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Post-Translational Control of Retinoblastoma Protein Phosphorylation

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POST-TRANSLATIONAL CONTROL OF RETINOBLASTOMA PROTEIN
PHOSPHORYLATION

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by

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The School of Graduate and Postdoctoral Studies
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Abstract

The retinoblastoma tumor suppressor protein (pRB) functions through multiple mechanisms to serve as a tumor suppressor. pRB has been well characterized to be inactivated through phosphorylation by CDKs. pRB dephosphorylation and activation is a much less characterized aspect of pRB function. In this thesis, I detail work to study the post translational control of pRB phosphorylation. Here I present work detailing efforts to generate a gene targeted mouse which disrupts PP1 binding to the C-terminus of pRB, allowing for detailed study of the mechanisms of pRB dephosphorylation. This work also details an examination of acetylation in the C-terminus of pRB, which disrupts CDK phosphorylation of pRB. I generated a site specific antibody to examine K873/K874 acetylation, and carried out characterization of this set of post-translational modifications. This work highlights the complex mechanisms surrounding pRB phosphorylation state and regulation of pRB activation.

Keywords: pRB, post-translational modifications, DNA damage, dephosphorylation, acetylation, Protein Phosphatase 1, gene targeted mouse

Co-Authorship Statement

All chapters in this thesis were written by Paul Stafford and edited by Dr. Fred Dick.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

°C: Degrees Celsius

µg: microgram

µL: microliter

µM: micromolar

^{32}P: Phosphorus-32 radioisotope of phosphorus

A: Alanine amino acid

APC: Anaphase promoting complex

ATP: adenosine triphosphate

bp: Base pair

BSA: Bovine serum albumin

CDK: Cyclin dependent kinase

CKI: Cyclin dependent kinase inhibitor

CO_2: carbon dioxide

CRF: Chromatin regulatory factor

C-terminal: carboxy terminal

DBD: DNA binding domain

DDR: DNA damage response

DMEM: Dulbecco's Modified Eagle Medium

DMZ: dimerization domain

DNA: deoxyribonucleic acid

DP: Differentiation Related Transcription Factor-1 polypeptide-1

DTT: Dithiothreitol

E: Embryonic day

E2F: E2 promoter binding factor

E7: early protein 7

EDTA: Ethylene diamine tetraacetic acid

ELISA: enzyme-linked immune sorbent assay

F: Phenylalanine amino acid

FBS: fetal bovine serum

xG: Gravity

G1: gap 1 phase of the cell division cycle

G2: gap 2 phase of the cell division cycle

GSE: Gel shift extraction buffer

GST: Glutathione S-transferase

GST-RB-LP: GST tagged large pocket domain of pRB

HA: Hemaglutinnin

HAT: histone acetyl transferase

HCl: hydrochloric acid

hESC: Human Embryonic stem cell

Hr(s): Hour(S)

IF: Immunofluorescence

IgG: immunoglobulin G min: minutes

IN: input

IP: Immunoprecipitation

IPTG: Isopropyl !-D-1-thiogalactopyranoside

K: Lysine amino acid

kb: Kilo base

KCl: Potassium Chloride

kD: Kilo dalton

LB: Luria Broth

LXCXE: Leucine-any amino acid-cysteine-any amino acid-glutamate

M: mitosis (when referring to the phases of the cell division cycle)

MB: marked box

MEF: mouse embryonic fibroblast

mESC: Mouse Embryonic stem cell

MgCl$_2$: magnesium chloride

min: minutes

mL: milliliter

N/A: not applicable

Na$_3$VO$_4$: Sodium Orthovanadate

NaCl: Sodium Chloride

NaF: Sodium Fluoride

NaN$_3$: Sodium azide

ng: nanogram

N-terminus: Amino Terminus

p107: Retinoblastoma-like protein 1

p130: Retinoblastoma-like protein 2

PAGE: Polyacrylamide gel electrophoresis

PBS: Phosphate buffered saline

pCAF: p300-CBP associated factor

PCR: polymerase chain reaction

PMSF: Phenylmethylsufonylfluuride

PP1: protein phosphatase 1

PP2A: protein phosphatase 2A

pRB: Human Retinoblastoma tumor suppressor protein

pRb: Mouse Retinoblastoma tumor suppressor protein

R: Arginine

RB1: Human retinoblastoma tumor suppressor gene

Rb1: Mouse retinoblastoma tumor suppressor gene

S: DNA synthesis phase of the cell division

SDS: Sodium dodecyl sulfate

TA: transactivation domain

Taq: *Thermus aquaticus*

TBS: Tris-Buffered Saline

TBST: Tris-buffered Saline with Tween20

TCEP: tris (2-carboxyethyl) phosphine

WB: Western blot

WB: western blot

Δ: lacking

ΔG: General Site Mutation

ΔL: ΔLXCXE mutation

ΔPP1: Protein Phosphate 1 Binding Site mutation

ΔS: Specific Site Mutation

Chapter 1

1   Introduction

1.1   Classical Roles of pRB

1.1.1   pRB Regulates the G1 to S Transition of the Cell Cycle

The retinoblastoma tumor suppressor protein (pRB) was identified as the product of the retinoblastoma susceptibility gene *RB1*, with mutations in this gene being the cause of inherited cancers of the retina which are termed retinoblastomas (Friend et al., 1986; Lee et al., 1987). pRB was classified as a tumor suppressor before it was apparent what the biological function of this protein was through evaluation of the genetics of retinoblastoma patients as well as study of oncogenic viruses (Dyson et al., 1989; Knudson, 1971; Murphree and Benedict, 1984; Whyte et al., 1988). Initial functional studies of pRB determined that pRB plays a critical role in regulating the cell cycle, by controlling the progression of the cell from G1 into the S-phase (DeCaprio et al., 1989). This regulation of the cell cycle is imparted through the regulation of a family of transcription factors known as the E2Fs through a physical interaction (Chellappan et al., 1991; Hiebert et al., 1992a; Lees et al., 1993) which was demonstrated to occur through the "small pocket" domain (res. 379-792) and the C-terminus of pRB (res. 793 – 928)(Qin et al., 1992)(Fig. 1.1). The E2Fs induce a transcriptional program at the G1-S transition that functions to drive the entry into S-phase and initiate DNA synthesis (Blake and Azizkhan, 1989; Dalton, 1992; Hiebert et al., 1989; Thalmeier et al., 1989). The expression of multiple different E2F family members is able to drive cells into S phase (DeGregori et al., 1997; Johnson et al., 1993) highlighting the importance of the E2F family in mediating the G1-S transition. The physical interaction though between pRB and E2Fs was found to lead to negative regulation of E2F target genes (Helin et al., 1993; Hiebert et al., 1992a), thus giving a mechanism to pRB's ability to regulate the G1-S transition of the cell cycle and a basis for pRB's tumor suppressive properties.
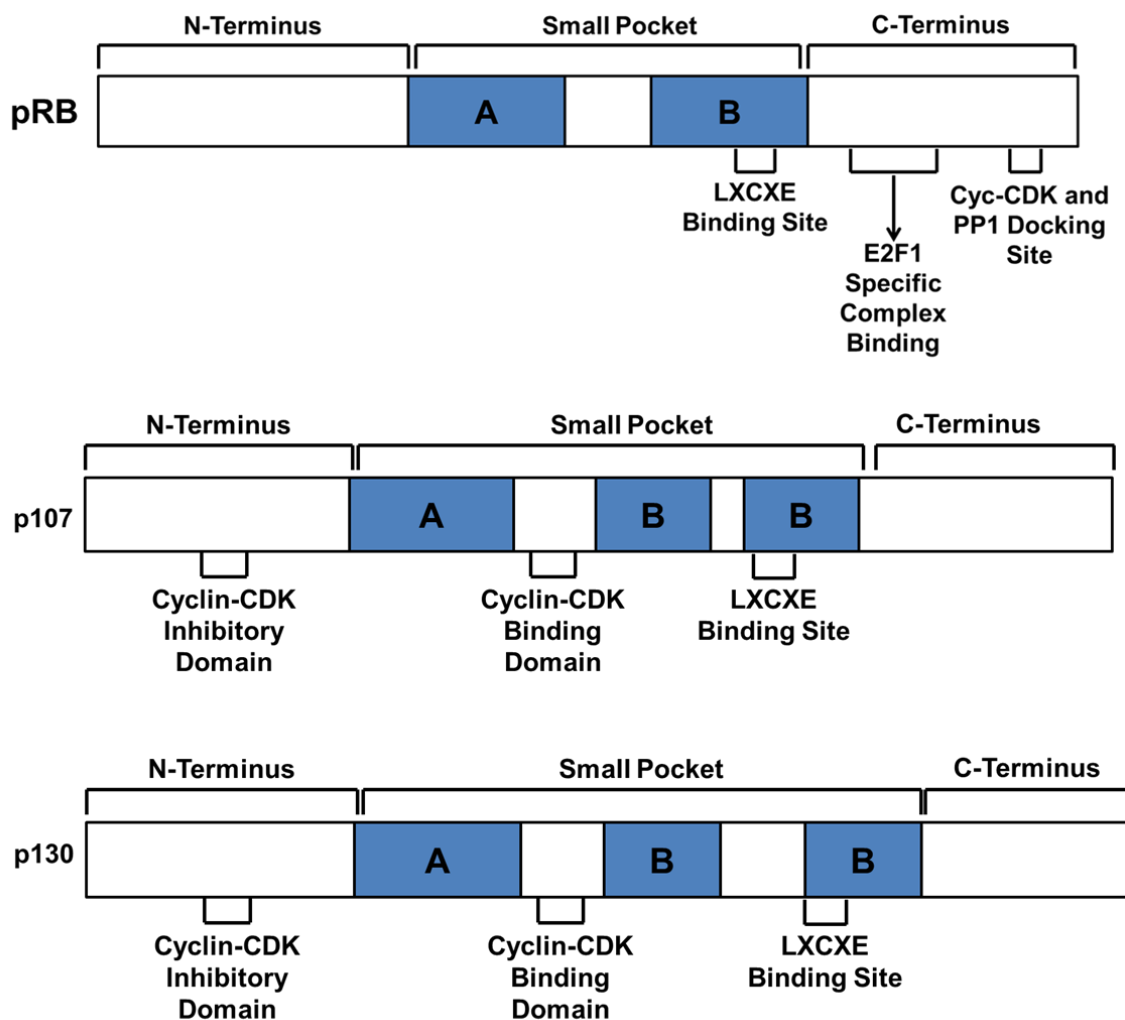
Figure 1.1: Structures of the Pocket Proteins

The pocket proteins are defined by the small pocket region consisting of the A and B cyclin fold domains that are conserved across all three members. The LXCXE binding cleft for LXCXE motif containing proteins is highly conserved across the pocket protein family. pRB is primarily distinguished structurally from the other pocket proteins members by a unique interaction with E2F1 in the C-terminus. The C-terminus of pRB also contains overlapping binding sites for Cyclin-CDKs and PP1. The other pocket protein family members, p107 and p130, are distinguished from pRB by the presence of a Cyclin-CDK binding domain in the small pocket itself and an insert in the B part of the small pocket. The N-terminus of p107 and p130 also contain a CDK inhibitory domain which is unique to these two family members.

1.1.2 The Pocket Protein Family

The pocket protein family consists of three highly related proteins, which are pRB, and the gene products of *RBL1 and RBL2*, p107 and p130 (Fig. 1.1) (Cobrinik et al., 1993; Ewen et al., 1991; Hannon et al., 1993; Mayol et al., 1993). Each family member contains a highly conserved region called the "small pocket" which is made up of an A and B domain consisting of single cyclin-like folds, which are separated by a flexible linker region(Lee et al., 1998) (Fig 1.1). This domain serves as the minimal binding domain of many viral oncoproteins(Hu et al., 1990) and is sufficient to repress transcription through interactions with E2Fs (Chow et al., 1996; Chow and Dean, 1996; Sellers et al., 1995). Viral oncoprotein binding to the small pocket has been attributed to the LXCXE motif. Crystallography has demonstrated that the LXCXE motif contained in viral oncoproteins makes contact with a shallow groove in the B domain of the small pocket (Lee and Cho, 2002; Lee et al., 1998) known as the LXCXE binding cleft. The small pocket, though being highly conserved across the family members and different species, still has subtle differences between the different family members, with p107 and p130 having insertions in the B domain and a longer linker relative to pRB(Classon and Dyson, 2001; Hurford et al., 1997; Mulligan and Jacks, 1998). The "large pocket" consists of the small pocket as well as the C-terminus and is the minimal growth suppressing domain and is sufficient to interact with the E2F family of transcription factors and inhibit E2F target gene transcription(Bremner et al., 1995; Hiebert, 1993; Hiebert et al., 1992b; Qin et al., 1992; Yang et al., 2002). This interaction is mediated through a physical association of the large pocket with the transactivation domain of the E2F (Lee et al., 2002). Specific amino acids in the transactivation domain of E2F make conserved contacts with amino acids in the A domain. Further contacts with the largely unstructured C-terminus of the large pocket further act to stabilize this interaction with E2Fs, leading to an inhibition of E2F transcriptional activity.

