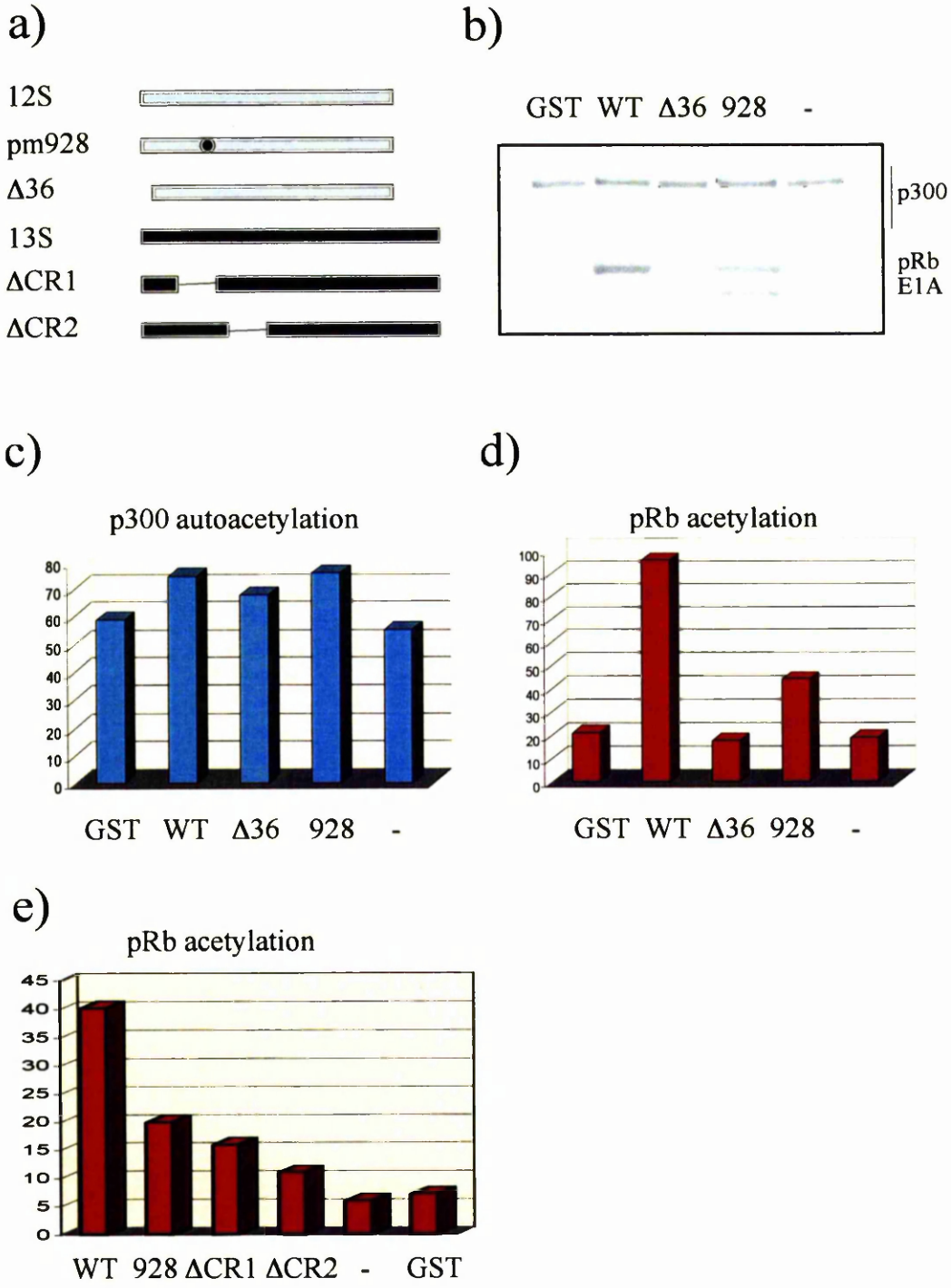

a)
12S
pm928
Δ36
13S
ΔCR1
ΔCR2
b)
GST WT Δ36 928 -
p300
pRb
E1A
c)
p300 autoacetylation
80
70
60
50
40
30
20
10
0
GST WT Δ36 928 -
d)
pRb acetylation
100
90
80
70
60
50
40
30
20
10
0
GST WT Δ36 928 -
e)
pRb acetylation
45
40
35
30
25
20
15
10
5
0
WT 928 ΔCR1 ΔCR2 - GST

Figure 3.8

E1A requires binding to both pRb and p300 to enhance pRb acetylation by p300 *in vitro*.

a) Schematic representation of E1A constructs used in the assay.

b) Phosphoimage of GST-Rb acetylated by Flag-p300$^{1135-2414}$. 2μg of GST-Rb was incubated in the presence of 1μg GST, GST-E1A, GST-E1AΔ36 or GST-E1A928, and acetylated by 0.3μg of Flag-p300$^{1135-2414}$. Acetylated proteins are labeled with ^{14}C-acetyl-coA. Lane indicated with (-) shows control with BC100 buffer alone.

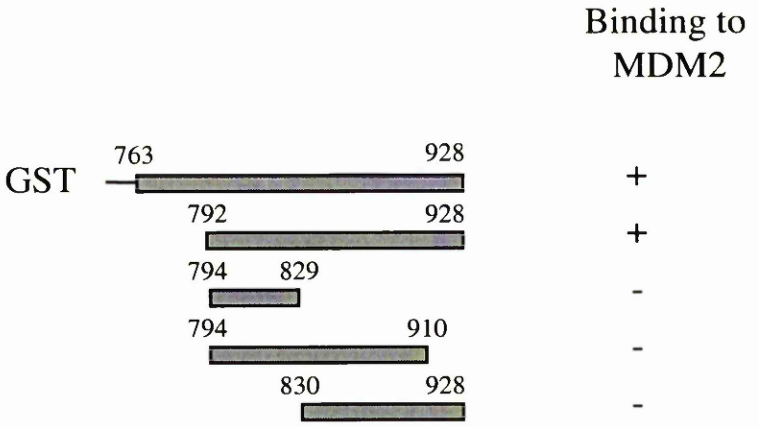
c) Quantitaion of p300 auto-acetylation from the phosphoimage in b).

d) Quantitaion of pRb acetylation from the phosphoimage in b).

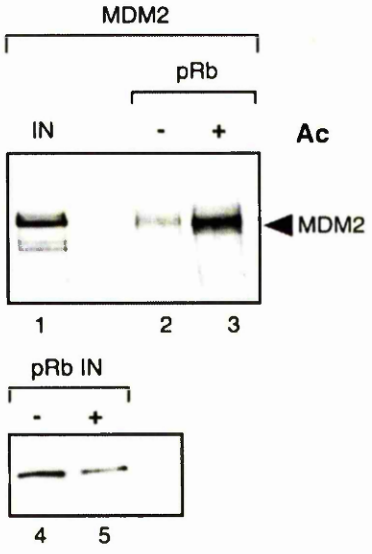
e) Quantitaion of pRb acetylation from another experiment using the same experimental procedure and conditions as described in b). Two other E1A mutants, ΔCR1 and ΔCR2, which are defective in pRb binding, were included in the assay. Control using BC100 alone is indicated as -. All three E1A mutants have reduced ability to enhance pRb acetylation by p300 *in vitro*.

Figure3.9

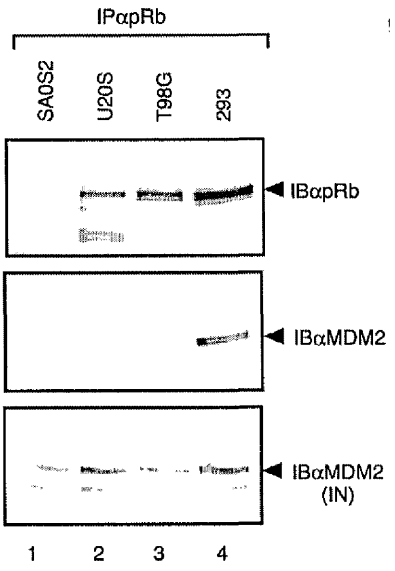
a)


Binding to MDM2
GST
763
928
+
792
928
+
794
829
-
794
910
-
830
928
-

b)


MDM2
pRb
IN
-
+
Ac
MDM2
1
2
3
pRb IN
-
+
4
5

c)


IPαpRb
SAOS2
U20S
T98G
293
IBαpRb
IBαMDM2
IBαMDM2
(IN)
1
2
3
4

d)

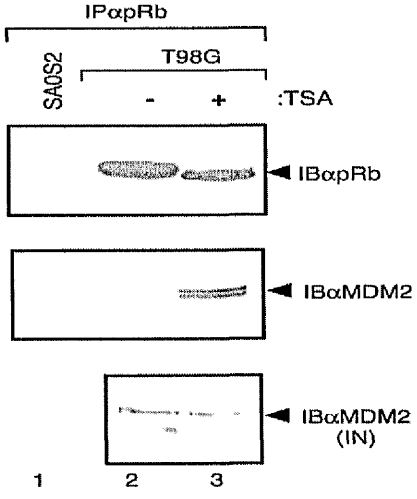

IPαpRb
SAOS2
T98G
-
+
:TSA
IBαpRb
IBαMDM2
IBαMDM2
(IN)
1
2
3

Figure 3.9

Acetylation of pRb enhances pRb binding to oncoprotein MDM2.

a) Summary of results of domain mapping of MDM2 binding to the C-terminal region of pRb. GST-Rb 792-928 was found to be the minimal domain required for binding to MDM2.

b) GST-Rb was acetylated by Flag-p300 (FL) *in vitro* using non-radiolabeled acetyl-CoA. Unacetylated pRb control was prepared in an identical acetylation reaction but omitting acetyl-CoA. After completion of the acetylation reaction, Flag-p300 was removed using Flag-beads (Sigma). The supernatant was immunoprecipitated using either anti-Ac-K antibody or anti-pRb antibody (C15) to purify the acetylated and unacetylated GST-pRb respectively. The lower panel shows input pRb used in the pulldown assay against *in vitro* transcribed and translated MDM2. Note that acetylated pRb binds more efficiently to MDM2 *in vitro* (compare lane 3 to 2, top panel).

c) Extracts from the indicated cell lines (SAOS2, U2OS, T98G and 293 HEK) were immunoprecipitated with anti-Rb antibodies (IF-8) and, thereafter analysed by SDS-PAGE and immunoblotted with either anti-Rb antibodies (G3-245, top panel), or anti-MDM2 (H-221, middle panel). A high level of co-immunoprecipitated MDM2 was seen in the pRB immunocomplex from 293 cell extracts. SAOS2 cells are $Rb^{-/-}$, and were used as negative control for the immunoprecipitation and immunoblotting. The bottom panel show input protein level of MDM2 from various cell lines.

d) Extracts prepared from T98G cells either treated with or without TSA (5μM) for 10-20h, and untreated SAOS2 cells were immunoprecipitated with anti-pRb (C15 and IF-8) antibodies and after electrophoresis immunoblotted with either anti-pRb G3-245 (top) or anti-MDM2 (middle) monoclonal antibody. An increased amount of MDM2 was found in the pRb immunocomplex from TSA-treated T98G cells. In lane 1, SAOS2 cell extract was used for control of the immunoprecipitation and immunoblotting. The bottom panel indicates the input of MDM2 protein level.

Figure 3.10

a)

870-KPLKKL-876

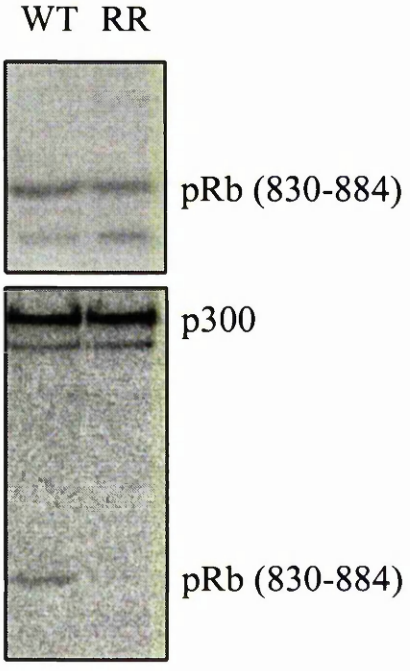
RR

QQ

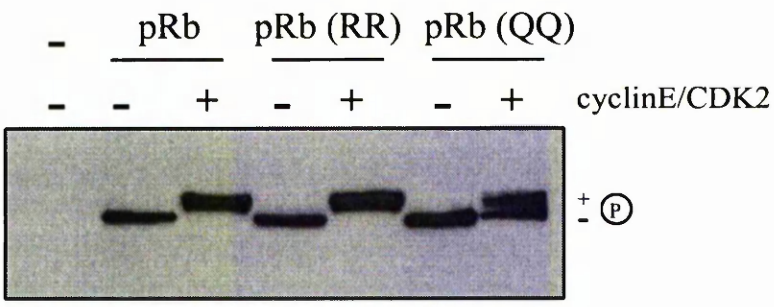
b)

KPL**KK**L	pRb
KAC**K**RK	MyoD
KGV**K**SP	E2F-1
KTP**K**SP	E2F-2
KGG**K**G-	H4
KGS**K**K-	H2B
KGK**K**--	GATA-1
RHK**K**L-	p53

c)



d)



e)

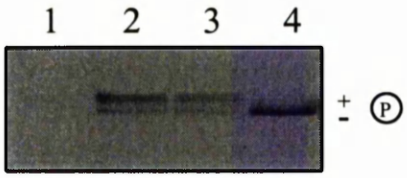


Figure 3.10

Acetylation at the pRb C-terminus (K873/874) influences pRb phosphorylation by cdk complexes.

a) The sequence motif previously indicated to be important in regulating pRb phosphorylation. The lysine resiudes 873, 874 were mutated to arginine (R) or glutamine (Q) as indicated.

b) Sequence comparison of a lysine sequence found to be acetylated in various proteins. A KXXK motif may confer a general consensus sequence for acetylation. The acetylated lysines are indicated in bold.

c) *In vitro* acetylation of the indicated derivatives (2μg) of GST-pRb (830-884), or GST-pRb (830-884)RR. The top panel shows the coomassie staining of the purified proteins (WT for GST-pRb (830-884); RR for GST-pRb (830-884)RR) and the lower panel shows the autoradiograph of the acetylated proteins.

d) *In vivo* phosphorylation of the indicated derivatives of full-length pRb, namely the wide type pRb, 873/874RR and 873/874QQ. SAOS2 cells were transfected with pRb (10μg) in the presence or absence of cyclinE/cdk2 (4μg each). Lane 1 is a control of untransfected SAOS2 cell. After 36 hour of transfection, 75μg of the whole cell extract was immunoblotted with anti-pRb (G3-245). The position of the hyper- (+) and hypo- (-) phosphorylated (P) pRb is indicated.

e) *In vivo* phosphorylation of pRb and mutant in U2OS cells. Same procedure as described in d), but without expressing exogenous kinases. Lane 1 is a control of untransfected cell extracts. Lanes 2 to 4 are derived from U2OS cells transfected with wild type pRb, 873/874RR and 873/874QQ (10μg) respectively.

Figure 3.11

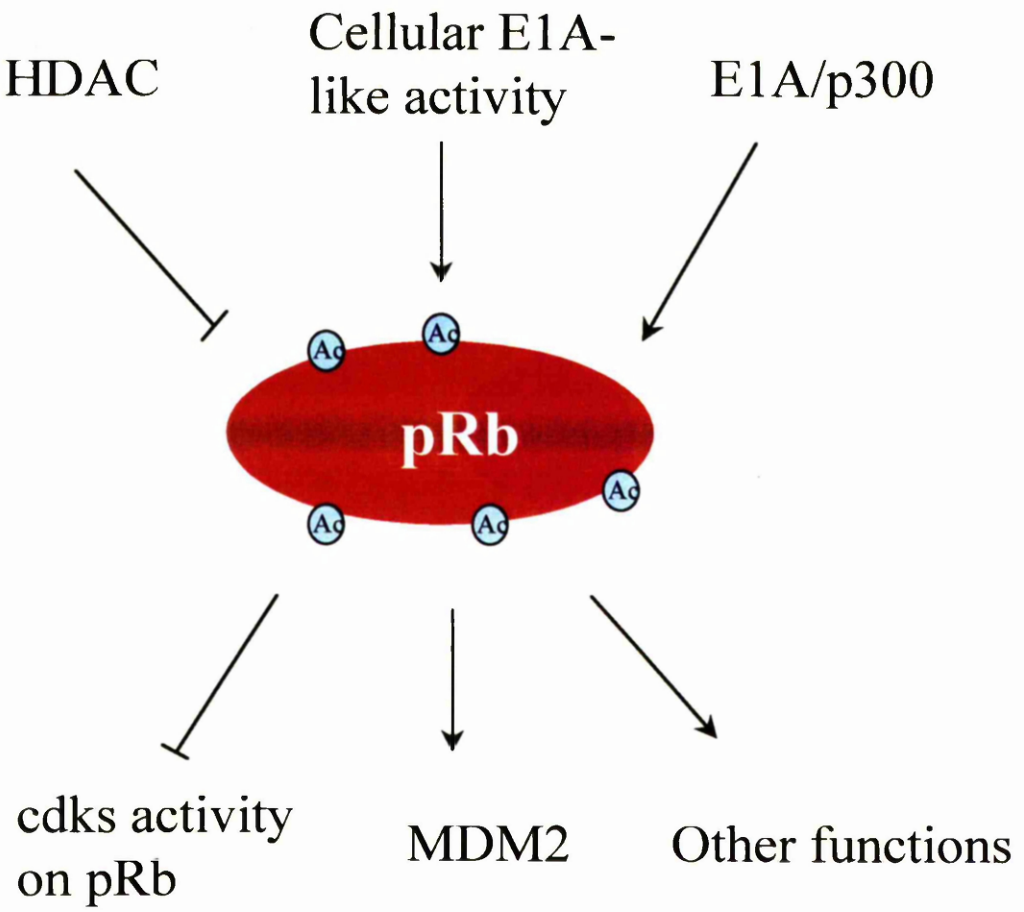

HDAC
Cellular E1A-like activity
E1A/p300
Ac
Ac
pRb
Ac
Ac
Ac
cdks activity on pRb
MDM2
Other functions

Figure 3.11 Summary of pRb acetylation.

Overall, the results suggest that E1A/p300 can positively stimulate pRb acetylation, and that the pRb associated HDAC activity may de-acetylate pRb (Smith *et. al.*, personal communication). Acetylated pRb binds more efficiently to MDM2, and acetylation in the C-terminal region of pRb also affects its phosphorylation by cyclin/CDK complex. A possible unidentified cellular E1A-like activity could exist in regulating pRb acetylation. In addition, acetylation might have other function(s) in controlling pRb activity.

Chapter 4: Functional Interaction of a Nucleosome Assembly Protein-2 and p300

Introduction

Transcription of the eukaryotic genome is a highly dynamic process in which genes are constantly turned on and off. Genomic DNA exists as chromatin, whose complex structure and highly condensed status acts as a significant barrier to sequence specific transcription factors by preventing them accessing and activating target genes (reviewed in 7, 54, 82, 157, 170). Until now, at least two mechanisms have been widely documented to remodel chromatin, thereby regulating target genes transcription (reviewed in 82). The first mechanism involves enzymatic activities, such as acetylation/deacetylation, methylation/de-methylation, and/or phosphorylation. In particular, HATs and HDACs, which, respectively, add and remove acetyl groups from the amino termini of the four core histones; hyperacetylation is usually (but not always) correlated with activation of transcription, whereas hypoacetylation is associated with repression of transcription. The second mechanism consists of ATPase-dependent chromatin remodelling complexes, which utilize energy from ATP hydrolysis to alter chromatin structure by changing the location and/or conformation of the nucleosomes.

The p300/CBP family is a group of co-activator proteins that act to nucleate the assembly of diverse co-factors into co-activator complexes (reviewed in 147). p300/CBP were shown to bind to a wide variety of transcription factors (55, 76, 87, 92, 97, 147, 164, 178), interact directly with the basal transcription machinery (124), and possess intrinsic HAT activity (12, 128). These proteins function as transcription co-activators through a process that is believed to bridge transcription factors with the basal transcription machinery, and by utilizing their HAT activity. The p300/CBP HAT domain can stimulate transcription when artificially

tethered to the AdML promoter as a Gal4- fusion protein (109). However, evidence is still lacking that p300/CBP acetylates histones on promoters *in vivo*. p300/CBP has been frequently found in complexes with other HATs, including pCAF (188), SRC-1 (154) and ACTR (26). Different HATs may act on specific substrates to achieve a co-ordinated transcriptional programme. Alternatively, a critical mass of HAT activity maybe required to achieve transcriptional activation. In this model, p300/CBP might be considered as an assembly platform for the formation of protein complexes, leading to transcriptional activation. Furthermore, p300/CBP can acetylate other non-chromosomal proteins, including TFIIE and TFIIF (68), which are part of the basal transcription machinery, suggesting alternative mechanisms of p300/CBP in transcriptional control.

Nucleosome assembly protein (NAP) is a family of histone chaperone-like proteins which have been attributed to roles in cell cycle function and transcriptional control (72, 79, 171). Mouse NAP-1 (also known as AP-1) (70) was first cloned from FM3A cells using a biochemical assay to purify an activity which facilitates the assembly of nucleosome-like structures *in vitro* (70). NAP shares similar activity as nucleosplasmin and N1/N2 in its ability to bind to core histones, and induce supercoiling into DNA in the presence of histones (70, 71, 171). Biochemical assays and immunoprecipitation experiments suggest that NAP preferentially binds to H2A/H2B heterodimer, and forms a 12S complex in the presence of the histone (71, 112). Similar to nucleoplasmin and N1/N2, NAP family proteins also contain clusters of negatively charged amino acids, which might assist nucleosome assembly *in vitro* (49, 136). Soybean, yeast and Drosophila NAP-1 were demonstrated to possess nucleosome assembly activity (22, 49, 72, 191). Yeast NAP-1 stimulates transcription factor binding and histone displacement on a nucleosome template, probably via a mechanism involving disruption of the histone octamer (171). In Drosophila, dNAP co-operates with dCAF-1 (*drosophila* chromatin assembly factor-1 containing fraction) to promote regularly spaced nucleosome arrays in an

ATP-dependent manner (22, 72). Interestingly, both yeast and *Xenopus* NAP-1 were shown to associate with Clb2 to perform Clb2-dependent mitotic functions, and suppress polar bud growth in budding yeast (79, 80). Human NAP-2 was identified by positional cloning at chromosomal location 11p15.5, a region implicated in several diseases including Wilms tumour (WT) and Beckwith-Wiedemann syndrome (BWS), in an effort to determine the genomic boundary for imprinting in the region (66, 136). However, hNAP-2 is biallelically expresssed, and was not found to be involved in the WT or BWS etiology (64, 134). NAP-2 shows significant sequence homology to NAP-1 and also possesses histone binding and nucleosome assembly activity (136). hNAP-2 is phosphorylated by Caesin kinase 2 (CKII), and phosphorylated hNAP-2 is retained in the cytoplasm in a complex with histones during the G0/G1 phase. Dephosphorylation of NAP-2 at the G1/S boundary triggers NAP-2 transport into the nucleus in S phase, suggesting a role in replication-dependent chromatin assembly (137). Consistent with this finding, hNAP, yNAP and dNAP were predominantly cytoplasmic, but showed nuclear localisation during the S phase of the cell cycle (72, 137). Another NAP family protein, TAF-1 (template activating factor 1, also known as *set*), was identified on the basis of its ability to stimulate adenovirus replication in a viral chromatin template (123). In addition, TAF-1 is the subject of chromosomal aberration in myeloid leukaemiogeneisis (123), implying that the NAP family of proteins may play a role in oncogenesis. Overall, NAP is a family of proteins which are highly conserved through evolution among different species, with common function in histone binding and nucleosome assembly.

In an effort to understand p300 cellular functions, a yeast two-hybrid screen was carried out to isolate p300-interacting proteins (Shikama *et. al.*, in press). Both NAP-1 and -2 polypeptides were identified to bind to p300 from a 10.5d.p.c mouse embryo cDNA library. Given that both NAP and p300 have been reported to act in chromatin remodelling and modification, experiments were carried out to investigate if these two proteins co-operate in transcriptional regulation. Domain mapping experiments suggested that NAP-2 binds

exclusively to the CH3 domain in p300. Conversely, p300 binds to two discrete regions in NAP, one in the central region, and the other in the C-terminal region. NAP family proteins may also dimerise and/or oligomerise *in vitro*. Domain-mapping experiments suggested that the N- and C-terminal of the protein is required for NAP-NAP interaction. Furthermore, NAP was found to augment the transcriptional activity of p300 targets, including p53 and E2F-1. Interestingly, NAP inhibits histone actylation by p300 *in vitro*, probably by binding directly to the histone tails. NAP was not found to have any intrinsic HAT activity, nor act as a substrate of acetylation by p300.

Results

Using LexA-p300$^{611-2284}$ as bait, one NAP-1 and two NAP-2 clones were identified as p300 interacting proteins from a 10.5 d.p.c. mouse embryo cDNA library (Shikama *et. al.*, in press). Other p300 interacting proteins identified included JMY (146). Immunoprecipitation experiments were performed in which over-expressed G4-p300$^{611-2284}$ was confirmed to interact specifically with HA-NAP-2 in SAOS2 cells (Shikama *et. al.*, in press). Using an anti-NAP-2 antibody against the C-terminus of the protein, it was demonstrated that endogenous NAP-2 associated with p300 in A31 cells (Shikama *et. al.*, in press). The mouse NAP-2 protein was shown to possess nucleosome assembly activity using a DNA supercoiling assay (Cairns *et. al.*, personal communication). In addition, p300 augmented NAP-2 function in the supercoiling assay.

The aim of this study was to define the domains of interaction between NAP-2 and p300, to decipher the functional significance of the interaction, and to explore the possible regulation of NAP-2 on p300 HAT activity. It is to be noted that two NAP-2 splice isoforms were isolated, named L5 and L7 (66, 136). L7 has additional 12 amino acids at the C-terminus of the protein (136). All work presented here used the NAP-2 L7 isoform (which is generally referred to as NAP-2), unless otherwise stated.

NAP-2 binds to the CH3 domain in p300

Original yeast two-hybrid screening experiments identified a p300 fragment from amino acid residue 611-2284 bound to NAP-2 (Shikama *et. al.*, in press). Mammalian two hybrid assays were performed to further locate the NAP-2 binding domain(s) on p300. Various Gal4-p300 fragments were tested for their binding to NAP2-VP16 (Fig 4.1b). The NAP-2 binding domain on p300 was mapped to the C-terminal region, from amino acid 1572-2284. The binding

domain was further resolved to a region between 1572-1906, which encompasses the CH-3 (Fig 4.1b). In the same experiment, Gal4-p300$^{1818\text{-}2080}$ and Gal4-p300$^{1906\text{-}2284}$ were not found to interact with NAP-VP16.

In order to substantiate the mammalian two-hybrid mapping data, an alternate approach was used to confirm the result. Various GST-p300 fusion proteins were over-expressed and purified from bacterial extracts. The NAP-2 polypeptide was generated and labeled with ^{35}S by *in vitro* transcription and translation. An *in vitro* binding assay was performed to identify the NAP-2 interaction domain on p300. Consistent with the findings of the mammalian two-hybrid assay, GST-p300$^{1572\text{-}1906}$ bound specifically to NAP-2, but not to lucifearse, which was used as a negative control for non-specific binding (Fig 4.2). Furthermore, two over-lapping fragments, GST-p300$^{1818\text{-}2080}$ and p300$^{1302\text{-}1737}$, did not bind to NAP-2 (Fig 4.2), nor did any other GST-p300 fusion proteins which lack the CH3 domain. Some of the GST-p300 fusion proteins (e.g. 1-595, 1818-2080, 1906-2284) were active in binding to other well-described p300-interacting proteins, such as p53 and E2F-1 (data not shown). GST-p300$^{1195\text{-}1673}$, which encompasses the HAT domain of p300, has active HAT activity (Bandara *et. al.*, personal communication). Therefore, all GST-p300 fusion proteins are active in at least some p300 functions. Their lack of binding to NAP is not simply due to possible structural defects of the truncated proteins. These results indicated that NAP-2 bound specifically to the CH3 domain of p300.

Both the central and C-terminal region of NAP-2 bind to p300

Similarly, *in vitro* pulldown assays were used to identify p300 binding domain(s) on NAP-2. Various NAP-2 derivatives were ^{35}S-labeled by *in vitro* transcription and translation. Luciferase was used as negative control for non-specific binding. GST-p300$^{1572\text{-}1906}$ was used to pulldown different NAP-2 derivatives, and GST- alone was used as control for non-specific binding. Binding results suggested that NAP-2 has two discrete binding domains for p300, one

in the central region from amino acid 110-230, and the other in the C-terminal region, encompassing amino acid 234-386 (Fig 4.3a, b and c). The N-terminal region from amino acid 1-123 only had a marginal level of binding to p300, which was probably due to non-specific binding. These results indicated that NAP-2 contains at least two distinct interaction domains for p300.

The N and C-terminal regions of NAP-2 can mediate homo- or hetero-dimerisation of NAP family members

Previously, NAP-1 was found to sediment to a position of approximately 5S on a sucrose gradient, which has been considered as evidence for a dimer in physiological ionic condition (71). Experiments were therefore carried out to test if NAP-2 can form homo- or hetero- dimers. Using the biochemical pulldown approach, various NAP-2 deletion polypeptides were tested to identify the putative dimerisation domain(s) *in vitro*. His-NAP-2 fusion proteins were purified from bacteria (Fig 4.4a). His-beads treated with bacterial lysate which did not over-express His-NAP-2 fusion protein were used as a negative control for non-specific binding. The results suggested that NAP-2 can interact with itself under the conditions tested (Fig 4.3e, lane 1). Furthermore, there were two discrete domains for dimerisation, one in the N-terminal from amino acid 1-123, and the other at the C-terminal from amino acid 234-386. An internal region from amino acid 110-230 was not required for NAP-NAP interaction (Fig 4.3d, e and f). It was also demonstrated that NAP-1 can bind to NAP-2 using the same assays (Shikama *et. al.*, in press). Taken together, these results indicated NAP family members can form homo- and/or hetero-dimers, and two binding domains are likely to be involved in facilitating these interactions.

NAP-2 inhibits p300-dependent histone acetylation

p300 is endowed with HAT activity, and acetylates all four core histones *in vitro* (12, 128). At least on some promoters, the intrinsic HAT activity is required for its transcription activation (109). Here, experiments were carried out to investigate if NAP-2 regulates the p300-dependent acetylation. First, NAP was shown not to be a substrate of p300 or p300/pCAF HAT activity (Fig 4.4b). By titrating in the indicated amount of bacterially purified His-NAP-2 fusion protein (either L5 or L7), NAP-2 inhibited histone acetylation (Fig 4.5a). NAP-2 also inhibited histone acetylation by another HAT, namely pCAF (Fig 4.5b). Furthermore, although NAP-2 inhibited histone acetylation by p300 *in vitro*, it did not inhibit the autoacetylation of p300, implying that NAP-2 might not affect the intrinsic HAT activity of p300 (Fig 4.5c).

NAP family of proteins are known to bind to histones (71), possibly via the two highly conserved acidic amino acid regions (49). These acidic residues were thought to interact with the basic tail-region of histones, in a charge-mediated interaction (49). A synthetic peptide containing the acidic stretch in the C-terminal, but not a control peptide covering the N-terminal of the protein, was found to inhibit p300 and pCAF-dependent acetylation *in vitro* (Fig 4.5d). Together with the observation that GST-histone tail fusions can interact with NAP (Shikama *et. al.*, Smith *et. al.*, personal communication), it suggested that NAP-2 bound to histone tails, possibly 'hiding' the lysines in the tail region, and thereby inhibiting histone acetylation. Consistent with this idea, NAP-2 was also found to inhibit histone deacetylation by HDACs *in vitro* (Smith *et. al.*, personal communication).