1.1.3 A Model of pRB and E2Fs at the G1-S Transition

pRB has been shown to be regulated in a cell cycle dependent manner and as mentioned above to be a central regulator of the cell cycle(DeCaprio et al., 1989; Huang et al., 1988; Takahashi et al., 1991). Pocket proteins, which lack intrinsic ability to bind DNA, bind to

E2F family members, thus localizing themselves at E2F transcriptional target genes. Pocket proteins though display different cell cycle roles due to differential expression and preference for specific E2F family members (Classon and Dyson, 2001; Henley and Dick, 2012). The localization of pocket proteins at E2F target genes thus enables active repression of these genes and prevents progression into the cell cycle. Upon entrance to the cell cycle, expression of the retinoblastoma protein increases and pRB localizes to E2F target genes, inhibiting the transcriptional activity of E2Fs. This is mediated by physically masking their transactivation domain as mentioned above, preventing transcription of genes required for progression into S-phase(Hurford et al., 1997; Lavia and Jansen-Durr, 1999; Takahashi et al., 2000). Activation of E2Fs, and progression into S-phase, is mediated by phosphorylation of pocket proteins in a cell cycle-dependent manner by cyclin/cyclin-dependent kinase (CDK) complexes. Mitogen stimulation of cells leads to activation of CDK complexes and phosphorylation of the pocket proteins (Buchkovich et al., 1989; DeCaprio et al., 1992; Lin et al., 1991; Mihara et al., 1989). The phosphorylation of pocket proteins leads to a release of E2F binding, allowing for the transactivation of E2F target genes (Burke et al., 2010; Chittenden et al., 1993; Mudryj et al., 1991). The activation of CDK complexes occurs in a feed-forward loop that is antagonized by CDK inhibitors (CKIs). As a result of a feed forward loop for CDK complexes, phosphorylation of pocket proteins is maximized, and thus cells irreversibly advance into S-phase (Fig 1.2)(Mittnacht, 1998; Sherr and Roberts, 1999; Sherr, 1994).

1.1.4 Regulation of Heterochromatin through LXCXE interactions with Pocket Proteins

As mentioned above, pocket proteins contain a highly conserved region in the small pocket that facilitates binding of viral oncoproteins, the LXCXE binding cleft. While highly conserved in viral oncoproteins, this LXCXE motif also has been found to be conserved with a host of cellular proteins and thus are able to bind in the LXCXE binding cleft (Dick, 2007). The majority of the proteins that bind through the LXCXE binding cleft function in the role of chromatin remodeling factors (CRFs), with notable interactions including HDAC1 and Suv39h1(Brehm and Kouzarides, 1999; Brehm et al., 1998; Nielsen et al., 2001). The association of these CRFs with pocket proteins allows for
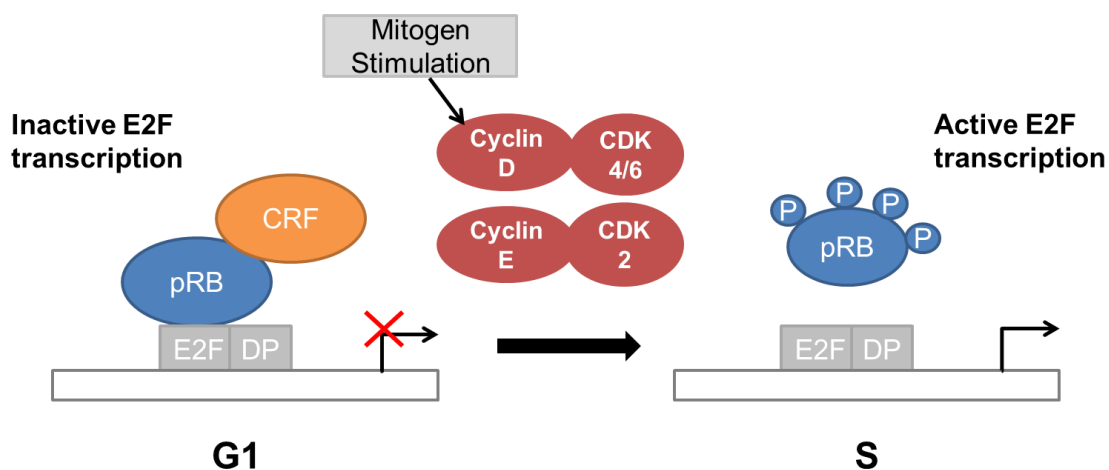
Figure 1.2: Model of pRB function in the Regulation of the G1-S transition of the cell cycle

In G1 pRB interacts with the transactivation domain of E2F/DP heterodimer and blocks their activation of E2F target genes. pRB-E2F-DP complexes bound to E2F target genes are capable of recruiting chromatin remodeling factors (CRFs) to further repress the activation of these genes through the generation of a repressive chromatin environment. As cells transition through G1 into S phase, cyclin dependent kinases are activated. This includes CDK4 and CDK6 with Cyclin D and CDK2 with Cyclin E. Cyclin/CDK phosphorylation of pRB phosphorylation leads to the release of E2F/DP heterodimers, allowing for active transcription of E2F target genes and progression of the cell into S-phase.

 recruitment of proteins that act to establish a repressive heterochromatin environment, allowing for establishment of heterochromatin at E2F target genes which have a pocket protein associated with them. This adds a further level of repression and regulation of E2F target genes in regards to our model of the pRB/E2F G1-S transition presented in Figure 1.2.

While CRFs are a predominant class of proteins that have interactions mediated through the LXCXE binding cleft, other classes of proteins bind and act through LXCXE interactions with pRB including those involved with DNA replication and differentiation(Chan et al., 2001a; Dick, 2007; Miyake et al., 2000; Nguyen et al., 2004). The preponderance of different protein complexes that interact with pRB through the LXCXE binding cleft are important to control of cell proliferation, though not through direct regulation of E2F target gene transcription as a gene targeted mouse developed in our lab, the *Rb1ΔLXCXE* (*Rb1ΔL*), which ablates the LXCXE binding cleft in pRB, do not have upregulated E2F target genes and are viable, fertile and born at nearly the expected Mendelian ratios(Isaac et al., 2006). Mouse embryonic fibroblasts (MEFs) from *Rb1*$^{\Delta L/\Delta L}$ mice though fail in properly establishing a full senescence program and seem to be defective for binding to PML proteins which facilitate the senescence program(Talluri and Dick, 2014; Talluri et al., 2009). Rb1$^{\Delta L/\Delta L}$ mice also exhibit defects in the ability to respond to TGF-β signalling during breast development (Francis et al., 2009). From these results, we can gather that a host of multimeric complexes centered on pRB/E2F interactions exist and that the proper regulation of these interactions may be critical in understanding pRB's ability to actively repress E2F target genes and lead to a stable cell cycle arrest.

1.2 Post G1-S Roles of the Retinoblastoma Protein are Tumor Suppressive

As mentioned above, the first regulatory paradigm discovered for pRB for regulation of the cell cycle is in regards to its role in mediating the G1-S transition in the cell cycle. Recent work in our lab and others have begun to discover other regulatory roles of pRB beyond the G1-S transition that may contribute to the tumour suppressive functions of pRB and appear to be unique to pRB alone.

1.2.1   A pRB/E2F1 "Specific" Interaction that Exists Outside of G1

As described above, pRB acts to repress E2F transcription through a physical association of the large pocket of pRB with the transactivation domain of E2Fs. Among the activator E2Fs, E2F1 demonstrates unique and possibly conflicting functions in regards to the other E2Fs. This is highlighted in a mouse genetic model where E2F1 has been ablated, which leads to defective apoptosis in thymocytes resulting in a defect in negative selection of T-cells (Field et al., 1996). This apoptotic defect is further highlighted as mouse genetic models ablating other E2Fs fail to show similar defects in regards to apoptosis (Chen et al., 2009; Humbert et al., 2000; Rempel et al., 2000). E2F1 induction of apoptosis relies on an induction of p53 and a physical association with a host of pro-apoptotic genes including p73, Caspase 8, and Bid (Cao et al., 2004; Kowalik et al., 1995; Nahle et al., 2002; Pediconi et al., 2003; Stanelle et al., 2002; Zhu et al., 1999). pRB interestingly has been shown to be localized with E2F1 at these pro-apoptotic promoters and localization of pRB and E2F1 across the genome during S-phase has been demonstrated through ChiP experiments(Ianari et al., 2009; Wells et al., 2003). Thus from overview of this data, pRB/E2F1 complexes seem to act in a unique manner relative to the canonical description of pRB/E2F interactions described above.

Through mutational studies of the large pocket of pRB, it was found that E2F1alone was able to bind to the C-terminus of pRB and that this interaction was unique within the pocket protein family to pRB/E2F1(Dick and Dyson, 2003). This interaction was found to be through distinct binding conformations with the C-terminus of pRB interacting with the marked box domain of E2F1(Julian et al., 2008). This interaction of the pRB c-terminus with the Marked box domain of E2F1 has been distinguished as the "Specific site" interaction while the large pocket binding of pRB to E2F1-4 has been distinguished as the "General site" (Fig 1.3). Interestingly, recent work has suggested that the specific complex is resistant to two canonical methods of disrupting pRB binding to E2Fs, with the viral oncoprotein E1A being unable to disrupt this complex (Seifried et al., 2008). Furthermore, this specific interaction was demonstrated to be resistant to CDK phosphorylation, allowing for a population of E2F1 to be bound by pRB even after phosphorylation(Cecchini and Dick, 2011). Interestingly, this complex was demonstrated
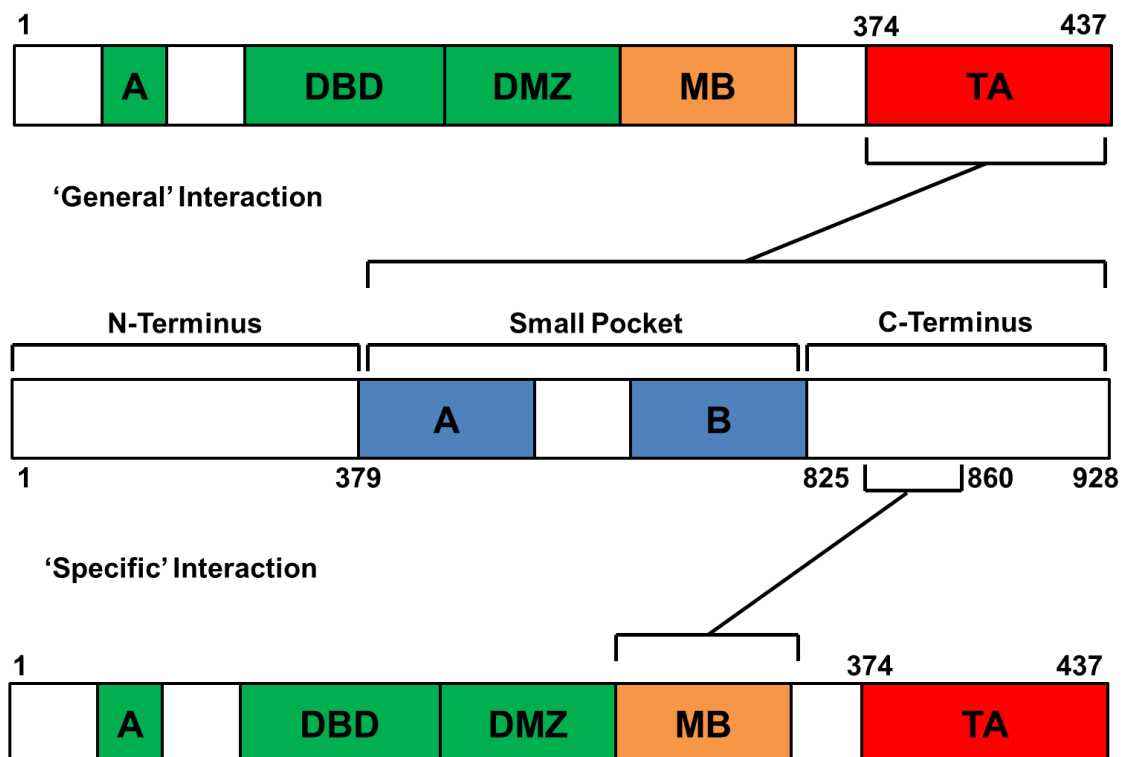
Figure 1.3: pRB has a Unique Interaction with E2F1

pRB is able to interact with E2F1 through two distinct interactions. The 'General' interaction is shared by all E2Fs that bind to pRB. This involves the large pocket, (the small pocket and the C-terminus), interacting with the transactivation domain of E2Fs. The 'Specific' interaction, which is unique for pRB among the pocket protein family, is through residues 825-860 of the pRB C-terminus with the marked box domain of E2F1. A -Cyclin A binding domain. DBD- DNA binding domain. DMZ- Dimerization domain. MB- Marked box domain. TA- Transactivation domain.

to be able to regulate the expression of p73 *in vivo*, and to modulate this response in the context of DNA damage signaling potentially give a mechanism to explain the pro apoptotic roles of pRB and E2F1 mentioned above(Carnevale et al., 2012; Julian et al., 2008). With the emergence of a multiple unique pRB/E2F1 interaction surfaces that appear to have functionally different consequences, understanding the regulation between these two unique complexes presents an exciting area for deeper understanding of pRB tumour suppressive functions.