NAP-2 and p300 co-operate in transcriptional activation

Since both NAP-2 and p300 were reported to possess chromatin remodelling activity in regulating transcription (171), experiments were performed to investigate if p300 and NAP-2 regulate transcriptional activity. Using a reporter gene assay, p300 and NAP-2 were

demonstrated to co-operate in transcription activation mediated by E2F-1 and p53 (Fig 4.6 and Shikama *et. al.*, in press). The reporter was stably integrated into U2OS cells to recapitulate the chromatin environment. Gal4-E2F-$1^{380-437}$, which contains the transcription activation domain of E2F-1 fused to the Gal-4 DNA binding domain, was used to activate the reporter. When NAP-2 or p300 were transfected independently with Gal4-E2F-1, only marginal transcription co-activation of 1.5-2 fold was observed. Co-transfection of all three constructs showed a significant co-activation of 8-9 folds over G4-E2F-$1^{380-437}$ alone (Fig 4.6). Similar experiments also suggested that NAP and p300 augment p53 transcriptional response (Shikama *et. al.*, in press). Furthermore, this co-operation can be blocked by a dominant negative p300 construct from amino acid 1572-1906, which encompasses the NAP-2 interaction domain on p300. In addition, NAP/p300 co-operates in up-regulating endogenous p21 (47) gene transcription by p53 in SAOS2 cells (Shikama *et. al.*, in press). These observations suggested that NAP-2 augments the transcriptional activity of at least two p300-dependent transcription factors.

Discussion

In this study, NAP-2 was identified as a novel p300 interacting partner. Given that both proteins have chromatin re-modelling activity (82), effort was concentrated to elucidate the functional consequence of NAP and p300 in transcriptional regulation. Indeed, NAP and p300 were found to augment E2F-1 and p53 dependent transcription activation. The result was substantiated with the observation that NAP-2 and p300 co-activated endogenous p21 gene expression in a p53-dependent manner (Shikama *et. al.*, in press). Previously, the p300/CBP family member p270 was found to be a component of the mammalian SWI/SNF complexes (34). NAP was also demonstrated to co-operate with the ISWI-ATPase complex in generating spaced nucleosome arrays (22, 72). The interaction between NAP and p300 might reflect another mechanism of co-operativity between HAT and ATPase remodelling activity in transcriptional regulation.

Domain mapping experiments demonstrated that NAP-2 specifically interacts with the CH3 domain in p300. A variety of cellular factors, including E2F-1 (164) and p53 (55), and viral oncoproteins, such as E1A (147), bind to p300 in similar region. Given that NAP augments E2F-1 and p53 transcription in a p300-dependent manner, it is unlikely that the NAP-p300 interaction will hinder the p300-E2F/p53 interaction. Indeed, E2F-1 and p53 may have multiple interaction domains within p300, including the CH1, CH3 and the C-terminal *trans*activation domain of p300 (data not shown). It will be interesting to investigate if NAP binding to p300 augments or alters the p300-E2F-1/p53 interaction, which could be another possible explanation to the activation observed with the E2F-1- and p53- dependent transcription.

p300 binds to at least two discrete regions on NAP-2. It is noted that both the central and the C-terminal domains in NAP-2, which were sufficient to bind to p300, possess stretches of highly conserved acidic amino acids. These acidic stretches may be important in mediating

NAP-2-histone interaction. Further experimentation will be required to characterise the precise region mediating the NAP-2/p300 interaction. Interestingly, in the binding assay performed, NAP-2 was found to complex with NAP-1 and NAP-2, possibly forming homo- or hetero-complexes with other family members. This is consistent with the previous observations suggesting that NAP protein might exist as dimer/oligomer under physiological conditions (71). When NAP was mixed with core histones, it sedimented as a 12S complex on a sucrose gradient (70, 71). The 12S complex was thought to consist of a histone octamer and two or three molecules of NAP (70). Two discrete regions were found to mediate dimer/oligomer formation, one in the N-terminal and the other in the C-terminal region of the protein.

In the current study, NAP-2/p300/histones were found to exist in a ternary complex (Shikama *et. al.*, in press). This was demonstrated by using an *in vitro* binding assay and by using a sucrose gradient. Furthermore, the NAP-2/p300 interaction was stabilized in the presence of histones. Therefore, NAP-2/p300 might form a tight complex with histones in the nucleosomes, which then stabilize chromatin in an activated state for transcription activation. Experiments were carried out to address whether NAP-2 can interact with the nucleosome in a nucleosome gel-shift assay (data not shown). However, the evidence was not conclusive if NAP-2 is in a stable complex with the nucleosome.

From the immunoprecipitation study, a low level of NAP-2 protein was in complex with p300 (Shikama *et. al.*, in press). Immunostaining experiments suggested that NAP-2 is predominantly cytoplasmic (72, 80, 136, 137). However, a significant amount of NAP re-located to the nucleus in S-phase, suggesting a role for NAP proteins in histone shuttling (72, 136, 137), and indicating a possible involvement in a replication-dependent nucleosome assembly. Indeed, the NAP family member TAF-1 was shown to activate adenoviral replication in a viral chromatin template (123). It remains to be tested if p300/NAP is involved in replication-dependent chromatin assembly. Interestingly, the NAP-2 protein was found to be phosphorylated

in cells and phosphorylation of NAP-2 may control its celluar localisation (137). CKII phosphorylates NAP-2 in HeLa and COS cells (137), and phosphorylated NAP-2 was retained in the cytoplasm. Consistent with the data, multiple consensus CKII phosphorylation sites do occur throughout the NAP-2 protein sequence (136). In yeast, NAP was found to be associated with Clb2 (79), and can be phosphorylated by cyclin B/p34^{cdc2} *in vitro* (80). Furthermore, NAP is required for Clb-2 mediated mitotic functions, including microtubule re-organisation during mitosis, and to suppress polar growth of the budding yeast (80). yNAP also interact with another kinase, the Gin4 kinase (4). Overall, phosphorylation control of NAP possibly occurs in a cell cycle dependent manner. It will be important to establish if the NAP-p300 interaction is also regulated by phosphorylation. Since NAP-2 was found to facilitate nucleosome assembly and co-operate with p300 in transcriptional regulation, both functions may require NAP protein to localise to the nucleus. NAP is only transiently transported into the nucleus during S phase of the cell cycle, and NAP predominantly resides in the cytoplasm. This potentially offers an explanation of low level of interaction between NAP-2 and p300 in various immunoprecipitation experiments performed by my colleagues in the laboratory.

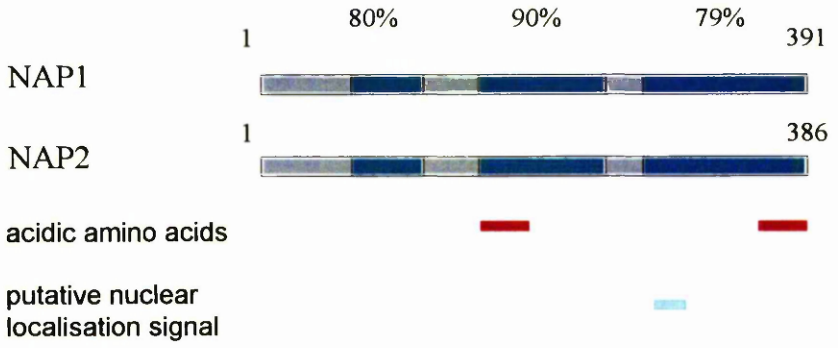
Since the intrinsic HAT activity is important for p300-dependent transcription co-activation (109), experiments were carried out to investigate if NAP regulates p300 HAT activity. First, NAP was demonstrated to possess undetectable HAT activity. In addition, NAP was not acetylated by p300 or pCAF *in vitro*. However, NAP inhibits histone acetylation by both p300 and pCAF *in vitro*. A synthetic peptide encompassing the C-terminal acidic stretch also inhibits histone acetylation *in vitro*. This observation at first seems to contradict with the findings that NAP/p300 co-operates in transcriptional activation since hypoacetylation of histones is generally correlated with transcriptional repression. Further analysis suggested that NAP does not inhibit the intrinsic HAT activity of p300. Indeed, NAP was known to bind to both acetylated and unacetylated histones with similar efficiency (112). The acidic amino acids

were shown to be required for histone binding (49). Therefore, the most likely explanation is that NAP binds to the N-terminal tail of histones, possibly via a charge-charge type of interaction, thereby 'hiding' the lysines in the histone tail from p300/pCAF HAT activity. The significance of this inhibition in term of NAP/p300-mediated transcriptional regulation is not clear.

In conclusion, p300 associates with a class of transcription regulator, NAP, which can contribute to chromatin remodelling. Here, p300 and NAP were demonstrated to activate transcription. Whilst it is well established that the HAT activity of p300 is required for transcriptional activation, at least on some promoters (109), the underlying mechanisms responsible for these effects are not clear. Moreover, while both ATP-dependent chromatin remodelling activity and HAT activity are known to be important for transcription regulation, the order in which these activities act on promoters is unclear. The current idea (82) is that the ATPase activity acts first, which causes a rapid interconversion of chromatin between an activated and a repressed state. The HAT activity is then targeted to the activated gene to 'fix' the chromatin in the active state to substantiate its activation. Although the generality of this model remains to be tested, our observations provide one mean to bridge together the ATPase activity with HAT, most probably via a direct interaction between NAP and p300 (Fig 4.7). NAP function is cell cycle regulated (112, 157), as well as the apparent association with H2A and H2B (71), indicating a histone chaperone function in transporting histones from the cytoplasm to nucleus. In the nucleus, NAP may act in concert with ATPase-dependent remodelling factors to re-structure nucleosome positioning. It may not be a coincidence that NAP interacts with the histone tails and the disordered regions of linker histone (112), since these are regions subject to post-translational modifications such as acetylation and the sites of action for transcriptional activators. Therefore, one might imagine that NAP recruits p300 to certain activated nucleosomes, allowing p300 to target specific nucleosomes to achieve a regulated transcriptional co-activation.

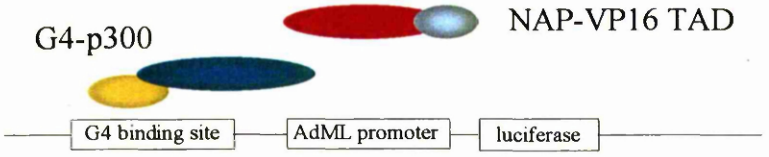
Figure 4.1

a)

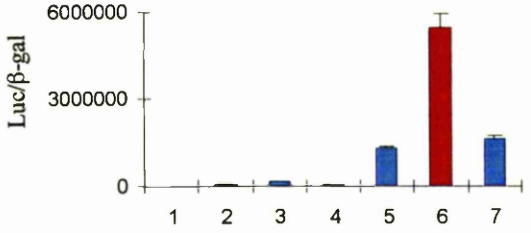


b)

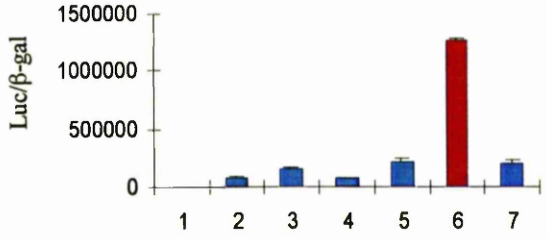
i)



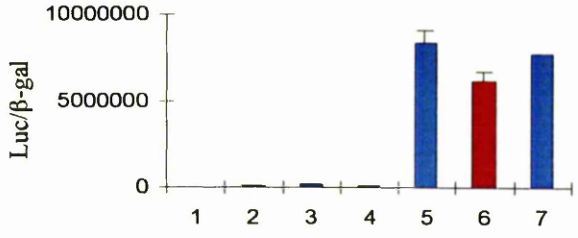
ii) G4-p300$^{1572-2284}$/NAP-VP16



iii) G4-p300$^{1572-1906}$/NAP-VP16



iv) G4-p300$^{1906-2284}$/NAP-VP16



v) G4-p300$^{1818-2080}$/NAP-VP16

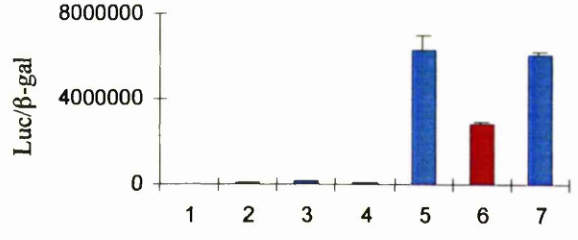


Figure 4.1

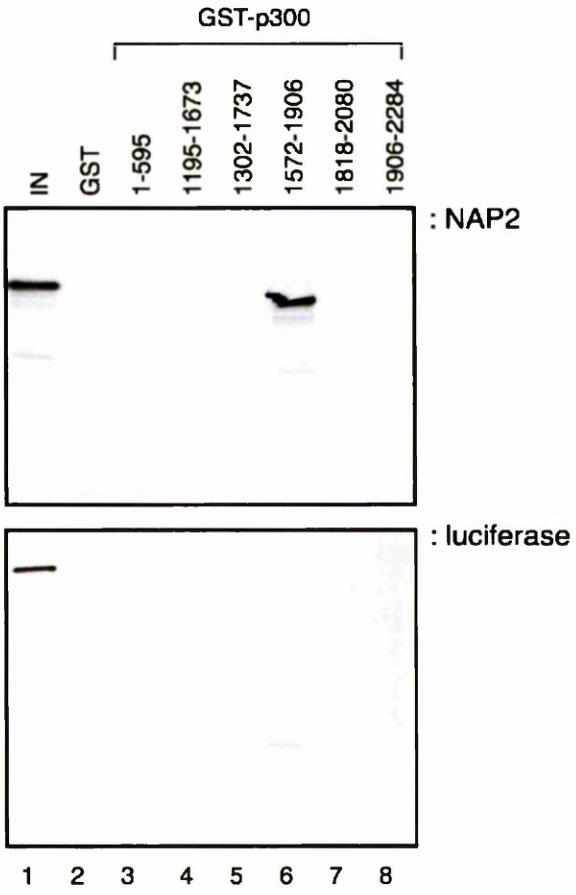
NAP-2 binds to the CH3 domain in p300.

a) Summary of functional domains and sequence similarity between NAP-1 and NAP-2. Mouse NAP-1 and NAP-2 share extensive homology. The numbers indicate the percentage of identify among the three shaded regions. Two stretches of acidic amino acids, one in the central region, and the other in the C-terminus of the protein, are indicated in red. A putative nuclear localisation signal (IKKKQKH) is indicated in blue.

b) A mammalian two-hybrid assay was performed in U2OS cells. The schematic representation of the assay is indicated in i). In ii), iii), iv) and v) pG4-p300$^{1572\text{-}2284}$, pG4-p300$^{1572\text{-}1906}$, pG4-p300$^{1906\text{-}2284}$, and pG4-p300$^{1818\text{-}2080}$ were tested if they interact with pVP16-NAP-2. Lane 1 is the basal activity of the reporter alone. Lanes 2 to 4 are controls when pG4 (1μg) was co-transfected with pVP-16, pVP-16-NAP-2, or non-specific DNA (all 0.5μg). Lanes 5 and 7 are controls when the indicated pG4-p300 derivatives (1μg) was co-transfected with pVP-16 or non-specific DNA (0.5μg). Lane 6 shows activity when the indicated pG4-p300 derivative was co-transfected with pVP-16-NAP-2 (0.5μg). The values shown are derived from duplicate readings (luciferase/β-gal activity). All experiments have been repeated at least three times to ensure the reproducibility. Similar results have been reproduced in C33A cells.

Figure 4.2

a)


GST-p300
IN
GST
1-595
1195-1673
1302-1737
1572-1906
1818-2080
1906-2284
: NAP2
: luciferase
1 2 3 4 5 6 7 8

b)

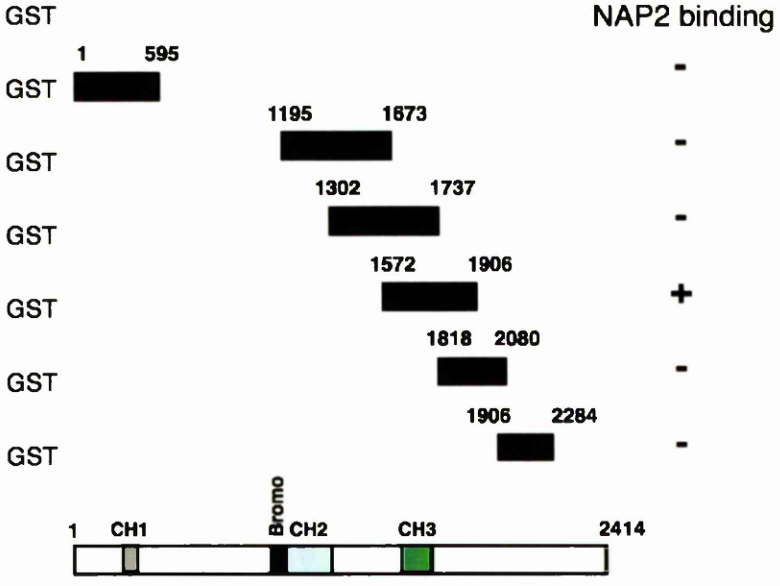

GST
NAP2 binding
1 595
GST
-
1195 1673
GST
-
1302 1737
GST
-
1572 1906
GST
+
1818 2080
GST
-
1906 2284
GST
-
Bromo
1 CH1 CH2 CH3 2414

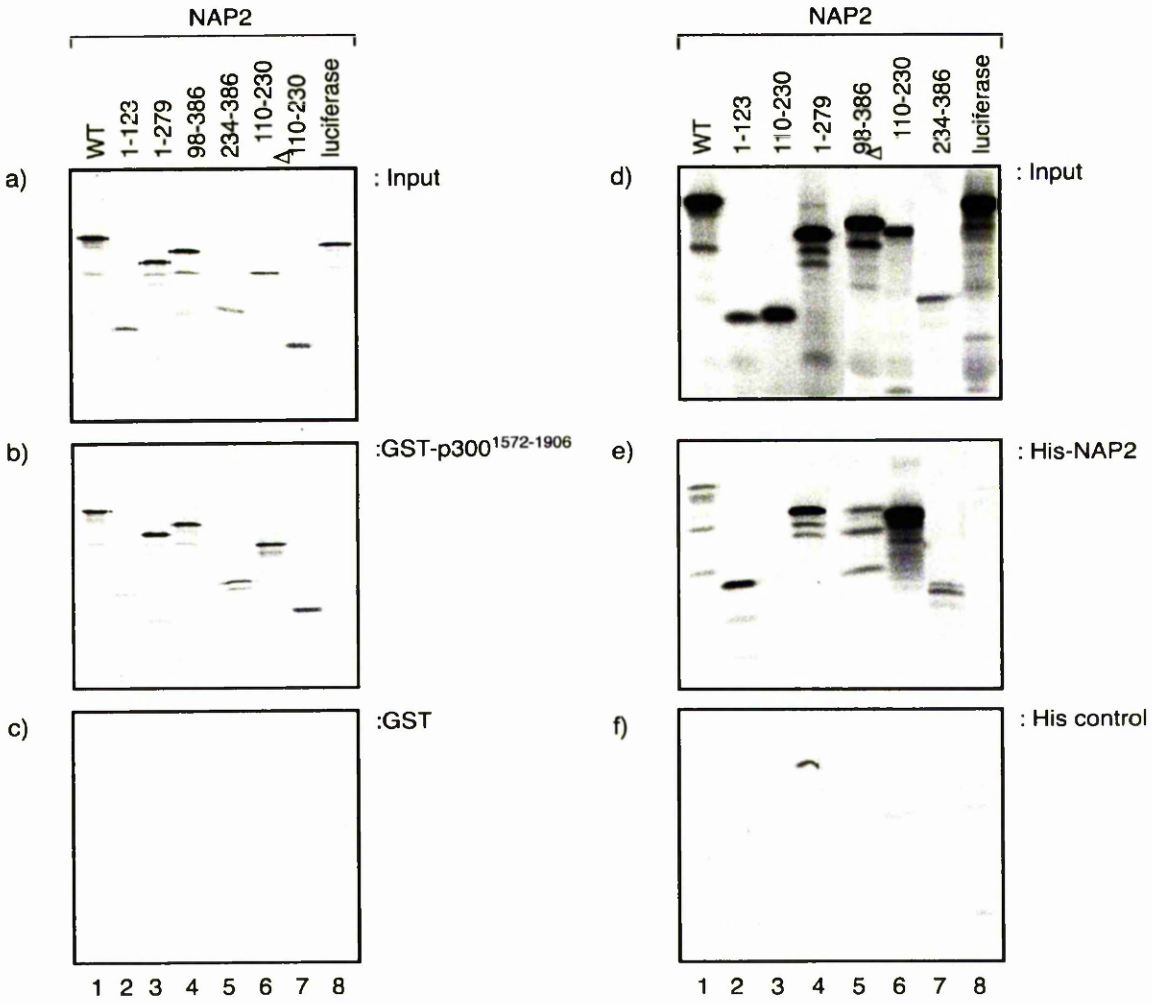
Figure 4.2

NAP-2 binds to the CH3 domain in p300.

a) Binding assay between the indicated GST-p300 fusion proteins and *in vitro* translated NAP-2 (top panel) or luciferase (bottom panel) in which about 5 μg of GST-fusion proteins was incubated with the *in vitro* translated products. The luciferase was used as a negative control for non-specific binding to the GST-fusion proteins.

b) Summary of the binding data in a). NAP-2 binds exclusively to the CH3 domain in p300, from amino acid 1737-1818.

Figure 4.3


NAP2
WT
1-123
1-279
98-386
234-386
110-230
Δ10-230
luciferase
a)
: Input
b)
:GST-p300[1572-1906]
c)
:GST
1 2 3 4 5 6 7 8
NAP2
WT
1-123
110-230
1-279
Δ98-386
110-230
234-386
luciferase
d)
: Input
e)
: His-NAP2
f)
: His control
1 2 3 4 5 6 7 8

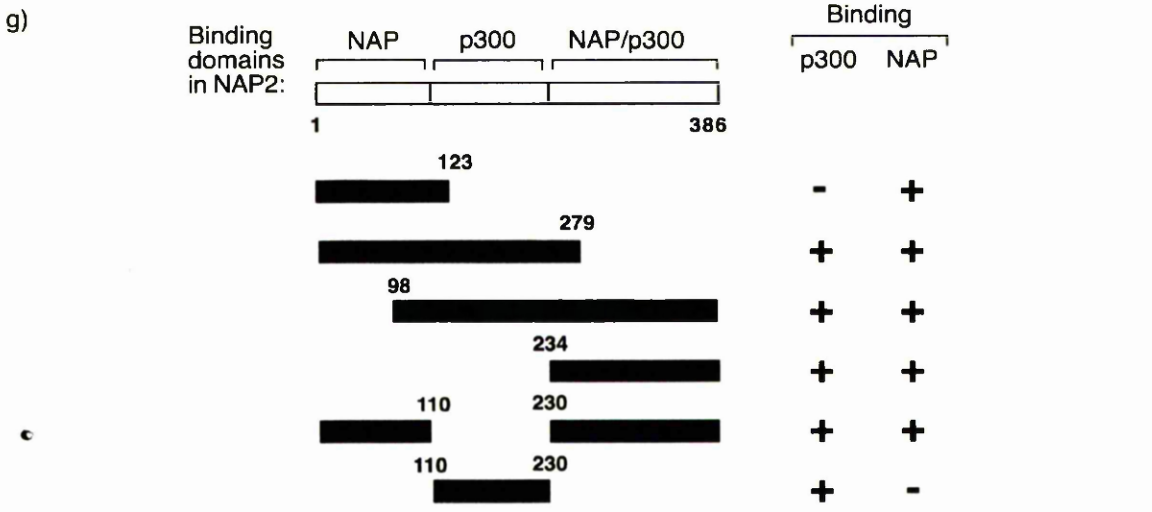

g)
Binding domains in NAP2:
NAP
p300
NAP/p300
1
386
Binding
p300
NAP
123
279
98
234
110
230
110
230
-
+
+
+
+
+
+
+
+
-

Figure 4.3

Domains in NAP-2 interact with p300, and NAP-2 oligomerisation domains.

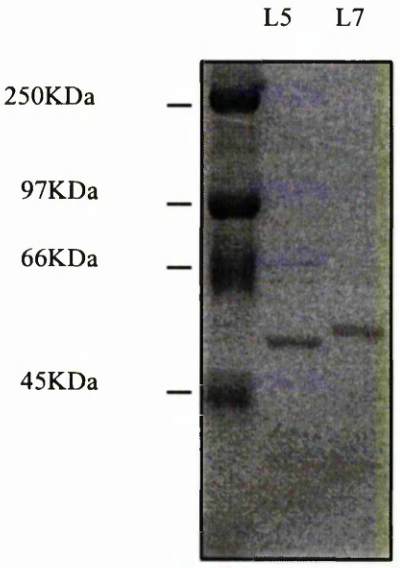
a), b) and c) The indicated regions of NAP-2 were *in vitro* translated as shown in a) and assessed for binding to GST-p300$^{1572\text{-}1905}$ (about 5μg) shown in b), or GST alone (about 5μg) shown in c).

d), e) and f) The indicated regions of NAP-2 were *in vitro* translated as shown in d) and assessed for binding to His-NAP-2 (about 5μg) shown in e) or His-control beads shown in f).

g) Summary of the binding domains in NAP-2 for p300 and NAP-2 protein.

Figure 4.4

a)


L5
L7
250KDa
97KDa
66KDa
45KDa

b)

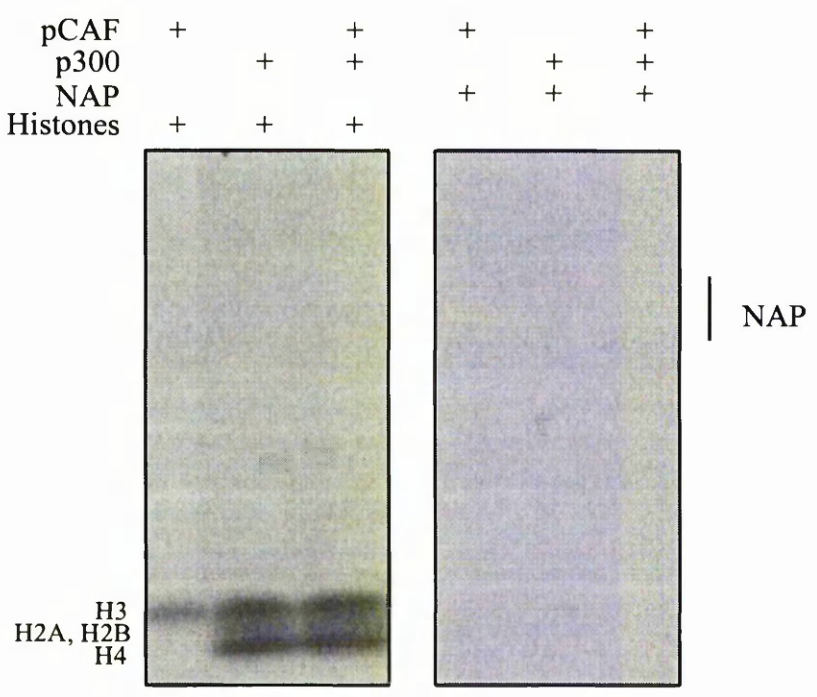

pCAF + + + +
p300 + + + +
NAP + + +
Histones + + +
NAP
H3
H2A, H2B
H4

Figure 4.4

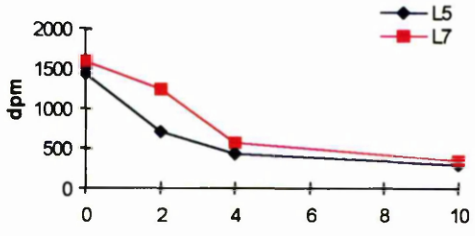
NAP is not acetylated *in vitro*.

a) Coomassie stain of His-NAP2 (L5 and L7) purified from bacteria.

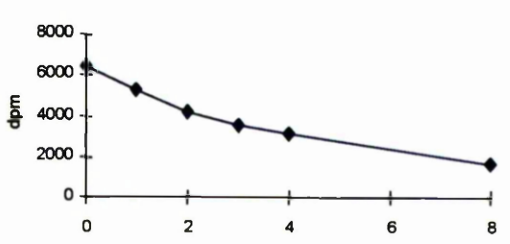
b) The left panel shows an autoradiograph where histone is acetylated by p300 and a combination of p300 and pCAF. H3 and H4 are preferentially acetylated compare to H2A and H2B. The right panel shows an autoradiograph where NAP was tested as a substrate for acetylation *in vitro*, using either p300 or p300 and pCAF as the enzyme source.

Figure 4.5

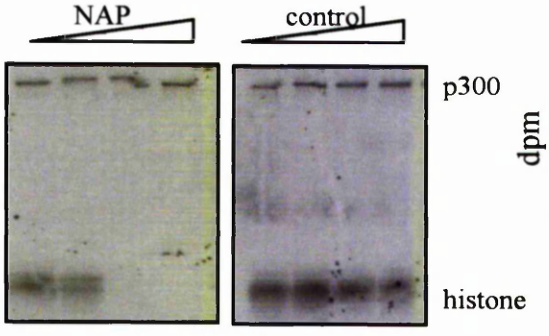
a)



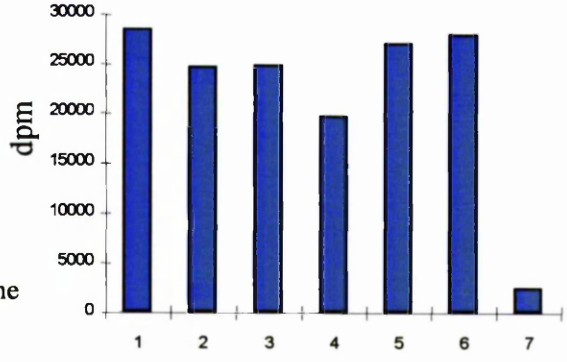
b)



c)



d)



e)

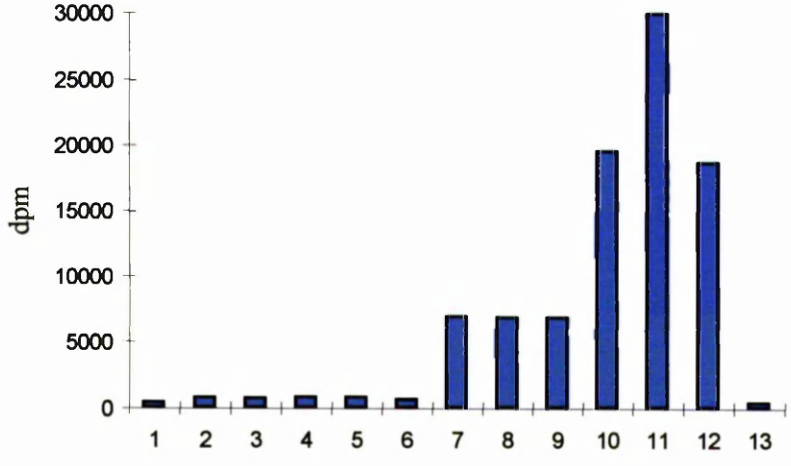


Figure 4.5

NAP-2 inhibits histone acetylation by p300 *in vitro*.

a) Both NAP-2 (L5) and (L7) isoforms inhibit histone acetylation by p300 *in vitro*. L5 and L7 fusion protein were purified from bacteria as His-tagged fusion. 2μg of chicken histones were acetylated by 0.5 μg of Flag-p300$^{1135\text{-}2414}$ in a standard 30μl acetylation reaction. The x-axis shows different units of L5 or L7 titrated into the reaction, with each unit represent 200ng of purified protein quantified by Bradford assay.

b) NAP-2 inhibits histones acetylation by pCAF. The assay is the same as described in a), but used Flag-pCAF as HAT source.