1.2.2   LXCXE Interactions with pRB Beyond G1

As mentioned above, many classes of proteins bind to pRB through the LXCXE binding cleft and help to facilitate a stable arrest in G1 of the cell cycle. As mentioned above, a gene targeted mouse model developed in our lab, *Rb1^{ΔL}*, are viable, though exhibit defects in scenarios of cell cycle exit including senescence, response to γ-irradiation and response to TGF-β signalling(Francis et al., 2009; Talluri et al., 2009). These mice, though while not succumbing to spontaneous formation of cancer, interestingly exhibited a form of genomic instability with an increase in aneuploidy (Isaac et al., 2006). From this study, the aneuploidy that accumulated in *Rb1^{ΔL}* MEFs was attributed to the fusions in the pericentromere of chromatin leading to aberrant progression through mitosis. As highlighted above, no defects in E2F target gene expression are observed in *Rb1^{ΔL}* MEFs, including E2F target genes involved in mitosis, including Mad2 and BubR1, which compose the spindle checkpoint(Isaac et al., 2006). While direct effects related to E2F transcription were ruled out, recent work from our lab, as well as other investigators have revealed that pRB is able to interact with the Condensin II complex subunit Cap-D3 and that this interaction is dependent on binding in the LXCXE cleft of pRB(Coschi et al., 2010; Longworth et al., 2008; Manning et al., 2010). Interestingly this interaction was able to mechanistically explain the earlier mentioned defects in pericentromeres of *Rb1^{ΔL}* MEFs and that proper regulation of this interaction is required for full pRB mediated tumour suppression (Coschi et al., 2010). With these findings, an unknown determinant of this mechanism is how in fact pRB localizes to the pericentromere without intrinsic ability to bind DNA. Recent observations from our lab have determined that pRB localization to the pericentromere of chromatin is dependent on pRB binding to E2F1

leading to Condensin II complex recruitment at the pericentromeres (Coschi et al., 2014). Furthermore, failure to recruit a complex of pRB/E2F1/Condensin II leads to an increase in genomic instability due to replicative stress (Coschi et al., 2014)

With the evidence of LXCXE dependent interactions of pRB that persist beyond G1 of the cell cycle, one question that persists is how this interaction is retained in an LXCXE manner. Prior observations suggest that phosphorylation of pRB by CDKs while disrupting pRB/E2F interactions through the general site also disrupt chromatin remodelers from binding to the LXCXE binding cleft (Harbour et al., 1999a; Knudsen and Wang, 1996). With these observations, speculation in the pRB field remains on how the LXCXE dependent interactions with pRB persist past the G1 phase of the cell cycle when pRB is extensively phosphorylated. By gaining a further understanding of the control and regulation of pRB phosphorylation, this offers a potential means to regulate and control these interactions.

1.3   Post-Translational Modifications of pRB

As described in the above mentioned sections, pRB plays host to numerous functions that comprise the roles of pRB as a prototypical tumour suppressor. To understand the various contrasting functions and interactions, it is necessary to further study the post-translational modifications of pRB to offer potential insights into the control of these different functions.

1.3.1   CDK Phosphorylation of pRB

As mentioned in previous sections, pRB has been observed to be phosphorylated by Cyclin/CDK complexes upon mitogen stimulation. The initial characterization of CDK phosphorylation of pRB was based on altered electrophoretic migration throughout the cell cycle with the observation of two unique species of pRB with regards to electrophoretic migration(Buchkovich et al., 1989). Through various biochemical and mass spectrometry methods, 15 CDK sites have been identified on pRB (Fig 1.4)(Brown et al., 1999; Connell-Crowley et al., 1997; Dephoure et al., 2008; Zarkowska and Mittnacht, 1997). In early G1 of cycling cells or G0 in non-cycling cells, few of these CDK sites are phosphorylated (Ezhevsky et al., 2001; Ezhevsky et al., 1997). This
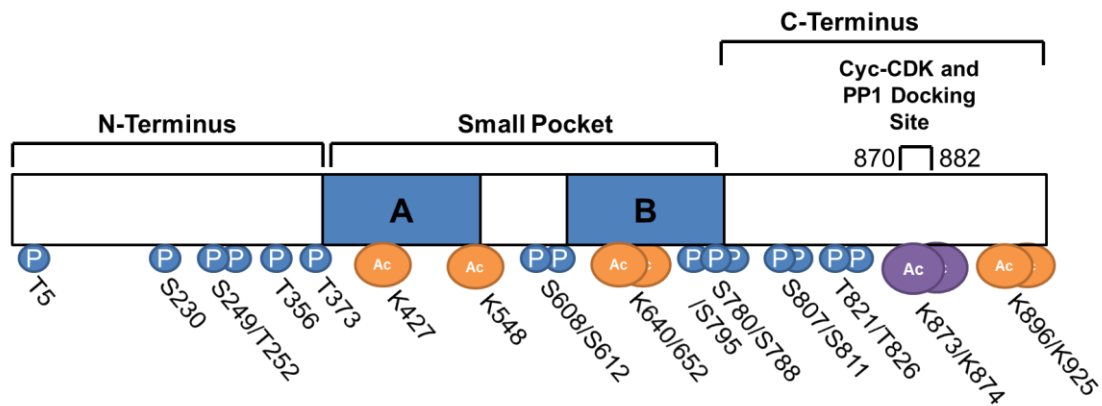
Figure 1.4: CDK Phosphorylation Sites and Lysine Acetylation Sites on pRB

pRB is extensively phosphorylated on CDK sites dispersed throughout the pRB primary structure with 15 known CDK phosphorylation sites confirmed through various biochemical and mass spectrometry means. Acetylation is found on both K873 and K874 in the C-terminus of pRB and is highlighted in purple. Acetylation has also been demonstrated on other residues of pRB, indicated in orange, though these are primarily only validated using mass spectrometry methods.

species of lightly phosphorylated pRB has been termed the hypo-phosphorylated form of pRB which is able to retain binding to E2Fs. These early phosphorylation events are catalyzed by activation of Cyclin D-CDK4/6 complexes which interact through a docking site in the C-terminus of pRB (Ezhevsky et al., 2001; Wallace and Ball, 2004). Following these initial phosphorylation events, as the cell progresses towards S phase of the cell cycle, activation of Cyclin E-CDK2 complexes leads to substantially more phosphorylation of pRB(Harbour et al., 1999b). This species of substantially phosphorylated pRB is characterized as the hyper-phosphorylated form of pRB and is unable to bind E2Fs through the general interaction, though retaining specific E2F1 binding as mentioned previously. This body of highlighted work suggests two basic structural conformations of pRB, that of the hypo-phosphorylated form and a conformation for the hyper-phosphorylated form of pRB. This potentially simplistic view of two pRB phosphorylated forms of functional consequence though has begun to be challenged through the emergence of recent works which suggest this is an oversimplification of pRB regulation by CDK phosphorylation. Highlighted below are works first suggesting that specific CDK phosphorylation sites on pRB may in fact have unique functional consequences in respect to pRB function and that the hypophosphorylated form of pRB which has previously described as the active form of pRB may in fact be attributed to a collection of mono-phosphorylated pRB isomers.

As pointed out above, pRB contains 15 CDK sites, and while initial work has focused on the hypo and hyper-phosphorylated forms of pRB, data has begun to emerge in regards to potential roles of single phosphorylation sites in regards to specific regulation of pRB function. Initial work to characterize phosphorylation sites suggested that the C-terminal phosphorylation sites were sufficient to disrupt pRB from E2Fs with more recent x-ray crystallography studies of pRB C-terminus with E2F1/DP1 peptides demonstrating a similar phenomenon(Chow et al., 1996; Knudsen and Wang, 1996; Rubin et al., 2005). Similar studies though for the N-terminus of pRB suggest that CDK sites in this region are also sufficient to disrupt pRB binding to E2Fs. A crystallography study using the pRB N-terminus with the small pocket suggests that T373 phosphorylation leads to folding of the N-terminus into the small pocket in an allosteric manner which may block both E2F and LXCXE cleft mediated interactions with

pRB(Burke et al., 2012). Furthermore, phosphorylation sites in the linker region of the small pocket have also been examined with interesting results. S612 has been suggested to be phosphorylated in scenarios of DNA damage, and although this site has been implicated as a CDK phosphorylation site, under this cellular context phosphorylation is catalyzed by Chk1/2 and leads to retention of E2F1 binding(Inoue et al., 2007; Zarkowska and Mittnacht, 1997). S608 which also lies in the linker region, using a phospho-mimetic and crystallography was observed to disrupt binding of E2Fs to pRB highlighting that two proximal phosphorylation sites may in fact have highly divergent functional roles(Burke et al., 2012).

More recently, insights into pRB phosphorylation by CDKs have begun to emerge that further gives credence to the idea that control and regulation of single phosphorylation sites may control the regulation of different discrete pRB functions. Through analysis using 2D gel electrophoresis, Narasimha and colleagues demonstrate that in the G1 phase of the cell cycle, pRB persists in distinct isomers of the mono-phosphorylated form with 14 distinct mono-phosphorylated pRB isomers present (Narasimha et al., 2014). It was noted that upon DNA damage, pRB remained in a mono-phosphorylated form which was mediated by CyclinD/CDK4/6 activity(Narasimha et al., 2014). Finally the authors noted that upon terminal differentiation of myoblasts into myotubules, pRB was uniquely present in an un-phosphorylated form(Narasimha et al., 2014).

From the above highlighted works, a more elaborate model of pRB phosphorylation begins to be elucidated wherein the simple model of hypo and hyper-phosphorylated pRB can be expanded to envision a system of multiple simultaneously existing species containing different arrangements of site specific phosphorylation. This potential model though begins to ask the question of how this tightly regulated system of distinct single phosphorylation population of unique pRB molecules is maintained in such a manner. To answer this potential question requires a full understanding of regulation of not only pRB phosphorylation but of pRB dephosphorylation which will be discussed in more breadth below.

1.3.2   Acetylation of pRB

pRB has been shown to be acetylated at K873/K874 in the C-terminus when bound by the p300/CBP co-activator complex(Chan et al., 2001b). Acetylation of these two lysine residues, which reside in a CDK docking site in the C-terminus of pRB, leads to a reduction in pRB phosphorylation by CDKs(Chan and La Thangue, 2001; Lowe et al., 2002; Wallace and Ball, 2004). Furthermore, acetylation of these two residues of pRB occurs in the cellular context of DNA damage and this leads to the accumulation of pRB/E2F1 complexes (Markham et al., 2006). These results suggest that pRB acetylation at these two sites may be beneficial in contexts of cellular arrest. This idea is further substantiated with the observations that pRB acetylation occurs in the cellular context of differentiation. With the use of C2C12 myoblasts, it was observed that pCAF, the p300/CBP associated factor, is recruited to pRB to acetylate K873/K874 and that this is required for transactivation of MyoD and proper differentiation (Nguyen et al., 2004). Similar effects were also observed for keratinocyte differentiation, with acetylation defective mutants unable to promote differentiation but were able to arrest cells in a SaOS2 arrest assay (Pickard et al., 2010).