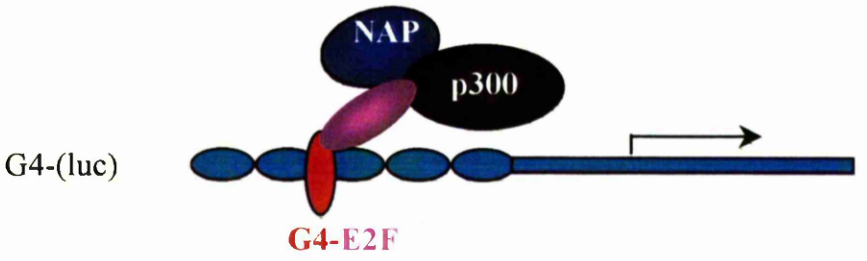
c) NAP-2 (L7) inhibits histone acetylation by p300 *in vitro*, but does not inhibit p300 autoacetylation in the same assay. The experiment was carried out in the same way as described in a). However, instead of using filter assays to quantify the acetylation level, a SDS-PAGE gel was used to analyze the samples, and the proteins acetylated was labelled with ^{14}C and visualized after autoradiography. NAP or BSA (in control reaction) was titrated into the reaction (0, 0.25, 0.5 and 1μg).

d) A NAP-2 peptide at the C-terminal region of the protein inhibits histone acetylation by p300 *in vitro*. A standard histone acetylation reaction was carried out as described in a). The basal level of acetylation is shown in lane 1. Lanes 2 to 4 show a titration (1, 2.5, 5μg) of a NAP-2 N-terminus peptide (142), which does not inhibit histone acetylation. Lanes 5-7 shows a titration of the NAP-2 C-terminal peptide (141) which shows significant inhibition of histone acetylation at 5 μg.

e) Lanes 1-3, 4-6, 7-9, 10-12 show a titration of 141, 142, Rb, and Histone H4 peptide (1, 2.5 and 5μg) respectively. Lane 13 shows the background level of dpm from the scintillation assay. Both 141 and 142 peptides show no significant level of acetylation. The pRb peptide (see chapter 3) shows a moderate level of acetylation. The peptide from the histone H4 tail was used as positive control for the acetylation reaction.

Figure 4.6

a)


NAP
p300
G4-(luc)
G4-E2F

b)

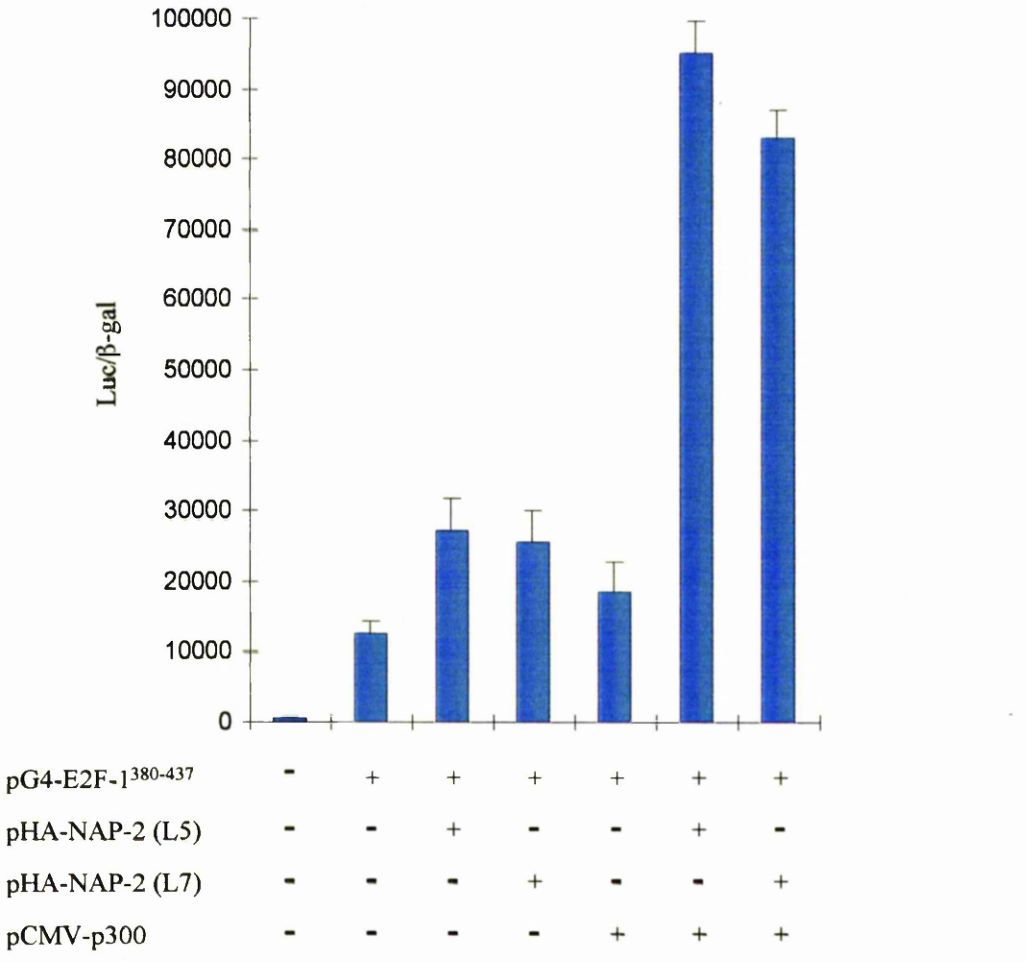

Luc/β-gal
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
pG4-E2F-1380-437 - + + + + + +
pHA-NAP-2 (L5) - - + - - + -
pHA-NAP-2 (L7) - - - + - - +
pCMV-p300 - - - - + + +

Figure 4.6

NAP-2 augments the p300-dependent transcription factor E2F-1.

a) Graphical representation of the reporter construct used in the assay. This reporter was integrated into the U2OS cells to recapitulate the chromatin environment of the genome.

b) The reporter was activated with the expression vector pG4-E2F-$1^{380-437}$ (100ng). Co-transfection with 5μg of pHA-NAP-2 (L5) or (L7), or 5μg of pCMV-p300 only caused marginal co-activation over pG4-E2F-$1^{380-437}$. However, co-transfection of all three plasmids together showed significant augmentation of the E2F-1 mediated activation of the reporter. The Y-axis shows the average value of duplicate readings and represents the normailised level of luciferase/β-galactosidase activity.

Figure 4.7

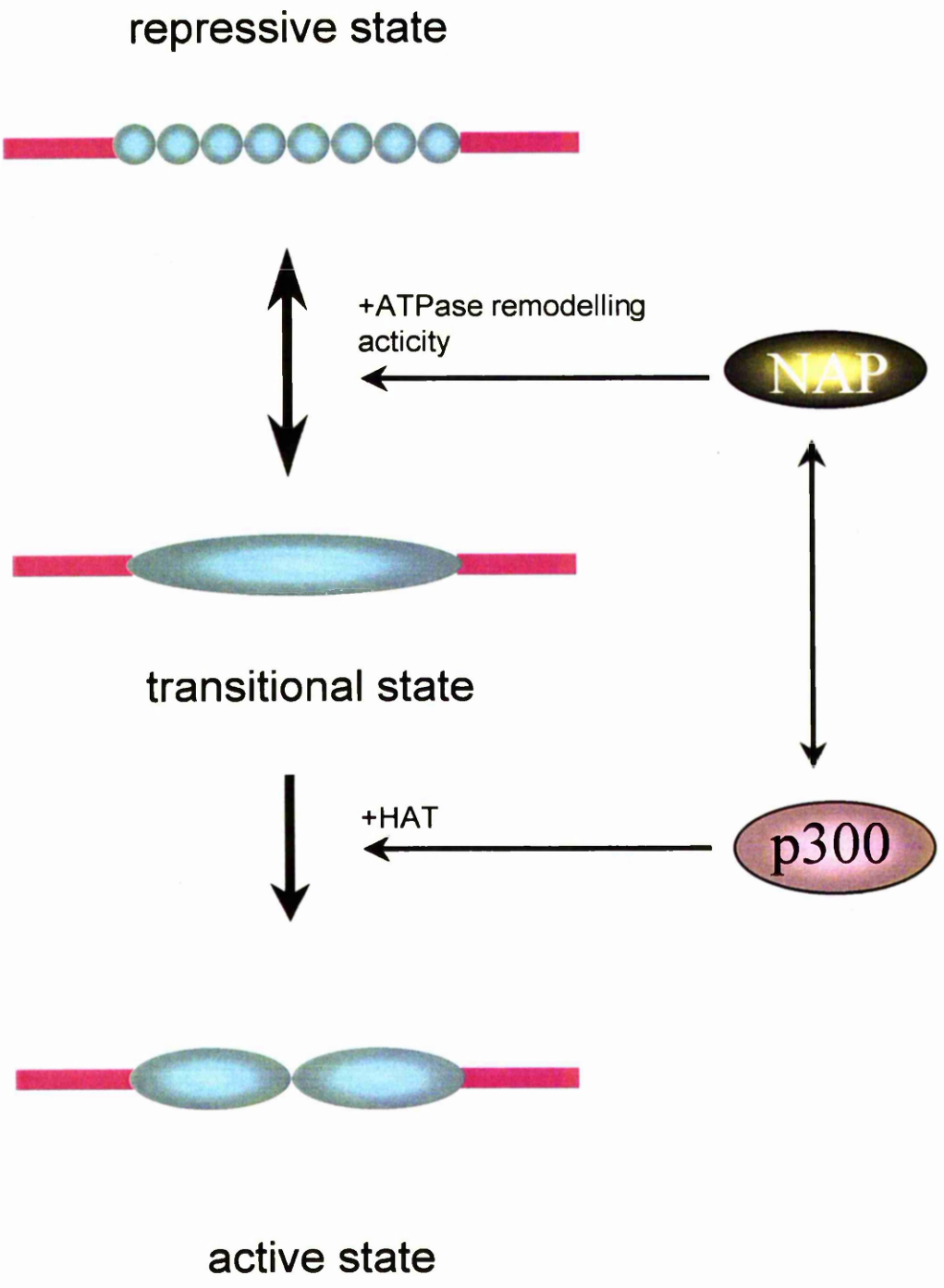

repressive state
+ATPase remodelling
acticity
NAP
transitional state
+HAT
p300
active state

Figure 4.7 A hypothetical model of p300 and NAP co-operate in transcriptional regulation.

A hypothetical model of ATP-dependent remodelling and acetylation as regulators of chromatin fluidity (reviewed in 82). In short, ATP-dependent remodelling complexes are suggested to increase the rate of interconversion between different chromatin states. In this case, NAP might co-operate with ISWI-related chromatin remodelling complexes to facilitate the switch of different chromatin configurations. Activating complexes and HATs are proposed to be recruited and preferentially bind to the activated states of the chromatin, and therefore 'fix' the chromatin configuration in an activating status. NAP may play a role in 'directing' p300 activity to 'fix' the chromatin configuration, possibly through its ability to physically interact with p300. This process leads to the transcriptional co-activation, as observed for the effect of NAP and p300 on p53 and E2F-1.

Chapter 5: Mutagenesis of the pRb pocket reveals that the cell cycle arrest function are separable from LXCXE binding

Introduction

The retinoblastoma protein (pRb) regulates the G1/S transition in the cell cycle (reviewed in 43, 175). The regulation of E2F-dependent transcription is thought to be an important aspect of pRb's function in cell cycle control (reviewed in 43, 62, 91). Previous studies have indicated that pRb has multiple modes in regulating E2F transcription (43). First, pRb binds to the *trans*activation domain of E2F, blocking its interaction with the basal transcription machinery. Second, it is increasingly apparent that E2F binding sites mediate transcriptional repression (30, 162, 171, 199). For example, B-myb, E2F-1 and p107 promoters possess repressive E2F sites whose occupancy co-relates with transcriptional repression (reviewed in 43, and references therein). In this respect, it is likely that the pRb family members play an important role not only in down-regulating E2F-dependent transcription by excluding the *trans*activation domain, but also in facilitating E2F- dependent transcriptional repression by assembling active repressive complexes with E2F.

The pocket domain of pRb was originally identifed as the region required for binding to viral oncoproteins. When the pRb pocket was artificially tethered to the promoter region as a Gal4- fusion, it repressed transcription, indicating its intrinsic transcription repressive activity (30, 177). Molecular candidates which could account for this active repressive function include CtIP (113), HBP1 (159), HDACs (19, 103, 106), and hSWI/SNF (196), which are all known to

bind to pRb in a pocket dependent manner. In particular, HDACs and h-SWI/SNF are classes of proteins with chromatin remodelling activity (60, 82). Upon treatment with trichostatin A (TSA), which is a deacetylase inhibitor (103), repression on Cyclin E and E2F-1 promoters is relieved (103). However, this effect is promoter dependent, as the repression on the TK and B-myb promoters is not relieved after TSA treatment. More recently, a pRb-HDAC-hSWI/SNF trimeric complex was demonstrated, and was shown to repress cyclins E and A gene transcription, arresting cells in the G1 phase of the cell cycle (196). Phosphorylation of pRb by cyclinD/cdk4 disrupts pRb/HDAC association, but the pRb-hSWI/SNF complex persists to repress the cyclin A gene transcription and prevent cell cycle progression beyond S phase (196). Therefore, a model was proposed that different pRb-associated repressive complexes maintain the order of expression of different sets of genes during the cell cycle, which in turn regulates exit from G1 and from S phase respectively. This might also explain the selective effect of TSA on different promoters.

HDACs, hSWI/SWF, CtIP and HBP1 all share a conserved LXCXE peptide motif, which was thought to be important in binding to pRb. Three dimensional structural analysis of the pRb pocket in complex with the LXCXE peptide from E7 oncoprotein illustrates that the LXCXE peptide binds to a conserved groove in the B box of the pocket (94). This LXCXE binding site is highly conserved among pRb homologs from different species, and is also conserved among family members p107 and p130 (94). E2F, on the other hand, binds to a more promiscuous region which is non-overlapping with the LXCXE peptide binding site (94). Consistently, biochemical evidence exists that E2F/pRb/HDAC ternary complex is stable (19). Interestingly, unlike viral oncoproteins such as E1A or E7, many cellular factors may not rely heavily on the LXCXE motif for binding to pRb (38). Mutation of the LXCXE binding pocket disrupts viral oncoprotein binding, whilst most of the cellular factors containing the LXCXE motif still interact with pRb (38). Furthermore, cyclin D/cdk4 disrupts pRb-HDAC interaction,

but fails to affect pRb-hSWI/SNF interaction, suggesting cellular factors use multiple surfaces for interaction with pRb, which also reflects the complexity of the regulation of pRb binding to target molecules (196).

In this study, I have investigated the consequences of mutating the LXCXE-binding motif in pRb. One such mutant is significantly compromised in binding to HDAC and other LXCXE-motif containing proteins, but is capable of binding to other targets, such as E2F and $TAF_{II}250$, which bind to pRb in a LXCXE-independent manner. Interestingly, this mutant can arrest SAOS2 cells in the G1 phase upon over-expression. Therefore, it suggested that HDAC maybe dispensable for pRb mediated G1 arrest.

Results

Mutational analysis of a conserved lysine patch in B domain of pRb

Originally, the investigation was focused on possible lysine residues which might be subject to acetylation in the B pocket domain. Sequence comparison among pRb family members and homologues from different species identified a group of highly conserved lysine residues in the B pocket of pRb (Fig 5.1c, d and e). The three dimensional structure of the small pocket domain of pRb bind to the LXCXE-containing peptide indicated that these lysines scatter around the LXCXE-binding groove (Fig 5.1e), and might function in recognising the LXCXE-peptide (94). In particular, K713 and K765 make contact with the backbone of the LXCXE peptide (94). K720, K722, K729 and K740 might contribute a cloud of positive charges which could facilitate LXCXE-peptide motif-containing proteins to bind to pRb (94). Acidic amino acid residues are frequently found to flank the LXCXE sequences (Fig 5.1f). Systematic site-directed mutagenesis was carried out until all the six lysines residues were changed to alanine (Fig 5.1a). Since all the lysine residues were on the surface of the protein (94), the change to alanine was predicted not to cause dramatic conformational change of the pRb molecule. All mutants were expressed to similar level as wild type pRb in various cell lines (Fig 5.1b and data not shown). As mentioned in chapter 3, K713 was found to be acetylated in a synthetic peptide (see chapter 3).