The observations recognized previously though in the literature could in fact be attributed to other acetylation sites on pRB, due to the lack of commercial site specific antibodies available for acetyl K873/K874 pRB. Mass spectrometry and global proteomics approaches have elucidated other less characterized acetylation sites on pRB which could be responsible for the mechanistic observations which have been ascribed to K873/K874 acetylation. A study by Choudhary and colleagues (Choudhary et al., 2009) treated A549, Jurkat and MV4-11 human cell lines with HDAC inhibitors and performed mass spectrometry analysis on lysates from these cells. From this study, the authors determined that K427, K548, K640, K652, and K896 on pRB were found to be acetylated in this context of HDAC inhibitor treated cells. Similar proteomic scale studies have identified K925 in the C-terminus as being acetylated, again with human cell lines that had been treated with HDAC inhibitors (Hornbeck et al., 2012). Less characterized acetylation sites within pRB are highlighted in Figure 1.4, where we can observe sites of acetylation throughout pRB. Interestingly, similar proteomic approaches were performed

in hepatocellular carcinoma tissue isolated from patients and strikingly from this study, only acetylation on K874 could be detected on pRB from these patient samples (Zhao et al., 2010). These findings offer insights that acetylation of pRB may be sensitive to context and cell type. The findings that K874 acetylation was present through mass spectrometry of Hepatocellular carcinoma tissue samples without K873 acetylation, in contrast to previous reports, highlights the potential for both acetyl K873/K874 to not be present in combination.

These results suggest that pRB acetylation may play a role in regulating the phosphorylation of pRB in contexts where stable cell cycle arrest is required such as in the scenarios of DNA damage or in differentiation. The work highlighted above though asks the general question of whether this is a widespread occurrence or limited to very specialized scenarios. Furthermore, most work was performed under *in vitro* conditions and thus does pRB acetylation play a functional consequence under physiological conditions remains to be answered.

1.4   pRB Dephosphorylation

As discussed in section 1.3.1 detailing pRB phosphorylation by CDKs, an emerging picture of highly regulated site specific phosphorylation is beginning to emerge with the potential for each individual phosphorylation site regulating specific functions and interactions of pRB. While the heavily studied role of pRB phosphorylation offers some insights into this regulation, the much less characterized area of pRB dephosphorylation offers an enticing means of regulating and controlling the distribution of specific pRB mono-phosphorylated isomers that have been shown to exist.

1.4.1   Regulation of pRB Dephosphorylation in the Cell Cycle

In a normal cycling cell, pRB becomes hyper-phosphorylated in late G1 and exists in this species until late in mitosis. In the transition between metaphase and anaphase, there is a sharp decrease in the activity of CDKs that result from the degradation of cyclin B by the anaphase-promoting complex (APC) (King et al., 1995). At this point, pRB is actively dephosphorylated primarily by the enzyme complex of Protein Phosphatase 1 (PP1) (Alberts et al., 1993; Ludlow et al., 1993). Evidence also exists that Protein Phosphatase

2 A (PP2A) may also play a role in mitotic dephosphorylation of pRB(Alberts et al., 1993). The end result of this targeted dephosphorylation of pRB is a re-establishment of a large population of hypo-phosphorylated pRB which can then re-engage and repress E2Fs (Fig 1.5).

From this work, pRB was determined to be actively dephosphorylated in anaphase by primarily PP1 but many questions remain in regards to how this process is specifically regulated. Both PP1 and PP2A typically reside in multimeric complexes and interestingly multiple different regulatory subunits for both enzymes have been shown to physically interact with pRB(Ceulemans and Bollen, 2004; Kolupaeva and Janssens, 2012).

1.4.2   Regulation of pRB Dephosphorylation in Conditions of Cellular Stress

As shown above, pRB dephosphorylation is a tightly regulated and coordinated event in a normal cell cycle context. Outside of this tightly ordered system of regulation, pRB can also be actively dephosphorylated in cellular contexts of stress such as oxidative stress, DNA damage and hypoxia. Once again, PP1 has been shown to play a major role in the dephosphorylation of pRB in response to cell cycle arrests induced by DNA damage or hypoxia (Dou and Lui, 1995; Krucher et al., 2006; Lentine et al., 2012). Upon DNA damage PP1 is activated to promote the dephosphorylation of pRB to a hypo-phosphorylated state that can maintain the interaction with E2Fs and block their transcriptional activity to mediate an acute arrest of the cell cycle. (Wang et al., 2001) In addition protein phosphatase 2 also interacts with and dephosphorylates pRB in some cellular contexts(Kolupaeva and Janssens, 2012). Thus the current understanding of pRB dephosphorylation in response to cellular stresses illuminates the potential complexity of regulating the various phosphorylation sites of pRB.

1.4.3   A Specific and Direct Interaction of PP1 with the C-Terminus of pRB

As mentioned above, pRB dephosphorylation appears to be a tightly regulated cellular program involving multiple different multimeric protein complexes. Previous studies carried out by our lab in coordination with another group determined that the C-terminus
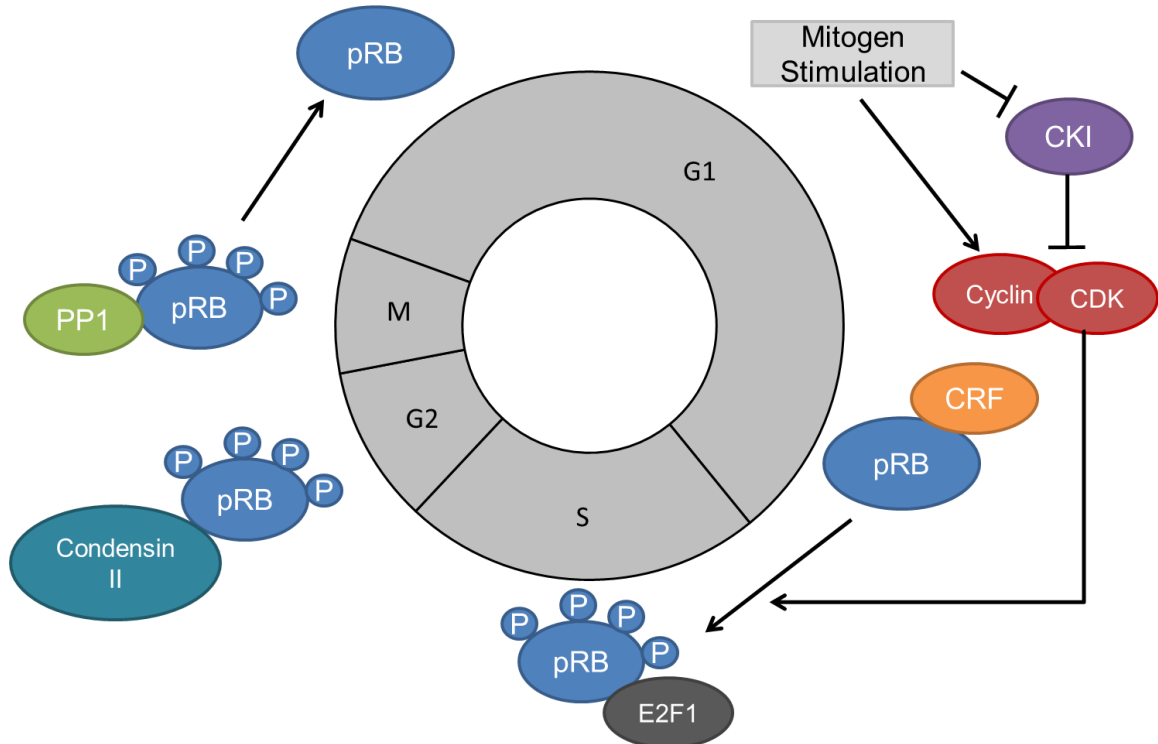
Figure 1.5: pRB Functions Throughout the Cell Cycle

As more research emerges on pRB, a broader understanding is beginning to emerge of pRB function outside of the G1-S transition of the cell cycle. As highlighted previously in **Fig 1.2**, pRB has historically been considered to mediate the G1-S transition of the cell cycle by controlling E2F target gene transcription. Emerging work also indicates the pRB/E2F1 complexes persist beyond G1 of the cell cycle through the 'Specific' interaction. A pRB LXCXE interaction with the Condensin II complex also persists beyond G1 of the cell cycle and mediates chromosomal condensation of pericentromeres at major satellite repeats. CKI- Cyclin Dependent Kinase Inhibitors.

of pRB contains a unique site that allows for direct interaction between PP1 and pRB(Hirschi et al., 2010). Analysis of a crystal structure of the $pRB_{870\text{-}882}$ C-terminus peptide bound to the α isoform of the PP1 catalytic subunit indicates that pRB binding is outside of the catalytic cleft of PP1. Furthermore, binding of pRB to PP1 occurs in a similar manner and location to that of the interactions that commonly occur between PP1 targeting subunits and PP1, with the interactions being mediated by a "RVxF" motif. This binding interaction and motif interestingly is not present in the other pocket protein family members, p107 and p130, indicating that this is a unique mechanism of regulation for pRB.

Intriguingly, the minimal binding domain of PP1 to pRB directly overlaps a previously identified binding site for Cyclin-CDK complexes in the C-terminus, and that binding of each of these complexes was competitive and mutually exclusive(Hirschi et al.). Furthermore, maximal catalytic activity from both Cyclin-CDK and PP1 complexes towards a pRB substrate were achieved when bound to the docking site in the C-terminus. As mentioned above, pRB is able to be acetylated at K873/K874, and previous works suggest that acetylation at these sites is responsible for decreased CDK activity in regards to a pRB substrate. Potentially acetylation at these sites may regulate binding and access to the docking site, further adding a potential layer of regulation and complexity to this mechanism.

1.5   Hypothesis and Objectives

As described above in the preceding sections of this chapter, while a large body of work has been established on general regulation of pRB phosphorylation, little has been explored in the regulation of pRB dephosphorylation and functional consequences of selective phosphorylation of pRB in regards to CDK phosphorylation. I hypothesize that the regulation of pRB dephosphorylation is critical in regulating pRB function and activity. Furthermore, I hypothesize that acetylation of lysines 873 and 874 in the pRB C-terminus potentially could play a role in the regulation of pRB dephosphorylation. To set up about trying to answer these questions regarding the regulation of pRB dephosphorylation I have outlined two specific aims in this thesis to address this question.

In the first aim, I detail work on generating and characterizing an antibody that recognizes pRB acetylation in the C-terminus on lysines 873 & 874 which sit directly within previously known CDK and PP1 binding sites within pRB. I validated this antibody and demonstrated that this antibody specifically recognized acetylation of lysines 873/874 of pRB using *in vitro* acetylation assays. Using this antibody, I examined the ability of pRB to be properly phosphorylated by CDK2. I then examined cellular conditions suggested from previous works on pRB acetylation to enrich for this modification and found no evidence using my antibody of the presence of acetyl-K873/K874 pRB under these outlined cellular conditions.

In the second aim, I present work to create a novel gene targeted mouse model of pRB which is defective for binding of PP1 to the pRB C-terminus. This mouse model, which I classify as the Rb1ΔPP1, would allow for a proper and discrete study of pRB dephosphorylation by PP1 and the potential mechanistic insights of this unique interaction with pRB. Through the attempt to generate this mouse model of pRb, I was unable to obtain viable mESCs which contained our targeted allele.

The results I present in this thesis detail the generation and characterization of an anti-acetyl pRB antibody and demonstrate limited functional impact of this specific post-translational modification. Furthermore, I have outlined the attempt to generate a mouse model for pRB which is deficient for dephosphorylation and have observed that this model was unable to be created through conventional gene targeting approaches.

1.6   References

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Chapter 2

2   Methods

2.1   Plasmids

All plasmids used in this thesis are described in Table 2.1. psCodon1-GST-RBLP has been previously reported (Cecchini and Dick, 2011). pBSKRB12KBSacI was a kind gift from Jean Wang and has been previously been reported (Chau et al., 2002)

2.2   Plasmid Construction

2.2.1   psCodon1-GST-RBLP-K873/K874A

PCR conditions and primers discussed here are detailed in 2.3.2 and Table 2.2. pFAD228 plasmid was used as a PCR template for RB379 and FD155 primers. This PCR product was isolated and digested with *Bam*H1/*Eco*R1. This digested product was then ligated into psCodon1-GST-pRB LP which had been digested with *Bam*H1/*Eco*RI. This lead to the insertion of a fragment of pRB containing K873/K874A mutations into a bacterial GST expression vector.

2.2.2   pRBΔPP1 Targeting Vector Construction

A targeting vector was constructed that allows for introduction of the pRBΔPP1 mutation (R869F F870R) in exon 25 of the mouse *Rb1* gene. A brief outline of the targeting vector construction is detailed in Figure 2.1 and an overview of how the construct facilitates recombination is provided in Figure 4.2. Our strategy for targeting was adopted from a previously published method targeting a nearby region (Chau et al., 2002). The pFAD326 Plasmid was constructed by Michael Thawites contained R869F F870R mutations in Exon 25 along with an insertion of an *Eco*RI restriction enzyme site in intron 24. A PGK-Neo cassette flanked by LoxP sites was inserted at a *Bgl*II site in intron 25. The pBSKΔBH plasmid was created by Fred Dick with a bluescript vector

Table 2.1 Description of Plasmids Used and Created

Plasmid Name	Genes encoded	Expression	Obtained/ Constructed	Resistance	Stock Number
psCodon1-GST-RBLP	GST, pRBLP	Bacterial	Fred Dick	Ampicillin	0526
pRB-DeltaPP1 Targeting Vector	*Rb1*	N/A	Paul Stafford*	Ampicillin	0683
pFAD326		Bacterial	Michael Thawites	Ampicillin	0684
pBSKRB12kbSacI		Bacterial	Jean Wang	Ampicillin	0685
pBSKΔBH		Bacterial	Fred Dick	Ampicillin	0686
pBSK-PGKNeoLox 2	Neomycin	Bacterial	Fred Dick	Ampicillin, Neomycin	0137
pCRII-Rb1ex24	*Rb1*	Bacterial	Fred Dick	Ampicillin, Kanamycin	0523
pFAD228	pRB K873/K874A	Mammalian	Fred Dick	Ampicillin	0220
psCodon1-GST-RBLP-K873/K874A	pRB K873/K874A	Bacterial	Paul Stafford*	Ampicillin	0687

(pBSK) modified to remove the *Bam*HI and *Hin*dIII sites from the multiple cloning site. A vector containing a 20 kb fragment of the 3' end of the *Rb1* gene (pBSK-RB12kbSacI) was digested with *Sac*I to obtain a 12 kb fragment, which was then ligated into the pBSKΔBH plasmid after a *Sac*I digest, to make the pFAD321 plasmid. The 326 plasmid and pFAD321 plasmids were then digested with *Bam*HI/*Hin*dIII, and ligated together to make the final targeting construct. The targeting construct was then maxi-prepped, and sequenced to confirm exon and neo cassette sequences and restriction mapped to confirm correct assembly.

2.3   PCR and Primers

2.3.1   PCR Conditions to Create GST-RB LP K873/K874A (GST Expression)

Master Mix per Reaction:

- 9 µL   H2O
- 1 µL Vent Polymerase Buffer
- 1 µL 2 mM dNTPs
- 1 µL 100 mM FD 155 Primer
- 1 µL 100 mM Rb379 Primer
- 1 µL Vent Polymerase
- 2 µL 200 ng/µL pFAD228

PCR Reaction Conditions:

1. 94°C   2 mins
2. 94°C   30 secs
3. 45°C   30 secs
4. 72°C   2 mins 30 secs
5. Go to Step #2, 34 times
6. 72°C   10 mins
7. 4°C   Indefinite
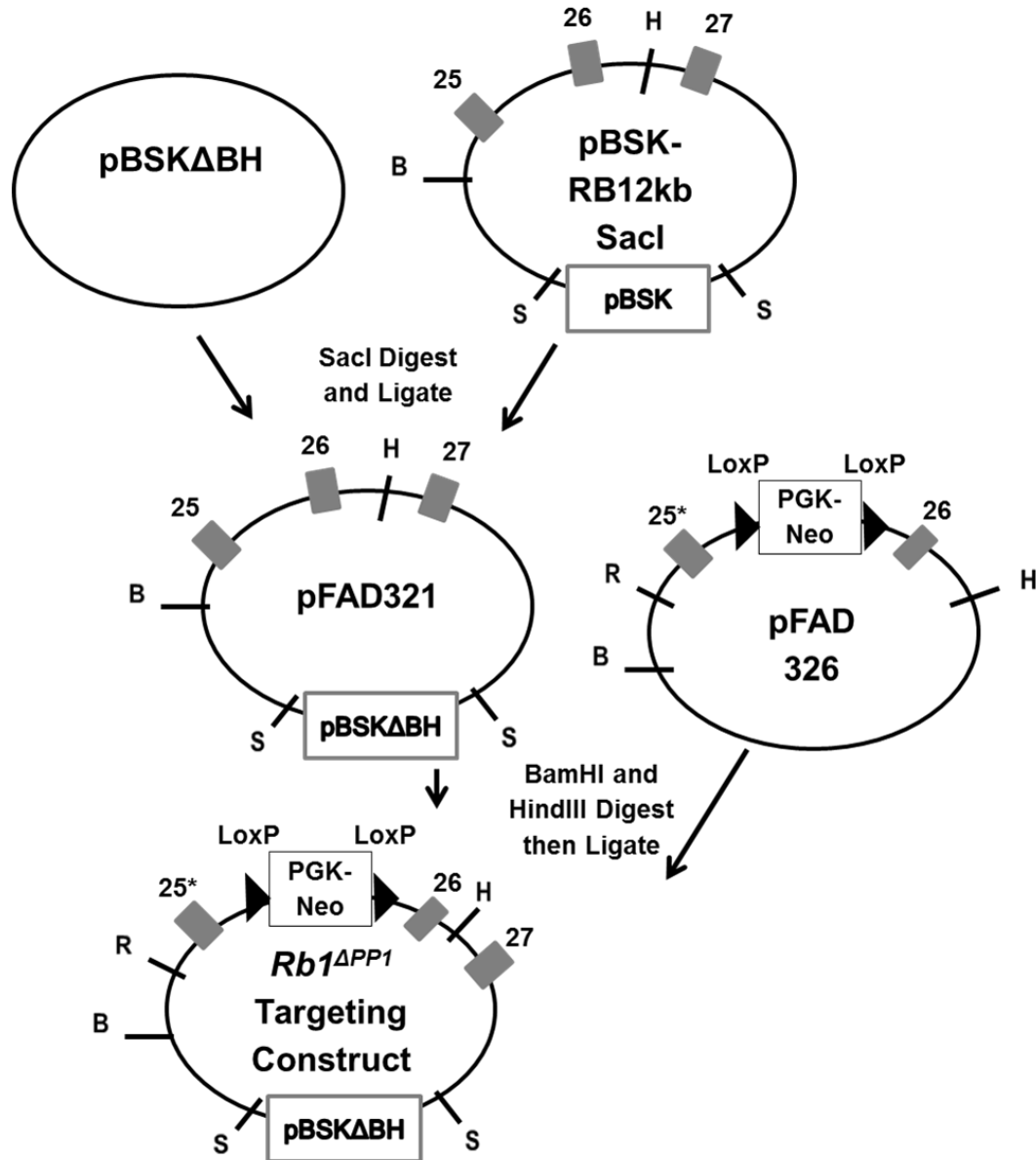
Figure 2.1: Schematic of the *Rb1ΔPP1* Targeting Construct

Schematic detailing the construction of the $Rb1^{ΔPP1}$ targeting construct. pBSK-Rb12kbSacI was cut with *Sac*I restriction enzyme and ligated into pBSKΔBH, a bluescript plasmid which had *Bam*HI and HindIII sites in the multiple cloning site removed. This plasmid, pFAD321 was cut with *Bam*HI and HindIII along with pFAD326 and were ligated together to create the $Rb1^{ΔPP1}$ targeting construct. B-*Bam*HI, H-HindIII, R-*Eco*RI, S-SacI, 25*-Exon 25 with ΔPP1 mutation, 25- Exon 25, 26-Exon 26, 27- Exon 27

Table 2.2: List of Primers

Primer Name	Oligonucleotide sequence	Obtained/Constructed
Rb379	GCGGGATCCGAAGAGGTGAATGTAATTCCTCC	Fred Dick
FD155	CGCGAATTCCTCATTTCTCTTCCTTGTTTG	Fred Dick

2.4　　Antibody Generation and Purification

Antibody generation and purification was performed as previously described(Taya et al., 2003). Synthetic peptides were generated against amino acids of the C-terminus of human pRB through Covance Discovery and Translational Services Inc. These peptides were 15 mer sequences (Residues 867-881) that flanked residues K873/K874 and were either unmodified or synthesized with acetylation of K873/K874. Two rabbits were immunized with the acetylated peptide following a standard 118 day protocol. The animals were subjected to a 3 week cycle of antigen boosts and test bleeds were taken 10 days following the boosts. Serum from initial test bleeds was used for initial ELISA screening to determine the specificity of the serum towards the modified epitope. Serum was then purified for anti-acetyl K873/K874 pRB antibody in 5 mL batches using a two-step immunoaffinity column chromatography procedure. Acetyl K873/K874 pRB and unmodified K873/K874 immunoaffinity columns were prepared using a NPPKPL-Ac-K-Ac-K-LRFDIEG peptide sequence used to immunize the rabbits along with an unmodified NPPKPL-K-KLRFDIEG. Peptides were reduced by incubation with an immobilized TCEP reducing gel (5 mL for 2.5 mg of peptide, Pierce) for 15 minutes at room temperature in gravity flow columns (Biorad). Reduced peptides were eluted in 0.5 mL fractions and the fractions containing the highest peptide concentrations, as determined by Bradford assay, were pooled and coupled to 4 mL each of Sulfolink Coupling gel (Pierce). Non-specific binding sites on the gel were blocked with 50 mL L-cysteine HCL and washed with 1M NaCl.

　　　Antibody purification then was carried out, first by clearing the serum of debris by centrifugation at 4°C at $1660 \times$ g for 10 minutes. The cleared serum was then passed through the immunoaffinity column coupled with modified acetyl-K873/K874 pRB peptide at 4°C with flow through being saved. The column was then washed with 25 mL of 1X PBS at 4°C. Bound IgG then was eluted from the column through a two-step process. Initially, 8 mL of 0.2M Glycine-HCl pH 2.5 was added to the column and 1 mL fractions were collected in microfuge tubes containing 95μL of 1.5M Tris-HCl pH 8.8 to immediately neutralize each fraction to ~ pH 7.3. Following this the second elution step was carried out where 8 mL of 0.2M Glycine-HCl pH 1.9 was added to the column and 1

mL fractions were collected into tubes containing 160µL of 1.5M Tris-HCl pH 8.8 to immediately neutralize each fraction to ~ pH 7.3. To remove non-acetyl-K873/K873 pRB antibodies, elution fractions containing IgG as determined by Bradford assay were loaded into the column coupled with unmodified peptide. This column was then subjected to a similar approach as described above for the modified column, wherein the flow through from the PBS wash step was isolated. Following purification, both columns were washed with 25 mL of 1X PBS at 4°C, then washed with 10 mL of 1X PBS with 0.05% Sodium azide at 4°C and stored for future use at 4°C. Purified acetyl-K873/K874 pRB antibody was dialyzed against 2L of 1xPBS and diluted with storage buffer (1xPBS, 20% Glycerol, 0.05% Sodium azide and 100 µg/mL BSA), and aliquoted and stored at -20°C.