The pRb 6A mutant is compromised in binding to LXCXE-motif containing proteins

Given that these lysine residues might function in recognising the LXCXE-peptide motif, biochemical pulldown assays were performed to test if the mutants were compromised in binding to LXCXE-motif containing molecules. The wild type pRb and the less severe mutants

(1A1 and 2A) efficiently bound to E7 and cyclin D (Fig 5.2 c iv) and v)). The binding to cyclin D was much less efficient than to E7, indicating pRb/cyclin D interaction is a low affinity event. The pRb 6A mutant was significantly compromised in binding to both E7 and cyclin D (Fig 5.2c iv) and v)). When the binding of pRb and mutants with E2F-1 was analysed, all mutants progressively lost affinity in binding to E2F-1. However, pRb 6A still maintains significant affinity in binding to E2F-1 (Fig 5.2c iii)). Since E7 and cyclin D contain the LXCXE motif, this suggested that pRb 6A is compromised in interacting with the LXCXE-motif containing protein. Furthermore, pRb 6A completely lost ability in binding to HDAC (Fig 5.2b). HDAC was known to contain an LXCXE like motif, which was thought to be important in mediating its interaction with pRb. Consistently, pRb 1A1, which is capable in binding to the LXCXE-peptide motif, retained efficient binding to HDAC (Fig 5.2b). Similarly, pRb 6A was found to have reduced affinity in binding to E2F-1 when GST-pRb 6A and *in vitro* transcribed and translated E2F-1 was used in the pulldown assay (Fig 5.2b). $TAF_{II}250$, which is another molecule which binds to pRb in an LxCxE-independent manner (144), was found to interact with pRb and mutants in a similar manner as E2F-1 (Fig 5.2b). Whilst the mutants progressively lost affinity in binding to $TAF_{II}250$, pRb 6A still retain significant affinity to bind to $TAF_{II}250$.

The pRb 6A represses E2F-1 transcription

The most important properties of pRb's tumour suppressive function were attributed to its ability to repress transcription from E2F-family proteins (reviewed in 43). Therefore, experiments were carried out to investigate if pRb mutants inhibit E2F-dependent transcription. All pRb mutants tested repressed E2F-1 mediated transcription (Fig 5.3c and data not shown). Similar repression was observed from the cyclin E-luc reporter (Fig 5.3c), and from an artificial E2F-luc reporter (data not shown). Furthermore, these observations were repeated with different cell lines, including C33A, U2OS and SAOS2 (Fig 5.3c and data not shown). In nearly in all

cases, mutants behaved similarly as the wild-type pRb in repressing E2F dependent transcrption. Since all the mutants were shown to retain ability to bind E2F, it was expected that they would repress E2F-dependent transcription. The pRb 6A mutant, which showed reduced affinity in binding to E2F-1, did consistently show slightly decreased efficiency in repressing E2F-1 transcription.

Given pRb's intrinsic transcriptional repressive ability is also relevant toward its growth suppressive function (177), a few representative mutants were fused to the Gal4-DNA binding domain and tested for their transcriptional repressor function. A Gal4-Tk-luc reporter and a Gal4-AdML-luc reporter were used in the assay (Fig 5.3 a and b). The Gal4-AdML-luc reporter was known to be TSA sensitive (103), indicating its regulation could be HDAC dependent. Both wild-type pRb and the pRb 1A1 mutant showed potent repressive activity. However, the intrinsic pRb repressive function was progressively lost with progressive mutation of lysine residues (Fig 5.3 a and b). The Gal4-pRb 6A mutant showed much reduced ability in transcriptional repression.

The pRb 6A induces G1 arrest in SAOS2 cells

Over-expression of pRb in a Rb-deficient osteosarcoma cell line SAOS2, was known to cause G1 arrest. Here, I investigated if the pRb 6A mutant retains such function. The cell cycle profile was monitored by the use of a CD20 cell surface marker to identify the transfected cells. In these experiments, transient expression of pRb and pRb 6A gave robust cell cycle arrest (Fig 5.4). The pRb 6A was even slightly more efficient than the wild-type pRb in mediating G1 arrest in those experiments. Overall, it demonstrated that mutation of the lysine patch has no effect on pRb's ability in mediating G1 arrest.

Discussion

The pRb protein is a master regulator of cell proliferation, differentiation and survival. A detailed structure-function analysis on pRb was complicated since many of its functional properties rely on the integrity of its pocket domain, which is a structure difficult to analyse by systematic mutagenesis. The recently published pRb structure shows an extensive interface between the A and B halves of the pocket, and a highly conserved groove in binding to LXCXE-peptide (94). A number of important amino acid residues were indicated to function in contacting the LXCXE-peptide. In particular, a patch of highly conserved lysine residues was found to circumvent the LXCXE-peptide binding groove, suggesting possible functional importance.

Whilst our initial investigation focused on these lysine as possible sites of acetylation (also see chapter 3), subsequent analysis has not yet resolved the question of their possible modification by acetylation. However, one of these mutants, pRb 6A, was compromised in binding to HDAC, therefore allowed a separate investigation on the functional importance of the reported pRb/HDAC interaction (19, 103, 106).

Most of pRb's tumour suppressive effect was ascribed to its ability to repress E2F-dependent transcription (43). Furthermore, pRb possesses intrinsic transcriptional repression activity (30, 177), which might involve its interaction with classes of molecules with chromatin remodelling activity, including HDACs and hSWI/SNF complexes (19, 103, 106, 196). In particular, HDACs were reported to associate with pRb and E2F-1 in a trimeric complex, and selectively repress certain E2F-responsive promoters. Subsequently, it was suggested that HDACs are required for the pRb active repressive complex to repress certain genes during G1/S transition, and contributes towards pRb's G1 arrest function.

A large number of pRb interacting molecules, including HDACs and hSWI/SNF, contain a motif known as the LXCXE, which was thought to be important in mediating the interaction (38). Mutational analysis of the pRb LXCXE-peptide binding groove showed that mutations in this domain are sufficient to block virus proteins, such as E7 and E1A, binding to pRb (38). This suggests viral oncoproteins heavily rely on this motif to bind to pRb and inactivate its function. Interestingly, most of the cellular factors seem not to be affected by these mutations, suggesting cellular factors may use multiple modes to bind to pRb, and the LXCXE is not the sole determinant for their interaction with pRb. Therefore, those pRb mutants were still capable of causing G1 arrest (38).

The conserved lysine patch is scattered around the LXCXE-binding groove. From the three dimensional structural studies, at least three functions were described to these lysines (94). First, K713 and K765 were found to directly contact the LXCXE-peptide backbone. Second, the other four lysines, including K720, K722, K729 and K740 were suggested to form an overall positive charge, which might 'guide' the LXCXE peptide into its binding groove. Indeed, most of the LXCXE-motif containing proteins have conserved acidic amino acids flanking the LXCXE sequence, which might interact with the highly positively charged lysine. Third, these lysine residues may function as phospho-receptor sites, and mediate intramolecular interaction when pRb is phosphorylated. One report demonstrates that phosphorylation at the C-terminal region of pRb initiates an interaction with this lysine patch, which subsequently leads to inactivation of pRb activity (59).

In this study, the most severe mutant, pRb 6A, was found to be significantly compromised in binding to various LXCXE-motif containing proteins, including E7, cyclin D and HDAC. However, this mutant still retains binding to E2F-1 and $TAF_{II}250$, though with slightly decreased affinity. While pRb 6A is sufficient to repress E2F-1 transcription, it is significantly compromised in the intrinsic repression activity when tethered to the promoters as a

Gal4-fusion. One of the explanations is that the loss of binding to HDAC compromises its intrinsic repressive activity. However, other explanations also exist. It has yet to be tested if this mutant binds to hSWI/SNF complexes, which is another mechanism of pRb's intrinsic repressive activity. Furthermore, as demonstrated by Dick *et. al.* (38), various pRb mutants which were compromised in LXCXE binding, while retaining HDAC binding, also showed similarly reduced repressive activity when assessed as a Gal4 fusion. The underlying mechanism was not investigated.

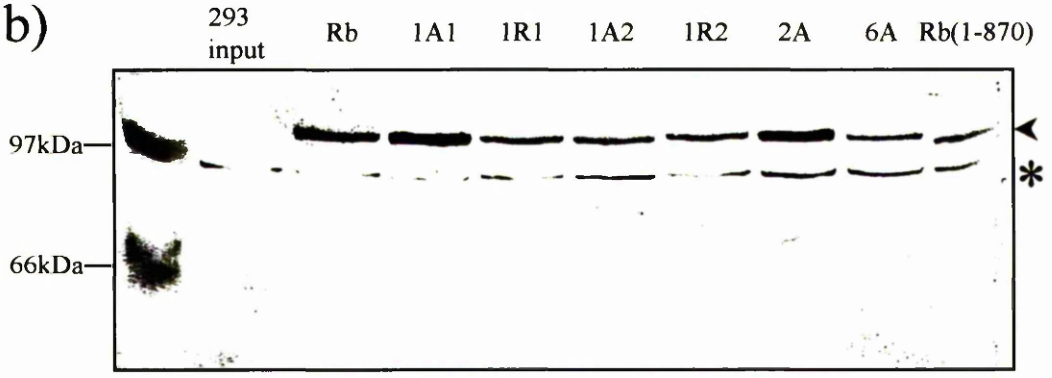
Nonetheless, the pRb 6A mutant is clearly defective in binding to HDACs and I tested if this mutant can cause G1 arrest. Previously, using the E2F DNA binding domain as a dominant negative to inhibit E2F-dependent transcription, it was shown that this was not sufficient to initiate several kinds of G1 arrest (197). Therefore, the active repressive complexes of E2F/pRb might contribute other functions for the G1 arrest. One of the candidates may be HDAC as it was shown to repress some E2F responsive genes required for G1/S phase transition (196). However, over-expression of pRb 6A, which lacks the ability to bind to HDAC, maintains potent G1 arrest ability. This leads to the possibility that HDAC is not essential for pRb mediated G1 arrest, at least in SAOS2 cells under over-expression condition. An alternate explanation could be that under over-expression conditions, pRb was expressed at an abnormally high level which might sequester other factors, thereby bypassing the need for HDAC. In a parallel study, HDAC or the pRb pocket domain was fused to the E2F-1 DNA binding domain, thereby artificially recruiting HDAC or pRb to the E2F-DNA binding sites (Smith *et. al.*, personal communication). Whilst the E2F/pRb hybrid was capable of mediating G1 arrest, the E2F/HDAC hybrid was deficient for such activity. Taken together, it suggests that HDAC may not be required for pRb mediated G1 arrest. It is possible that HDAC is specifically involved in certain forms of G1 arrest mediated by pRb. It also remains to be tested if HDAC is involved in other aspects of pRb function, such as pRb's role during differentiation and anti-apoptosis.

Figure 5.1

a)

Mutants	Substitution
1A1	K713A
1R1	K713R
1A2	K765A
1R2	K765R
2A	K713A, K765A
3A	K713A, K720A, K722A
4A	K713A, K720A, K722A, K765A
5A	K713A, K720A, K722A, K729A, K765A
6A	K713A, K720A, K722A, K729A, K740A, K765A

b)



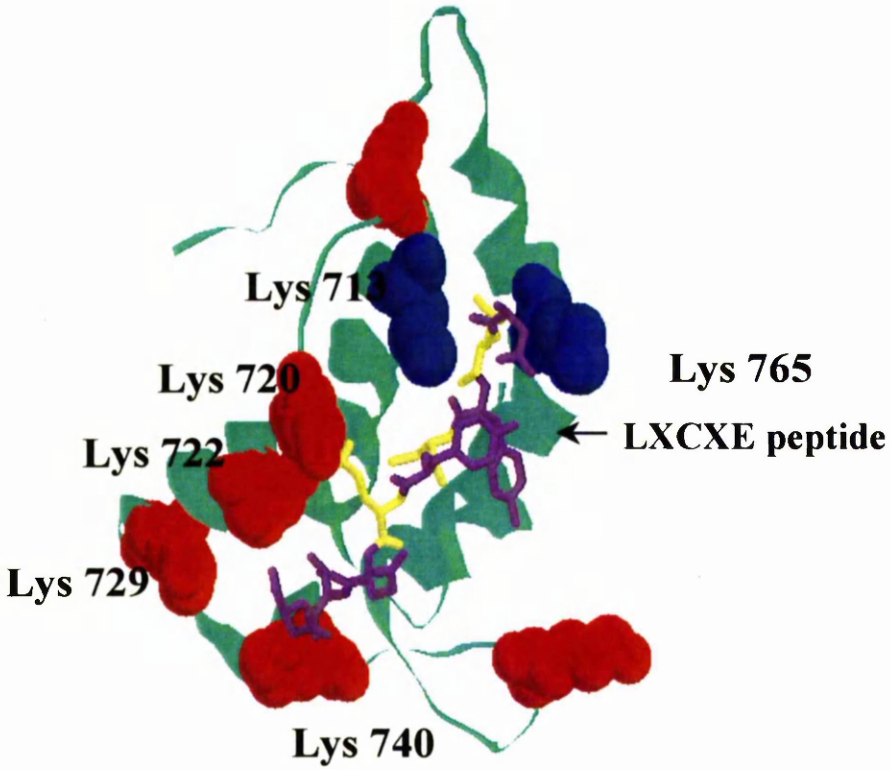
c)

```
     Human  (710) GICKVK-NIDLKFKIIVTAYKDLPHAVQETFKRVLIKEEEYDS-----IIVFYNSVFMQRLKT
     Mouse  (672) GICKVK-NIDLKFKIIVTAYKDLPHAAQETFKRVLIREEEFDS-----IIVFYNSVFMQRLKT
       Rat  (680) GICKVK-NIDLKFKIIVTAYKDLPHAAQETFKRVLIREEEFDS-----IIVFYNSVFMQRLKT
Drosophila  (643) IYIRVKRMEDPKFSDIMRAYRNQPQAVNSVYREVFIDINEDGEPKVKDIIHFYNHTYVPLMRQ
      Frog  (682) GICKAK-NIDLRFKTIVTAYKGLTNTNQETFKHVLIRDGQHDS-----IIVFYNLVFMQKLKS
Consensus         GICKVK NIDLKFKIIVTAYKDLPHA QETFKRVLIREEEFDS     IIVFYNSVFMQRLKT
```

d)

```
 pRb(712) CKVKNIDLKFKIIVTAYKDLPHAVQETFKRVLIKEEEYDSIIVFYN-----------------SVFMQRLKT
p107(853) AKVTKEERTFQEIMKSYRNQPQANSHVYRSVLLKSIPREVVAYN-----------------------KNIN
p130(897) AKVTKEDRSFQNIMRCYRTQPQARSQVYRSVLIKGKRRNSGSSESRSHQNSPTELNTDRASRDSSPVMRSNST
Consensus AKVTKEDRSFQ IMKAYR QPQA S VYRSVLIK   RDSIA                    S  M KN T
```

e)



f)

Consensus	LxCxE
Ad5 E1A	PEVIDLTCHEAGFPPSDD
HPV16 E7	PETTDLYCYEQLNDSSEE
SV40 T	NEENLFCSEEMPSSDD
CtIP	HQAAELECEEDVIPDSP
BRM	AEVERLTCEEEEEKIFGR
BRG1	AEVERLTCEEEEEKMFG
HDAC1	SDKRIACEEEFSDSEE
HDAC2	SDKRIACEEEFSDSEE
UBF	DPSQSLLSLEKPEDLLEE
Cyclin D1	MEHQLLCCEVETIRRAY

Figure 5.1 Mutagenesis of pRb B pocket lysine residues.

a) Designated mutants nomenclature is listed, accompanied by the corresponding changes that have been introduced into pRb.

b) An immunoblot showing all mutants were expressed to similar levels in 293 HEK cells. 10μg of pCDNA3-9E10 pRb and mutants were introduced into 293 HEK cells and 100μg of the total cell extracts were used for the immunoblot. The band corresponding to pRb were indicated with an arrow. (*) indicates a non-specific band which occur in all samples.

c) An alignment of pRb B pocket sequences from various species. Lysine residues which were mutated, were highly conserved among different species and they are indicated in red.