2.5   Enzyme Linked Immunosorbent Assay

ELISA was adapted from a previously described method for testing antibody specificity(Taya et al., 2003). ELISA screening was performed to test the specificity of acetyl-K873/K874 antibody toward acetyl K873/K874 pRB substrate. Synthetic peptides from Covance Discovery and Translational Services generated for acetyl-lysine 873/874 and non-acetylated pRB were used in this subsequent assay. 0.3 µg of peptides (or TBS in the negative control) were added in triplicate to the wells of a flat bottom 96 well plate (Falcon). The plate was incubated for 5 hours at room temperature to adsorb the peptides to the bottom of the wells after which the supernatant was removed and 50 µL of Blocking buffer (1x PBS, 3% BSA) was added for 2 hours at room temperature. Wells were washed 3 times in TBST after which 30 µL of a titration (1/10, 1/100, 1/1000, 1/10000, 1/100000, 1/1000000, 1/100000000 in blocking buffer) of either serum or purified anti-acetyl K873/K874 antibody was added to the wells and incubated for 1 hour at room temperature. Wells were then washed 5 times in TBST after which 30 µL of 1:10000 diluted goat-anti rabbit secondary antibody coupled to alkaline phosphatase (Sigma) in blocking buffer was added to the wells for 1 hour at room temperature. The plate was then washed 3 times with TBST, followed by the addition of 50 µL of Akaline Phosphatase Yellow (para-Nitrophenylphosphate) Liquid Substrate system for ELISA (Sigma). Colorimetric analysis was then performed by reading absorbance at 405 nm on Wallac-Victor plate reader.

2.6   Peptide SPOT Membrane Assay

Peptide arrays of the pRB C-terminal region for both mouse and human consisting of 122 and 123 15-mer peptides each consisting of a one amino acid overlap were synthesized in the Li lab as spots on a nitrocellulose membrane (Reineke and Sabat, 2009). Peptide sequences for both the mouse and human arrays are outlined in Table 2.3. To carry out epitope characterization of the indicated pRB antibodies, the membrane was rehydrated in 100% ethanol for 1 minute, then distilled water was added to 50% and the membrane was incubated at room temperature for 15 minutes. The membrane then was washed in distilled water 3 times for 15 minutes each followed by 3 washes with Tris buffered saline-0.1% Tween20 (TBST- 20 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween20) for 10 minutes each. The membrane was then blocked for 3 hours at room temperature by incubation in 5% non-fat milk TBST. The membrane was then washed 3 times for 10 minutes each in TBST and incubated with the indicated primary antibody. pRB rabbit polyclonal M153 antibody (Santa Cruz) was diluted 1:500, pRB mouse hybridoma monoclonal antibody RB4.1 was diluted 1:10 from culture supernatant, pRB rabbit antibody generated in our lab against the final 15 amino acids of the human pRB sequence C15 was diluted 1:500 and pRB Sheep antibody M136 generated in our lab against the mouse C-terminus was diluted 1:500. All primary antibodies used were diluted in 5% non-fat milk TBST. Following primary antibody treatment, the membrane was blocked for 3 hours at room temperature with 5% non-fat milk TBST. The membrane was then washed 3 times for 10 minutes with TBST and incubated with peroxidase secondary antibody (anti-mouse for RB4.1, anti-rabbit for Santa Cruz M153 and C15, and anti-sheep for M136) at a 1:10000 dilution in 5% non-fat milk TBST. The membrane was then washed 3 times for 10 minutes in TBST and developed using chemiluminescence detection solution (0.1M Tris pH 8.5, 390 μM courmaric acid, 2.46 mM luminol, 0.02% H_2O_2) and exposure to Amersham hyperfilm (GE Healthcare).

Table 2.3: List of Human and Mouse pRB Peptides for SPOT Array

Peptide #	Human Peptide	Mouse Peptide
1.	FPSSPLRIPGGNIYI	SSSPLRIPGGNIYIS
2.	PSSPLRIPGGNIYIS	SSPLRIPGGNIYISP
3.	SSPLRIPGGNIYISP	SPLRIPGGNIYISPL
4.	SPLRIPGGNIYISPL	PLRIPGGNIYISPLK
5.	PLRIPGGNIYISPLK	LRIPGGNIYISPLKS
6	LRIPGGNIYISPLKS	RIPGGNIYISPLKSP
7.	RIPGGNIYISPLKSP	IPGGNIYISPLKSPY
8.	IPGGNIYISPLKSPY	PGGNIYISPLKSPYK
9.	PGGNIYISPLKSPYK	GGNIYISPLKSPYKI
10.	GGNIYISPLKSPYKI	GNIYISPLKSPYKIS
11.	GNIYISPLKSPYKIS	NIYISPLKSPYKISE
12.	NIYISPLKSPYKISE	IYISPLKSPYKISEG
13.	IYISPLKSPYKISEG	YISPLKSPYKISEGL
14.	YISPLKSPYKISEGL	ISPLKSPYKISEGLP
15.	ISPLKSPYKISEGLP	SPLKSPYKISEGLPT
16.	SPLKSPYKISEGLPT	PLKSPYKISEGLPTP
17.	PLKSPYKISEGLPTP	LKSPYKISEGLPTPT
18.	LKSPYKISEGLPTPT	KSPYKISEGLPTPTK
19.	KSPYKISEGLPTPTK	SPYKISEGLPTPTKM
20.	SPYKISEGLPTPTKM	PYKISEGLPTPTKMT
21.	PYKISEGLPTPTKMT	YKISEGLPTPTKMTP
22.	YKISEGLPTPTKMTP	KISEGLPTPTKMTPR
23.	KISEGLPTPTKMTPR	ISEGLPTPTKMTPRS
24.	ISEGLPTPTKMTPRS	SEGLPTPTKMTPRSR
25.	SEGLPTPTKMTPRSR	EGLPTPTKMTPRSRI
26.	EGLPTPTKMTPRSRI	GLPTPTKMTPRSRIL
27.	GLPTPTKMTPRSRIL	LPTPTKMTPRSRILV
28.	LPTPTKMTPRSRILV	PTPTKMTPRSRILVS
29.	PTPTKMTPRSRILVS	TPTKMTPRSRILVSI
30.	TPTKMTPRSRILVSI	PTKMTPRSRILVSIG
31.	PTKMTPRSRILVSIG	TKMTPRSRILVSIGE
32.	TKMTPRSRILVSIGE	KMTPRSRILVSIGES
33.	KMTPRSRILVSIGES	MTPRSRILVSIGESF
34.	MTPRSRILVSIGESF	TPRSRILVSIGESFG
35.	TPRSRILVSIGESFG	PRSRILVSIGESFGT
36.	PRSRILVSIGESFGT	RSRILVSIGESFGTS
37.	RSRILVSIGESFGTS	SRILVSIGESFGTSE
38.	SRILVSIGESFGTSE	RILVSIGESFGTSEK
39.	RILVSIGESFGTSEK	ILVSIGESFGTSEKF
40.	ILVSIGESFGTSEKF	LVSIGESFGTSEKFQ
41.	LVSIGESFGTSEKFQ	VSIGESFGTSEKFQK
42.	VSIGESFGTSEKFQK	SIGESFGTSEKFQKI
43.	SIGESFGTSEKFQKI	IGESFGTSEKFQKIN
44.	IGESFGTSEKFQKIN	GESFGTSEKFQKINQ
45.	GESFGTSEKFQKINQ	ESFGTSEKFQKINQM

Peptide #	Human Peptide	Mouse Peptide
46.	ESFGTSEKFQKINQM	SFGTSEKFQKINQMV
47.	SFGTSEKFQKINQMV	FGTSEKFQKINQMVS
48.	FGTSEKFQKINQMVS	GTSEKFQKINQMVSN
49.	GTSEKFQKINQMVSN	TSEKFQKINQMVSNS
50.	TSEKFQKINQMVSNS	SEKFQKINQMVSNSD
51.	SEKFQKINQMVSNSD	EKFQKINQMVSNSDR
52.	EKFQKINQMVSNSDR	KFQKINQMVSNSDRV
53.	KFQKINQMVSNSDRV	FQKINQMVSNSDRVL
54.	FQKINQMVSNSDRVL	QKINQMVSNSDRVLK
55.	QKINQMVSNSDRVLK	KINQMVSNSDRVLKR
56.	KINQMVSNSDRVLKR	INQMVSNSDRVLKRS
57.	INQMVSNSDRVLKRS	NQMVSNSDRVLKRSA
58.	NQMVSNSDRVLKRSA	QMVSNSDRVLKRSAE
59.	QMVSNSDRVLKRSAE	MVSNSDRVLKRSAEG
60.	MVSNSDRVLKRSAEG	VSNSDRVLKRSAEGG
61.	VSNSDRVLKRSAEGS	SNSDRVLKRSAEGGN
62.	SNSDRVLKRSAEGSN	NSDRVLKRSAEGGNP
63.	NSDRVLKRSAEGSNP	SDRVLKRSAEGGNPP
64.	SDRVLKRSAEGSNPP	DRVLKRSAEGGNPPK
65.	DRVLKRSAEGSNPPK	RVLKRSAEGGNPPKP
66.	RVLKRSAEGSNPPKP	VLKRSAEGGNPPKPL
67.	VLKRSAEGSNPPKPL	LKRSAEGGNPPKPLK
68.	LKRSAEGSNPPKPLK	KRSAEGGNPPKPLKK
69.	KRSAEGSNPPKPLKK	RSAEGGNPPKPLKKL
70.	RSAEGSNPPKPLKKL	SAEGGNPPKPLKKLR
71.	SAEGSNPPKPLKKLR	AEGGNPPKPLKKLRF
72.	AEGSNPPKPLKKLRF	EGGNPPKPLKKLRFD
73.	EGSNPPKPLKKLRFD	GGNPPKPLKKLRFDI
74.	GSNPPKPLKKLRFDI	GNPPKPLKKLRFDIE
75.	SNPPKPLKKLRFDIE	NPPKPLKKLRFDIEG
76.	NPPKPLKKLRFDIEG	PPKPLKKLRFDIEGA
77.	PPKPLKKLRFDIEGS	PKPLKKLRFDIEGAD
78.	PKPLKKLRFDIEGSD	KPLKKLRFDIEGADE
79.	KPLKKLRFDIEGSDE	PLKKLRFDIEGADEA
80.	PLKKLRFDIEGSDEA	LKKLRFDIEGADEAD
81.	LKKLRFDIEGSDEAD	KKLRFDIEGADEADG
82.	KKLRFDIEGSDEADG	KLRFDIEGADEADGS
83.	KLRFDIEGSDEADGS	LRFDIEGADEADGSK
84.	LRFDIEGSDEADGSK	RFDIEGADEADGSKH
85.	RFDIEGSDEADGSKH	FDIEGADEADGSKHL
86.	FDIEGSDEADGSKHL	DIEGADEADGSKHLP
87.	DIEGSDEADGSKHLP	IEGADEADGSKHLPA
88.	IEGSDEADGSKHLPG	EGADEADGSKHLPAE
89.	EGSDEADGSKHLPGE	GADEADGSKHLPAES
90.	GSDEADGSKHLPGES	ADEADGSKHLPAESK
91.	SDEADGSKHLPGESK	DEADGSKHLPAESKF
92.	DEADGSKHLPGESKF	EADGSKHLPAESKFQ
93.	EADGSKHLPGESKFQ	ADGSKHLPAESKFQQ
94.	ADGSKHLPGESKFQQ	DGSKHLPAESKFQQK

Peptide #	Human Peptide	Mouse Peptide
95.	DGSKHLPGESKFQQK	GSKHLPAESKFQQKL
96.	GSKHLPGESKFQQKL	SKHLPAESKFQQKLA
97.	SKHLPGESKFQQKLA	KHLPAESKFQQKLAE
98.	KHLPGESKFQQKLAE	HLPAESKFQQKLAEM
99.	HLPGESKFQQKLAEM	LPAESKFQQKLAEMT
100.	LPGESKFQQKLAEMT	PAESKFQQKLAEMTS
101.	PGESKFQQKLAEMTS	AESKFQQKLAEMTST
102.	GESKFQQKLAEMTST	ESKFQQKLAEMTSTR
103.	ESKFQQKLAEMTSTR	SKFQQKLAEMTSTRT
104.	SKFQQKLAEMTSTRT	KFQQKLAEMTSTRTR
105.	KFQQKLAEMTSTRTR	FQQKLAEMTSTRTRM
106.	FQQKLAEMTSTRTRM	QQKLAEMTSTRTRMQ
107.	QQKLAEMTSTRTRMQ	QKLAEMTSTRTRMQK
108.	QKLAEMTSTRTRMQK	KLAEMTSTRTRMQKQ
109.	KLAEMTSTRTRMQKQ	LAEMTSTRTRMQKQR
110.	LAEMTSTRTRMQKQK	AEMTSTRTRMQKQRM
111.	AEMTSTRTRMQKQKM	EMTSTRTRMQKQRMN
112.	EMTSTRTRMQKQKMN	MTSTRTRMQKQRMNE
113.	MTSTRTRMQKQKMND	TSTRTRMQKQRMNES
114.	TSTRTRMQKQKMNDS	STRTRMQKQRMNESK
115.	STRTRMQKQKMNDSM	TRTRMQKQRMNESKD
116.	TRTRMQKQKMNDSMD	RTRMQKQRMNESKDV
117.	RTRMQKQKMNDSMDT	TRMQKQRMNESKDVS
118.	TRMQKQKMNDSMDTS	RMQKQRMNESKDVSN
119.	RMQKQKMNDSMDTSN	MQKQRMNESKDVSNK
120.	MQKQKMNDSMDTSNK	QKQRMNESKDVSNKE
121.	QKQKMNDSMDTSNKE	KQRMNESKDVSNKEE
122.	KQKMNDSMDTSNKEE	QRMNESKDVSNKEEK
123.	QKMNDSMDTSNKEEK	

2.7   Cell Culture

C33A human cervical carcinoma cells were obtained from the American Type Culture Collection (ATCC). Cells were thawed from stocks stored at -150°C and plated onto 10cm plates cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with penicillin (100U/mL), L-glutamate (2 mM), streptomycin (50 µg/mL) and 10% fetal bovine serum (FBS). Cells were grown in a humidified incubator at 37°C with 5% CO2.