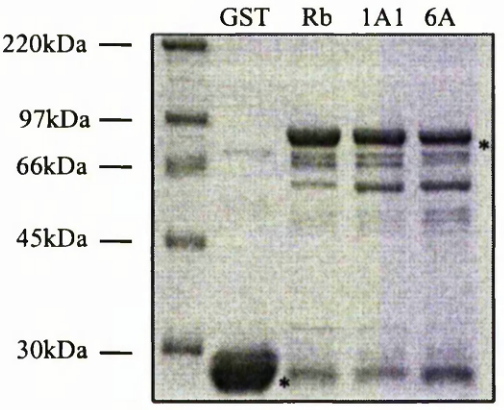
d) An alignment of pRb B pocket sequences from the three family members. The most conserved K713 is indicated in red. K720, K722 which show conservation in overall charge among the family members, are indicated in orange. The other three lysines, K729, 740, 765 are not conserved among the family members, are indicated in blue.

e) A diagram of an LXCXE peptide derived from E7 bound to pRb. This diagram is generated with Rasmol software using the co-ordinates as published (94). All lysines being mutated in the study and the LXCXE peptide are indicated. K713, 765 (blue) were reported to contact LXCXE peptide backbone. All other lysine that clusters around the LXCXE binding groove is shown in red.

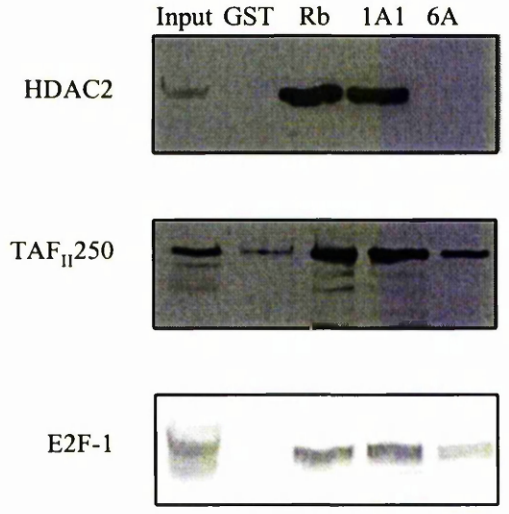
f) Sequence alignment of LXCXE-containing proteins. The LXCXE motif was boxed in green. Various acidic amino acids flanking the LXCXE motif are indicated in blue.

Figure 5.2

a)


GST
Rb
1A1
6A
220kDa
97kDa
66kDa
45kDa
30kDa
*
*

b)


Input
GST
Rb
1A1
6A
HDAC2
TAF$_{II}$250
E2F-1

c)

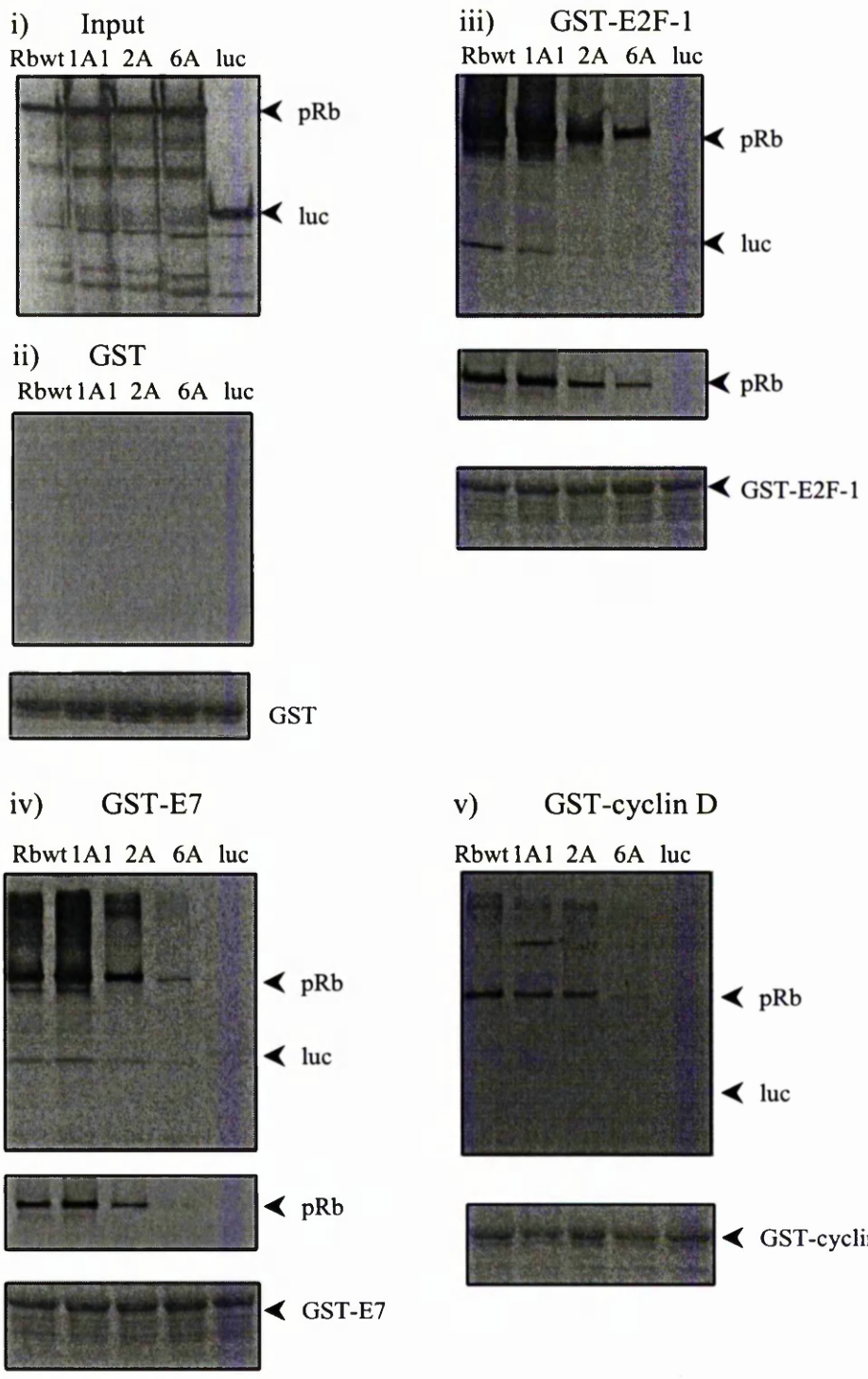

i) Input
Rbwt 1A1 2A 6A luc
pRb
luc
iii) GST-E2F-1
Rbwt 1A1 2A 6A luc
pRb
luc
pRb
GST-E2F-1
ii) GST
Rbwt 1A1 2A 6A luc
GST
iv) GST-E7
Rbwt 1A1 2A 6A luc
pRb
luc
pRb
GST-E7
v) GST-cyclin D
Rbwt 1A1 2A 6A luc
pRb
luc
GST-cyclin D

Figure 5.2 pRb 6A mutant has significantly reduced ability in binding to LXCXE-motif containing proteins.

a) Coomassie blue staining of the purified GST, and GST-pRb and mutant recombinant proteins.

b) 5μg of the indicated GST-fusion proteins were used in the pulldown assay for HDAC2 (top panel), $TAF_{II}250$ (middle panel) and E2F-1 (bottom panel) respectively. For HDAC2 and E2F-1 pulldown, each indicated GST fusion protein was incubated with 1mg of HELA nuclear extract, followed by immunobloting with the respective antibodies. For the $TAF_{II}250$ pulldown, each indicated GST fusion protein was incubated with 200μg of sf9 cell extract which have over-expressed HA-tagged $TAF_{II}250$, using a baculovirus. Subsequently, the samples were analysed by western blot using anti-HA antibody.

c) Analysis of pRb and mutants binding to E2F-1, cyclin D and E7.

   i) pRb and the indicated mutants, and the luciferase were generated by *in vitro* transcription and translation. They were labelled with ^{35}S. This autoradiograph showed all proteins were generated to similar level for the experiment.

   ii) GST (5μg) alone were used as negative control for the pulldown assay. No pRb or any mutants, or the luciferase were found to associated with GST fusion protein (top panel). The bottom panel show GST protein level (coomassie stained) used in the assay.

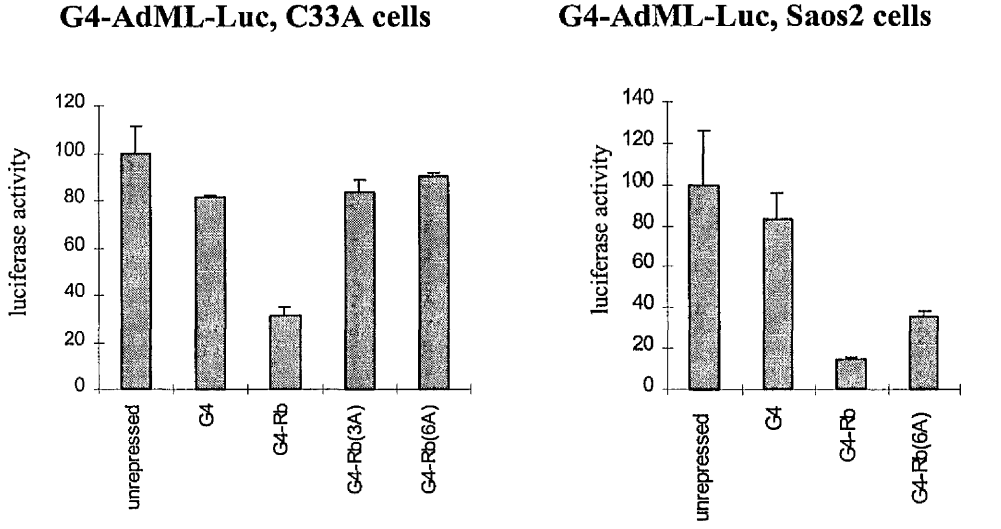
   iii) GST-E2F-1 (5μg) was used to pulldown pRb and the mutants. The top panel shows a long exposure of the autoradiograph (4 days). The middle panel shows a short exposure of the same autoradiograph (12 hours). The bottom panel shows the GST-E2F-1 protein level (coomassie stained) used in the assay.

   iv) GST-E7 (5μg) was used to pulldown pRb and the mutants. The top panel shows a long exposure of the autoradiograph (4 days). The middle panel shows a short exposure of the same autoradiograph (12 hours). The bottom panel shows the GST-E7 protein level (coomassie stained) used in the assay.

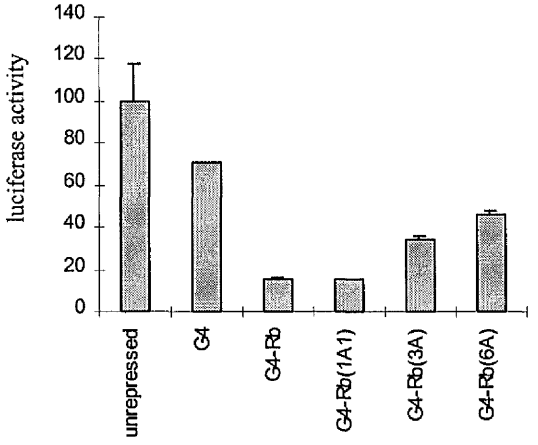
v) GST-cyclin D (5μg) was used to pulldown pRb and the mutants. The top panel shows a long exposure of the autoradiograph (4 days). The bottom panel shows the GST-cyclin D protein level (coomassie stained) used in the assay.

Figure 5.3

a)



b)



c)

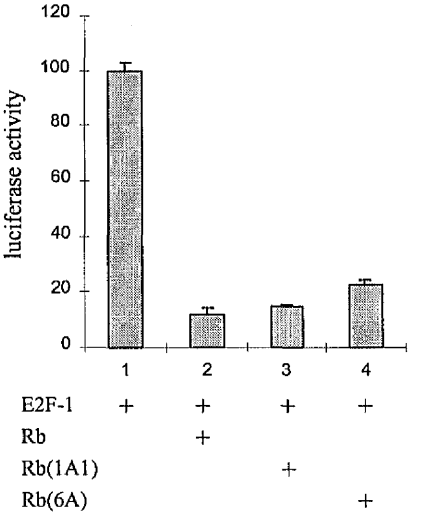


Figure 5.3 Transcriptional repression mediated by pRb and the mutants.

a) 10μg of the indicated G4- and G4-pRb and mutants constructs were transfected into C33A and SAOS2 cells respectively. The unrepressed state of 1μg of the Gal4-AdML-luc reporter was normalised to 100% of luciferase activity.

b) Same transfection condition was used as described in a). In this case, the repression on a Gal4-Tk-luc reporter was analysed using G4-Rb and mutants.

c) 1μg of cyclin E-luc was transfected into C33A cells. The reporter was activated upon co-transfection of 100ng of pCMV-E2F-1. The repressive effect of pRb and mutants on E2F-1 mediated transcription was analysed by co-transfection of the corresponding expression vector as indciated (10μg each). The normalised activity of (luc/β-gal) of E2F-1 alone was normalised as 100% of luciferase activity.

Figure 5.4

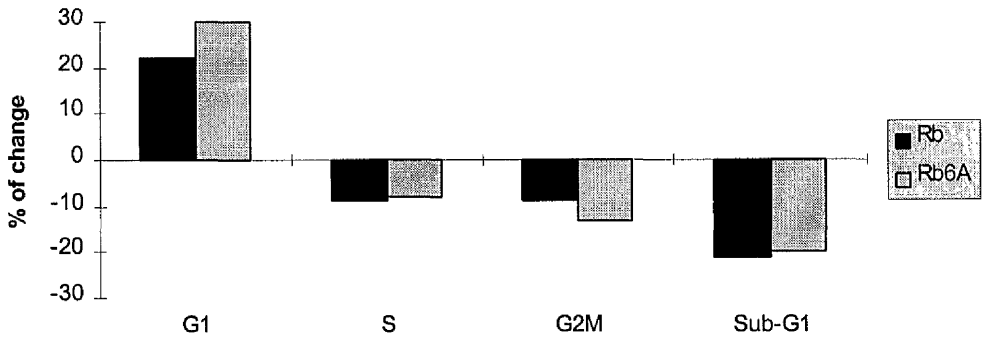


Figure 5.4 pRb and pRb 6A arrest SAOS2 cells in G1 phase of the cell cycle.

SAOS2 cells were transfected with 10μg of pRb or pRb 6A constructs, together with 10μg of pCMV-CD20. Cells were fixed and stained for CD20 with fluorescein-conjugated antibody, and for DNA content with propidium iodide. The percentage of change of the cells (compare to a mock sample which were transfected with 10μg of pCDNA3 alone) in different phases of the cell cycle were shown in the diagram.

Chapter 6: Summary

Acetylation: more than the chromosome

The isolation of proteins with nuclear HAT activity is a key step forward in understanding the regulatory role of nucleosomes in transcription (reviewed in 21, 82, 170 and references therein). Although the connection between histone acetylation and transcription was observed at least 30 years ago, the enzymes responsible for such action remained elusive for a long time. Acetylation of multiple sites in the core histone tails was known to associate with transcriptional activity in isolated nuclei and partially purified chromatin preparations. Constitutively inactive heterochromatin shows a lack of acetylation in histone tails, suggesting deacetylation is linked with gene silencing. Substitution of the lysine residues in the H4 tail with arginine, which cannot be acetylated, virtually abolishes transcription of inducible genes in yeast. Conversely, substitution of the lysines with glutamate, which might mimic the acetylated state of lysine, can bypass the need for acetylation. When the first few nuclear HATs were identified, it was very encouraging that many of them were previously identified transcriptional regulators. Immediately, one question arises as to why there are so many HATs if their sole role is to modify nucleosomes.