2.8   Recombinant Protein Purification

Recombinant GST Protein purification was performed as previously described(Cecchini and Dick, 2011). Recombinant Proteins used for downstream applications were generated using the following protocol. Plasmids encoding the desired recombinant protein were transformed into competent BL21 Gold *E.coli* cells and plated onto 10 cm dishes containing LB growth media with agar and supplemented with ampicillin (100 µg/mL) and incubated for 16hrs at 37°C. Single colonies were isolated and grown in 25 mL of LB media supplemented with 100 µg/mL ampicillin and grown for 16 hrs at 37°C shaking at 200 RPM in a C-24 New Brunswick Orbital Shaker. Cultures were transferred to 1 L of LB media supplemented with 100 µg/mL ampicillin and grown for 4hrs at 25°C shaking at 200 RPM. Cultures were then induced with 100 µM IPTG and were transferred to a 16°C incubator and left shaking at 200 RPM for 16 hrs in a G-25 New Brunswick Shaker. Cell cultures were then centrifuged at $1660 \times g$ for 45 min at 4°C to pellet cells. Cell pellets were then either stored at -80°C or purification was performed immediately.

Cell pellets (either fresh or frozen) were washed with 1X Phosphate Buffererd Saline (PBS) and then suspended in GST Lysis Buffer (20 mM Tris pH 7.5, 0.5 M NaCl, 0.5 mM EDTA, 10% Glycerol, 0.5% NP-40, 20 mM DTT and 0.25 mM PMSF). Suspended cell pellets were subsequently sonicated at amplitude 3 with a 20 sec pulse 3 times. Cellular debris was then pelleted by centrifugation at $1660 \times g$ for 30 mins at 4°C and supernatant was isolated. The supernatant was then combined with 50% slurry of glutathione-sepharose beads (GE healthcare) and then rocked for 1hr at 4°C. Glutathione beads were subsequently centrifuged at $106 \times g$, 4°C for 2 mins with the resulting supernatant removed and then washed 3 times with High Salt GST Lysis Buffer (20 mM

Tris pH 7.5, 1 M NaCl, 0.5 mM EDTA, 10% Glycerol, 1% NP-40, 20 mM DTT and 0.25 mM PMSF). The beads were then washed 5 times with lysis buffer and finally washed once with KCL Buffer (20 mM Tris pH 7.5, 0.1 M KCl, 10% Glycerol, 0.02% NP-40, 20 mM DTT and 0.25 mM PMSF. GST tagged recombinant proteins were then eluted from Glutathione beads with the addition of 500 µL of GST Elution Buffer (50 mM Tris pH 8.0, 0.1M KCl, 0.1 mM EDTA, 10% Glycerol, 0.02% NP-40, 20 mM DTT, 0.25 mM PMSF and 200 mM Glutathione) for 1 hr rocking at 4°C. Beads were then centrifuged at $106 \times g$ for 5 mins at 4°C and supernatant was dialyzed against 1000x excess KCL buffer at 4°C for 16 hrs. Protein samples were then aliquoted, snap frozen and stored at -80°C until required for use.

2.9   GST Pulldown Binding Experiments

Recombinantly produced proteins were used for these assays and this procedure is detailed above in 2.8. This procedure was carried out as previously reported (Dick et al., 2000). In general 20 µL of Glutathione-sepharose beads were washed with 400 µL of Low Salt GSE Buffer (20 mM Tris pH 7.5, 200 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT and 0.1% NP-40) twice before being centrifuged at $106 \times g$, 4°C 3mins. The beads were then suspended in 400 µL Low Salt GSE Buffer and 200 µg of cell extract was added along with 3 µg of the desired GST tagged recombinant protein. This mix was then rocked for 1 hr at 4°C and subsequently washed with twice with Low Salt GSE Buffer. Beads were then resuspended 100 µL of 1X SDS-PAGE sample Buffer (62.5 mM Tris pH 6.8, 10% Glycerol, 2% SDS, 72.5 mM β-ME and Bromophenol Blue) and boiled for 5 mins at 95°C. Beads subsequently were centrifuged at $20800 \times g$ for 1 min and supernatant was loaded on SDS-PAGE gel of indicated percentage for optimal resolution for Western blotting and GST protein loading quantification.

2.10   Western Blotting and Immunoprecipitations

Western Blotting was used to determine protein abundance in samples and has been previously described (Cecchini and Dick, 2011). In general, protein samples were electrophoresed on 8% polyacrylamide gels unless otherwise stated. Gels were run in 1X SDS-PAGE running buffer (25 µM Tris Base, 200 µM Glycine and 0.1% SDS) at 150-

200 volts using a Biorad PowerPac HC 250 V to achieve proper resolution of desired protein targets. Proteins were then transferred onto a nitrocellulose membrane (GE Amersham Hybrid ECL) for 1hr on ice at 100 V in 1X Western Blot transfer buffer (48 µM Tris Base, 368 µM Glycine, 0.1% SDS and 20% Methanol). Membranes were then blocked in 1X TBST (20mM Tris pH7.5, 150 mM NaCl and 0.1% Tween-20) with 3% skim milk powder for 1hr rocking at room temperature. Membranes were then treated with primary antibody solution in 1x TBST with 3% skim milk powder at indicated working dilutions described below, overnight rocking at 4°C. Membranes were subsequently washed 3 times for 10 mins with 1X TBST and then treated with secondary antibodies of indicated species conjugated to HRP diluted 1:2000 in 1X TBST with 3% skim milk. Membranes were left shaking for 1hr at 25°C before being washed 3 times for 10 mins with 1X TBST. Membranes were incubated with ECL solution (Supersignal West Dura Extended Duration Substrate, Thermo Scientific) sufficient to properly cover membrane for 2 mins. Membranes were then developed using ChemiDoc apparatus (BioRad) with ImageLab software (BioRad) to achieve proper exposure for indicated proteins.

2.11   *In Vitro* Acetylation Assay

GST-pRBLP recombinant proteins were used for this assay and were produced as described above in **2.8**. This method was adapted from previously reported methods (Avvakumov et al., 2003; Kuninger et al., 2007). 3.5 µg of recombinant protein were added to Acetylation Buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.1% NP-40, 1 mM PMSF, 1 mM DTT), with 10 µM of acetyl-CoA and the volumes were made up to 20 µL with Acetylation buffer. Indicated reactions were then supplied 1 µg of recombinant pCAF HAT domain (Cayman Chemicals) to act as the acetyltransferase. Acetylation reactions were carried out for 10 mins at 30°C on a rotating platform. Following this, samples were snap frozen to be used in downstream assays or the reactions were stopped with the addition of SDS-PAGE sample buffer and electrophoresed on 8% polyacrylamide gels.

Table 2.4: Description of Antibodies

Antibody Name	Epitope	Species	Supplier / Source	Application
RB4.1	pRB	Mouse	Hybridoma	WB, IP
M153	pRB	Rabbit	Santa Cruz (sc-7905)	WB, IP
Ac-pRB	K873/K874 ac-pRB	Rabbit	Paul Stafford*	WB, IP
4G12	Ac-Lysine	Mouse	Millipore	WB
M136	pRB	Sheep	Dick Lab/Michael Thwaites	IP
07-631	CDK2	Rabbit	Upstate	IP
C15	pRB	Rabbit	Dick Lab	WB

2.12   *In Vitro* Kinase Assay

The CDK2 *in vitro* kinase assay was carried out according to a previously described method (Forristal et al., 2014). Nuclear extracts from proliferating C33A cells were collected as described above. 100 µg of extract was then added to 50 µL of Protein G dynabeads pre-bound with 4 µg of rabbit IgG or 4 µg of α-CDK2 for 1.5 hrs at 4°C. The beads were then washed twice in Kinase buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$ and 1 mM DTT). Washed beads were subsequently resuspended in 30 µL of Kinase buffer, 1 µL of γ-ATP^{32} (10 µCi), 2 µg of recombinant GST-pRBLP and the volume was made up to50 µL with Kinase buffer. The kinase reaction was carried out for 20 mins at 30°C and then stopped with the addition of 5x Laemmli SDS Page Buffer. Samples were then electrophoresed on 8% polyacrylamide gels and stained with Coomassie brilliant blue solution to resolve protein bands. The gels were then dried and imaged to allow for comparison of protein loading. The gel was then exposed to Amersham Hyperfilm MP (GE healthcare) for proper resolution of radiolabelled substrates and autoradiography was performed.

2.13   Immunopurified-Kinase Assay

Recombinant GST-pRB LP was prepared as discussed above in 2.8. Acetylated GST-pRB LP was prepared as described in 2.11. CDK2 was immunoprecipitated from proliferating C33A cells using 4 µg of α-CDK2 antibody (Upstate) or rabbit IgG that had been prebound by rocking with protein G dynabeads (Invitrogen) at 4°C for 1 hour. 6 µg of acetyl-K873/K874 antibody and M136 antibody which had been purified against the unmodified peptide discussed in 2.11**,** were prebound to protein G dynabeads for 1 hour rocking at 4°C. 5.5 µg of either modified or unmodified GST-pRB LP protein or GST was added to each IP for 1 hour rocking at 4°C. Each set of IPs was washed twice with Kinase buffer (50 mM Tris pH 7.5, 10 mM MgCl2 and 1 mM DTT). CDK2 IP beads were resuspended in 30 µL of Kinase buffer and combined with acetyl K873/K874 pRB or unmodified pRB IP beads which had been resuspended with 30 µL of Kinase buffer, with 12 µL added to the kinase reaction. 1 µL of γ-ATP^{32} (10 µCi) was added to each reaction and the final volume was brought up to 50 µL with the addition of Kinase buffer. Kinase reactions were then carried out as described in **2.9**. 12 µL of each IP acetyl-

K873/K874 pRB or unmodified pRB IP was set aside for Western blot analysis as described above.

2.14   mESC Cell Culture

Cell culture for mESCs was performed to standard protocols as previously described (Sicinski et al., 1995). mESCs were cultured in DMEM media with 4500 mg/L Glucose, 2 mM glutamine, 0.1 mM Non-Essential amino acids (Gibco #11140-050), 0.1 mM β-mercaptoethanol (Sigma M7522) diluted in PBS, Leukemia Inhibitory Factor (LIF, Millipore, ESG1106) 10^5 Units/L media, 50 µg/mL G418 (Sigma SLBB2604) and 15% Fetal Bovine Serum (FBS). mESCs were cultured on plates coated with 0.1% gelatin solution (Swine skin type II, Sigma G2500) with mitomycin c treated MEFs which were plated the night before and left at 37°C to adhere to the gelatin coated plate. The mESCs were grown in a humidified incubator at 37°C with 5% CO_2 and sub-cultured until optimal density was reached on a 10 cm dish. Cells were trypsinized with 0.25% Trypsin/0.04% EDTA (GIBCO) with media being changed 1 hour prior to treatment with trypsin.