So far, HATs can be divided into two groups: the type A HATs, which are nuclear, and active on chromosomal histones, with a role in transcriptional control; and type B HATs, which are cytoplasmic, and mainly acetylate newly synthesised histones. The nuclear HATs can be further divided into sub-families exemplified by pCAF/GCN5 (188), p300/CBP (12, 128), $TAF_{II}250$ (116), SRC-1 (154) and MOZ (147). These sub-families of HATs show very low levels of sequence homomology, and were frequently found to interact with each other in multi-protein complexes (reviewed in 52). Therefore, another question is why multiple HATs are

needed in co-activator complexes. Part of the answer could be that the HATs may act on non-histone targets. Indeed, a growing list of factors involved in transcription, including p53 (56), E2F-1, -2, -3 (110, 111), EKLF (195), GATA-1(17), HIV-Tat (78), MyoD (132), HNF4 (153), c-Myb (161), TFIIE, TFIIF (68) and HMGI(Y) (121) were found to be acetylated by p300/pCAF. Furthermore, proteins involved in other cellular processes, such as nuclear transport (e.g. importin-α family protein) (13), and microtubule organisation (e.g. α-tubilin) were acetylated too. Most of the HATs contain a homologous region known as the bromodomain, which was recently shown to recognise acetylated peptide motif (39, 74). Therefore, the presence of multiple HATs may function to recognise certain acetylated pattern on nucleosomes or protein complexes.

In this thesis, I reported pRb, and its family member p107, which are well known tumour suppressors, are acetylated by p300, but not by pCAF. This also demonstrated the specificity of the enzymes for substrate modification. Unlike most of the other factors which were reported to be acetylated (such as p53, E2F, MyoD, c-Myb, and GATA-1), pRb is not a DNA-binding factor, but a pleiotropic transcription regulator, with ability both to activate and repress transcription (reviewed in 18, 120). A further complexity arises when multiple domains on pRb can be acetylated. Acetylation of pRb is dependent upon the integrity of its pocket, as various tumour-derived pRb mutants, such as Δ21, Δ22, and 706F mutants (chapter 3 and data not shown), had diminished level of acetylation. Using an extensive panel of GST-pRb derivatives, two discrete regions in the C-terminal region, and the A domain of the protein, were found to be acetylated. In particular, the C-terminal region is heavily acetylated. Since these regions are intact in the pRb tumour-derived mutants mentioned above, and invariably all the mutants were known to have altered conformation, these results in turn suggested that acetylation of pRb could be dependent on its proper folding.

Nearly all the transcription factors known to be acetylated show enhanced DNA binding activity upon acetylation (17, 36, 110, 111, 132, 195). This is consistent with the view that acetylation is stimulatory towards transcription. In the case of p53, acetylation at the C-terminus might cause a conformation change in the protein, thereby relieving an inhibitory interaction between the C-terminus and the DNA binding domain (56). The underlying mechanism of the enhanced DNA binding for other factors was not investigated. Acetylation can also regulate protein-protein interactions. For example, acetylated dTCF had reduced affinity in binding to armadillo (172), and acetylated ACTR is inhibited to associate with nuclear receptors (52). These are unusual cases where acetylation is inhibitory to transcription. In this thesis, I reported that acetylation of pRb stimulates its binding to proto-oncoprotein Mdm2, providing another example whereby acetylation can be involved in regulating protein-protein interaction. Furthermore, one report suggested acetylation regulates the stability of E2F-1 (110). Since ubiqutination, Sumoylation, and acetylation all occur on lysine residues (198), it is possible that in some cases, these modifications might antagonise each other and play different roles in regulating protein stability. Analysis of acetylated E2F-1 showed it has a more stable half-life (110). Interestingly, p53 acetylation was found to be stimulated under certain DNA-damaging conditions (139), which were also conditions known to stabilise p53. The functional consequence of TFIIE and TFIIF acetylation, and the autoacetylation of some HATs, such as pCAF and p300/CBP, has not yet been characterised. Nonetheless, an overwhelming picture suggests that many non-histone proteins can be acetylated, and acetylation might mimic phosphorylation in regulating different aspects of protein activity, and participate in a variety of cellular processes.

pRb acetylation by p300 reveals additional level of control

I reported two functional consequences upon pRb acetylation. First, acetylated pRb had higher affinity in binding to oncoprotein MDM2. Most of the known functions of MDM2 were ascribed to its ability to regulate p53 stability (53, 101). Biochemical analysis also showed an interaction of MDM2 with the pRb/E2F pathway (64, 156, 183). Both pRb and E2F/DP were reported to interact with MDM2 (101, 183). MDM2 binds to the C-terminal region of pRb, which is also the region acetylated by p300 *in vitro*. Using biochemical pulldown assays, I demonstrated that MDM2 preferentially associated with the acetylated form of pRb. Consistently, MDM2 preferentially associated with pRb in cells treated with TSA. Further support came from the observation that there were relatively more MDM2/pRb immunocomplexes in 293 HEK cells. 293 HEK cells are transformed with E1A, which is an agent identified to stimulate pRb acetylation. In the literature, evidence exists that MDM2 functions to inactivate pRb by sequestering it away from E2F family protein. At face value, it makes sense that a growth-promoting signal, such as E1A, enhances pRb acetylation, thereby inactivating its function by stimulating its association with MDM2.

In this thesis, I also reported that acetylation of pRb might regulate pRb phosphorylation. In an analysis of the pRb acetylation domains in the C-terminus, one region was identified which shared a low level of homology with other acetylated substrates. Furthermore, this region was previously reported to contain a consensus sequence which was required for cyclin-cdk2 mediated phosphorylation of pRb (1). Mutagenesis of the lysine residue in this region showed that K873, 874 are possible targets of modification. Furthermore, when these lysines were mutated to glutamate, which might mimic the acetylated state, the mutants have defects in phosphorylation. Therefore, it suggests that there could be 'cross-talk' between

phosphorylation and acetylation. In this case, acetylation of pRb on K873, 874 may inhibit its phosphorylation by cyclin-cdk2.

E1A redirects normal acetylation patterns

Both p300 and pRb were identified as major cellular polypeptides that associate with viral oncoprotein E1A. Intriguingly, E1A was reported both to enhance and inhibit p300/pCAF-mediated acetylation (2, 25). First, E1A co-immunoprecipitates with p300/pCAF, which has active HAT activity (12). Ait-Si-Ali *et al* (2) demonstrated that p300/CBP HAT activity peaks at G1/S transition, suggesting the HAT activity is required to drive cells into S phase. Furthermore, cyclinE/cdk2 was found to phosphorylate p300 in the C-terminus, which in turn activates p300 HAT activity (48). Similarly, E1A enhances p300 HAT activity, suggesting that the viral oncoprotein might mimic cellular signals in activating p300 HAT function, thereby driving cells into S phase. However, contradictory evidence demonstrated that E1A represses histone and p53 acetylation *in vitro* (25). Noticeably, a very high molar ratio of E1A to histone was used in those assays. The different outcomes observed with E1A could be due to the different format of the assays employed. I was very intrigued with E1A's ability to regulate pRb acetylation because E1A was known to mediate a ternary complex between p300 and pRb. In addition, this complex is important for efficient transformation function of E1A. I hypothesized that E1A enhances pRb acetylation, via recruiting p300 and pRb into a trimeric complex, which might have an active function in E1A-mediated cellular proliferation. Indeed, biochemical analysis suggests that pRb acetylation is stimulated by E1A. A maximum of ten-fold stimulation of pRb acetylation was observed. Moreover, E1A mutants defective in binding to either p300 or pRb failed to enhance pRb acetylation. With the same assay, E1A was found to affect histone acetylation in a concentration dependent manner. At low levels, it enhanced histone acetylation to a maximum of two-fold. However, as reported in other publications (25, 58), in high concentrations, it repressed

histone acetylation. It is noted that at similar concentrations of E1A, there was no repression of pRb acetylation. In comparative studies, I demonstrated that E2F-1 and p53 acetylation is repressed by E1A. E1A, p53 and E2F-1 share over-lapping binding sites on p300, and E1A can inhibit p53 and E2F-1 binding to p300. Therefore, I suggest that E1A has a unique function in re-directing p300/CBP HAT activity, presumably in a way which will favour cellular growth and proliferation. As with pRb, E1A actively recruits pRb into complex with p300, and stimulates pRb acetylation. Consequently, acetylated pRb might favour cellular growth. Given that there are multiple acetylation sites on pRb, it remains to be seen if E1A favours certain acetylated state(s) of pRb. As with E2F-1 and p53, E1A might act as a competitive inhibitor to prevent their association with p300, thereby inhibiting their acetylation. Consistently, *in vitro* acetylation assays carried out on E2F-1 suggested that it is a poor substrate when the minimal HAT domain of p300 was used as the enzyme source. However, when the p300 enzyme also contains the E2F interaction domain, a much more efficient acetylation of E2F-1 was observed. It was reported that p53 acetylation was stimulated under certain DNA damaging condition (100, 139), implying that the acetylated p53 might function in growth arrest or apoptosis. Therefore, it is likely that E1A prevents acetylation events which might contribute to growth arrest.

p300 in transcription

In order to understand p300 function, a yeast two-hybrid screen was carried out by Shikama *et. al.* (in press) to identify p300 interacting proteins. One interesting clone identified encodes nucleosome assembly protein NAP-2. To this end, I was interested in understanding if NAP and p300 co-operate in transcriptional regulation. Using biochemical pulldown assays, I identified that NAP binds to the CH3 domain on p300. Conversely, p300 binds to two discrete regions on NAP. Further, NAP can dimerise or oligomerise, and two independent domains on NAP may mediate this dimerisation. The functional consequence of this dimerisation has not

been investigated. Using transient transfection assays, I demonstrated that NAP and p300 augment in transcription activation of an E2F-responsive reporter. Together with the observations of my colleagues, we concluded that NAP and p300 synergise in transcriptional activation, at least on certain E2F-1 and p53 responsive promoters. In order to understand the underlying mechanism, various biochemcial assays were performed, showing NAP, p300 and histones can associate in a complex (Shikama *et. al.*, in press). Furthermore, the NAP-p300 interaction seems to be enhanced in the presence of histones. Therefore, a simple model suggests that NAP might 'help' or even target p300 to certain regions of the promoter. Since p300 HAT activity is important in transcriptional control, I investigated if NAP can affect this enzymatic function. Surprisingly, *in vitro* assays suggested that NAP inhibits histone acetylation by p300. It was not investigated if this also happens *in vivo*. However, NAP does not inhibit the intrinsic HAT activity of p300. Therefore, a more likely scenario is that NAP binds to the histone tails, and 'hides' the tails from HAT. In a natural promoter context, it is possible that NAP specifically recruits p300 to certain promoter regions, allowing p300 to acetylate neighbouring nucleosomes for transcriptional modulation. Furthermore, NAP was reported to co-operate with the ISWI family of ATPase remodelling complexes in generating regularly spaced nucleosomal arrays (70). Together with our finding that p300 associates with NAP, it is possible that NAP might function as a 'bridge' to associate the ATPase activity and HAT activity, thereby co-ordinate these dual enzymatic functions in transcriptional regulation.

Since both NAP and p300 functions were known to be cell cycle regulated (2, 79, 80, 137), it is possible that these two proteins co-operate in regulating certain classes of genes whose expression is cell cycle dependent. It is therefore not surprising to find that NAP and p300 augment E2F-1 and p53 dependent transcription. Further analysis will be needed to test the generality of this hypothesis. Apart from transcription, these protein complexes could also

participate in chromatin remodelling events related to replication (123), which still remain to be explored.

p300 has at least three different modes of regulating transcription: I) as a bridging protein to associate DNA-binding factors to the transcriptional apparatus, II) through its HAT activity, III) as a assembly platform for multi-protein complex formation. In this study, we suggest another mechanism for how this pleiotropic protein can be targeted for transcriptional control.

Rb/HDAC: cell cycle arrest or not?

Abundant evidence in the literature ascribes pRb's tumour suppressive effect to the inhibition of E2F-dependent transcription (reviewed in 43, 62, 91, 99, 120, and references therein). pRb was found to interact with the *trans*activation domain of E2F, providing one mechanism of how pRb can inhibit E2F transcriptional activity. However, many E2F DNA binding sites are repressive in nature, whose occupancy correlates with transcriptional repression. pRb actively represses transcription when artificially tethered to the promoter region (30, 177). Using the E2F DNA binding domain as a dominant negative to competitively inhibit E2F dependent transcription, it was insufficient to cause several types of G1 arrest (including TGFβ–, contact inhibition- and p16- induced cell cycle arrest) (197). Therefore, one hypothesis suggested that the E2F/pRb complex recruits other factors to actively repress transcription, which may be more relevant to pRb's function in regulating E2F target genes. Recently, two classes of proteins, 1) HDACs and 2) h-SWI/SNF, were shown to bind to pRb to actively repress E2F target genes (196). Both proteins possess chromatin-remodelling activity, which may account for the underlying repressive mechanisms. Here, by utilizing the three-dimensional structure information on the pRb pocket region (94), a series of pocket mutants were generated.

One such mutant (pRb 6A) lacks the ability to bind to the LXCXE motif, therefore providing an invaluable tool in analysing the functional consequence of pRb/HDAC interaction.

First, I demonstrated that the pRb6A mutant had undetectable binding to HDAC. Importantly, this mutant is capable of binding to E2F-1 and $TAF_{II}250$, suggesting its overall structure is maintained. Consistently, this mutant repressed E2F-dependent transcription in reporter gene assays. Since pRb can block the recruitment of the preinitiation complex of TFIIA/TFIID by E2F (138), this provides a mechanism whereby the observed repression might be HDAC independent. In order to further analyse the repressive properties of pRb6A, a Gal4-6A fusion was generated and this fusion construct showed much reduced repression when compared to the wild type Gal4-Rb. This difference could be due to the inability of the mutant to recruit HDACs for active repression. When the pRb6A mutant was tested for its ability to cause cell cycle arrest in SAOS2 cells, it is almost more effective than the wild type pRb. So far, an interesting possibility arises is that HDAC may not be required for pRb-mediated G1 arrest in SAOS2 cells. A second explanation could be that under over-expression conditions, pRb protein maybe expressed at an abnormally high level, which might bypass the need for HDAC for cell cycle arrest. Further experiments will be required to resolve these issues. Using another approach to answer the above question, E2F/HDAC and E2F/pRb hybrids were generated and tested for their ability to cause cell cycle arrest (Smith *et. al.*, personal communication). If HDAC is important to actively repress E2F target genes for the G1 arrest, we reasoned that it might bypass the need of pRb by fusing HDAC with the DNA binding domain of E2F, therefore artificially targeting HDAC to E2F target genes. However, whilst the E2F/pRb hybrid is potent in causing G1 arrest in SAOS2 cells, E2F/HDAC hybrid is defective for such function. This suggests that HDAC alone may not be sufficient for G1 arrest, and pRb must provide additional functions for the observed effects. It is possible that HDAC might contribute toward pRb's growth arrest property under certain specific conditions. As discussed previously, pRb was

found to be acetylated. Indeed, *in vitro* assays also suggest that the pRb associated HDAC activity can de-acetylate the acetylated pRb (Smith *et. al.*, personal communication). It has not been investigated if acetylation of pRb is cell cycle regulated. A third possibility is that pRb/HDAC might regulate other aspects of E2F function. Ecotopic expression of E2F-1 was shown to cause extensive apoptosis in SAOS2 cells (65). p300 augments E2F-1 mediated apoptosis (92), though the involvement of HAT under those circumstances was not known. pRb can counteract E2F-1 mediated apoptosis (65). It is possible that HDAC might be involved in this process, perhaps by co-operating with pRb in rescuing E2F-dependent apoptosis. Further experimentation will be needed to verify, and eliminate these possibilities.

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