2.15   Southern Blot Screening

The targeting construct was linearized by a NotI digestion, and provided to the London Regional Transgenic and Gene Targeting facility where mouse embryonic stem cells (mESCs) were electroporated and grown in neomycin selection medium. Southern blot screening of mESCs was carried out as previously reported (Cecchini et al., 2014; Isaac et al., 2006). Electroporated mESCs were selected and underwent clonal expansion onto 24 well culture plates. Cells were lysed in Tail Lysis Buffer (50 mM Tris pH 8.0, 100 mM NaCl, 0.1% SDS, 0.1 mM EDTA) with 200 µg/µL of Prot K overnight at 37°C. Genomic DNA was then isolated through phenol chloroform extraction and quantified using a nanodrop. 10 µg of DNA was digested overnight at 37°C with 20 units of the desired restriction enzyme. The next morning, restriction digests were spiked with 20 units of restriction enzyme and left to digest at 37°C for 8 hours. Digested genomic DNA was then electrophoresed in a 0.8% agarose gel without Ethidium bromide (EtBr) overnight at 20V. The agarose gel was then stained in a TAE (40 mM Tris acetate, 1mM

EDTA, pH 8.2) with Ethidium Bromide and visualized to determine migration distances of the ladder and presence of digested DNA in the lanes. The gel was then washed 2 times for 30 minutes each shaking at room temperature in Wash Buffer 1 (1.5M NaCl, 0.5M NaOH). Following this the gel was washed for 5 minutes in Milli-Q water and then the gel was washed 2 times for 30 minutes each shaking at room temperature in Wash Buffer 2 (1 M Ammonium acetate, 20 mM NaOH). The gel was then placed in a Stratagene Posiblot apparatus and the transfer was carried out overnight at room temperature onto a nitrocellulose membrane (GE NitroPure 45 micron nitrocellulose membrane). The membrane was then dried at room temperature for 1 hour and then baked at 80°C for 1 hour. The membrane was then rehydrated with Hybridization Buffer (0.2% SDS, 25 mM Na_3PO_4, 0.1% Sodium pyrophosphate, 4 mM EDTA, 8 mM Tris Base, 600 mM NaCl, 0.2% Ficoll 400, 0.2% polyvinylpyrrolidone , 0.2% Bovine serum albumin), placed in a glass cylinder and rotated at 65°C for 2 hours. During this time, the indicated probe was labelled and quantified as discussed below in 2.15.1. The hybridization buffer then was changed and fresh buffer was added with 1,000,000 CPM of ^{32}P-α dCTP labelled probe per mL of hybridization buffer. The membrane was left to incubate with probe while rotating overnight at 65°C. The next day the membrane was washed 3 times for 10 minutes at room temperature with Low Stringency Buffer (0.1% SDS, 300 mM NaCl, 30 mM Sodium Citrate pH 7.0). The membrane then was washed 3 times for 10 minutes each at 65°C with High Stringency Buffer (0.1% SDS, 30 mM NaCl, 3 mM Sodium Citrate pH 7.0). The membrane then was exposed to Amersham hyperfilm (GE Healthcare) for autoradiography.

2.15.1   Southern Blot: Labeling and Purifying the Probe

Reactions are scaled for 25 ng of extracted probe DNA but can be scaled up as needed.

Labeling Reaction**:** Using Agilent Prime-It II Random Primer Labeling Kit (Agilent, 300385) contents

25 ng of DNA

10 μL Random Primers

Add H_2O up to 34 µL

5 min @ 95°C

Then Add:

10 µL 5x dCTP buffer

5 µL of ^{32}P-α dCTP

1 µL Klenow

 Place in 37°C water bath for 30 mins

Purifying the Probe:

Fill a 3 mL syringe with glass wool to 1 mL mark, being careful not to tightly pack the wool

Add Sephadex G-50 Beads suspended in TE buffer to the top of the glass wool to 1.5 mL mark, making sure beads are even over the wool

Centrifuged column at $210 \times g$ for 1 minute and check levels and quality of sephadex layer. Add more and centrifuge as needed.

Add labeled probe to column with 100 µL volume max/column, centrifuge ($210 \times g$, 1 min) syringe in a conical tube and collect flow through which is labeled probe.

Add 10 µg sheared of salmon sperm DNA per 100 µL of flow through

Heat for 5 mins at 95°C, quantify activity of the probe by liquid scintillation.

2.16   References

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Chapter 3

3 Study of pRB K873/K874 Acetylation

3.1 Analysis of pRB Antibody Specificity in the pRB C-Terminus

To study post-translational modifications of the pRB C-terminus, a comprehensive understanding of epitope locations for antibodies that will be used for Western blotting and immunoprecipitations (IPs) is critical for interpreting these results. As mentioned above in the introduction, a specific portion of the pRB C-terminus is of interest to my research due to the presence of the PP1 and CDK docking site, and the presence of the K873/K874 residues that have been demonstrated to be acetylated. To study this region of pRB, we mapped epitopes for the antibodies that we commonly use. The antibodies that I examined for analysis were the following; mouse monoclonal hybridoma RB4.1, rabbit polyclonal pRB antibody M153 from Santa Cruz, pRB rabbit antibody C15 generated in our lab against the final 15 amino acids of the human pRB C-terminus and a sheep pRB antibody M136 generated against the 136 amino acids of the mouse pRB C-terminus.

To map epitopes of the various pRB antibodies available, I performed a peptide SPOT assay. This consisted of an array of 122 or 123 peptides of the mouse and human pRB C-terminal regions respectively. Each peptide is 15 amino acids long and each had a one amino acid difference from its neighbour providing full coverage of all the 15 amino acid peptides possible from the C-terminus of pRB. With these peptides spanning the entire C-terminus, I incubated the membrane with each of the indicated antibodies and visualized the membranes using standard western blotting procedures. From this analysis for each respective antibody, specific epitopes or regions of preferred binding were observed for each antibody. For RB4.1, I observed from the spot pattern depicted in Fig 3.1A that the epitope for this antibody corresponds to amino acids 822-836 in the human sequence and 815-830 in the mouse sequence, which is illustrated in Fig 3.2A. For the pRB C15 antibody, I observed from the peptide array in Fig 3.1B that binding could be attributed to the C-terminus encompassing amino acids 908 to 928 in the human

sequence. It was also observed that it was bound to one mouse peptide as shown in Fig. 3.1B, with this peptide corresponding to amino acids 906-920 in the mouse pRB C-terminus. Presented in Fig 3.2B is a schematic detailing C15 binding to human pRB C-terminus corresponding to residues 908-928 and binding in the mouse pRB C-terminus, corresponding to residues 906-920. Both polyclonal antibodies exhibited widespread binding throughout the C-terminus but did have regions of preference. From analysis of the array presented in Fig 3.1C for M153, we observed peptides that were recognized specifically. Detailed in Fig 3.2C in a diagram of the pRB C-terminus is the regions of binding I observed for M153. While it is important to note that binding was observed in regions encompassing the PP1-CDK docking sites as well as acetylation sites K873/K874, many other prominent epitopes existed in the C-terminus including strong epitopes in the early C-terminus. Through analysis of the array presented in Fig 3.1D for M136, we again observed that there was a wide swath of binding throughout the C-terminus of pRB with epitopes encompassing the PP1-CDK docking site and K873/K874 acetylation sites. The epitopes for M136 were more concentrated on this region and displayed minimal binding to the early portion of the pRB C-terminus.

3.2   Generation and Characterization of Anti-Acetyl K873/K874 pRB Antibodies

As described in my introduction, the pRB protein has been shown to be acetylated in the C-terminus on lysine residues 873 and 874 (Chan et al., 2001). This acetylation has been shown to occur within a region of pRB that contains binding motifs for Cyclin-CDK complexes as well as PP1 (Hirschi et al., 2010), thus the potential exists that these modifications may act in a regulatory manner to control CDK phosphorylation or PP1 dephosphorylation of pRB. Though these post-translational modifications of pRB present a unique and exciting means of regulation of pRB, no commercially available antibodies exist for any combination of K873/K874 acetylation. Therefore, to further characterize pRB K873/K874 acetylation and the potential regulatory nature of these post-translational modifications, we set out to create an antibody that was specific for these
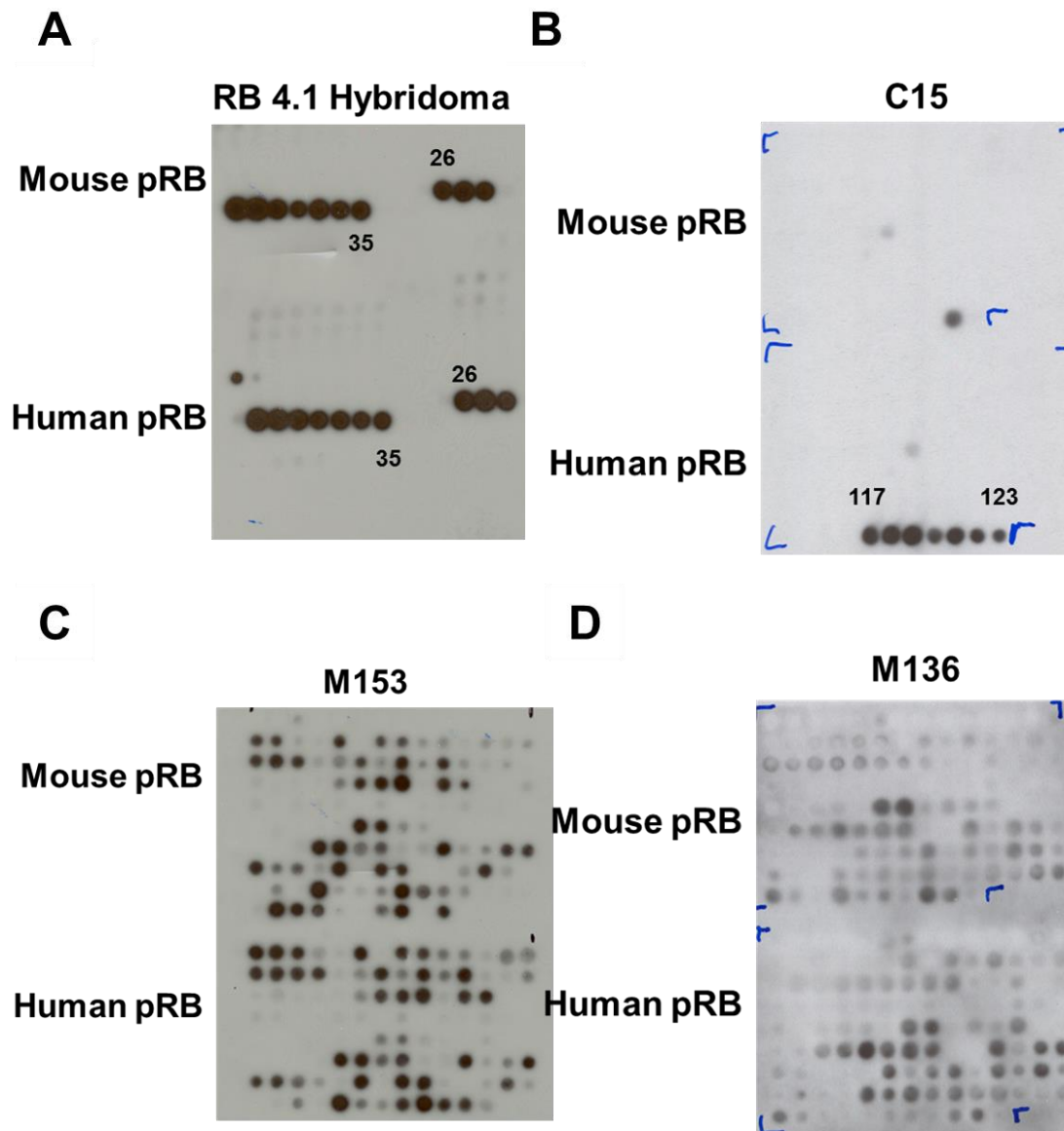
Figure 3.1: Characterization of pRB Antibodies

A) Peptide spot membrane of human and mouse pRB assayed for mouse derived monoclonal antibody RB4.1 epitopes. Peptides which demonstrated reactivity are indicated by their peptide number. **B)** Peptide spot membrane of human and mouse pRB probed with a rabbit antibody generated in our lab against the 15 C-terminal amino acids of human pRB. **C)** Peptide spot membrane of human and mouse pRB for rabbit derived polyclonal antibody M153 from Santa Cruz. **D)** Peptide spot membrane of human and mouse pRB probed with a goat antibody generated in our lab against the C-terminal 136 amino acids of mouse pRB. N=1 for all peptide arrays
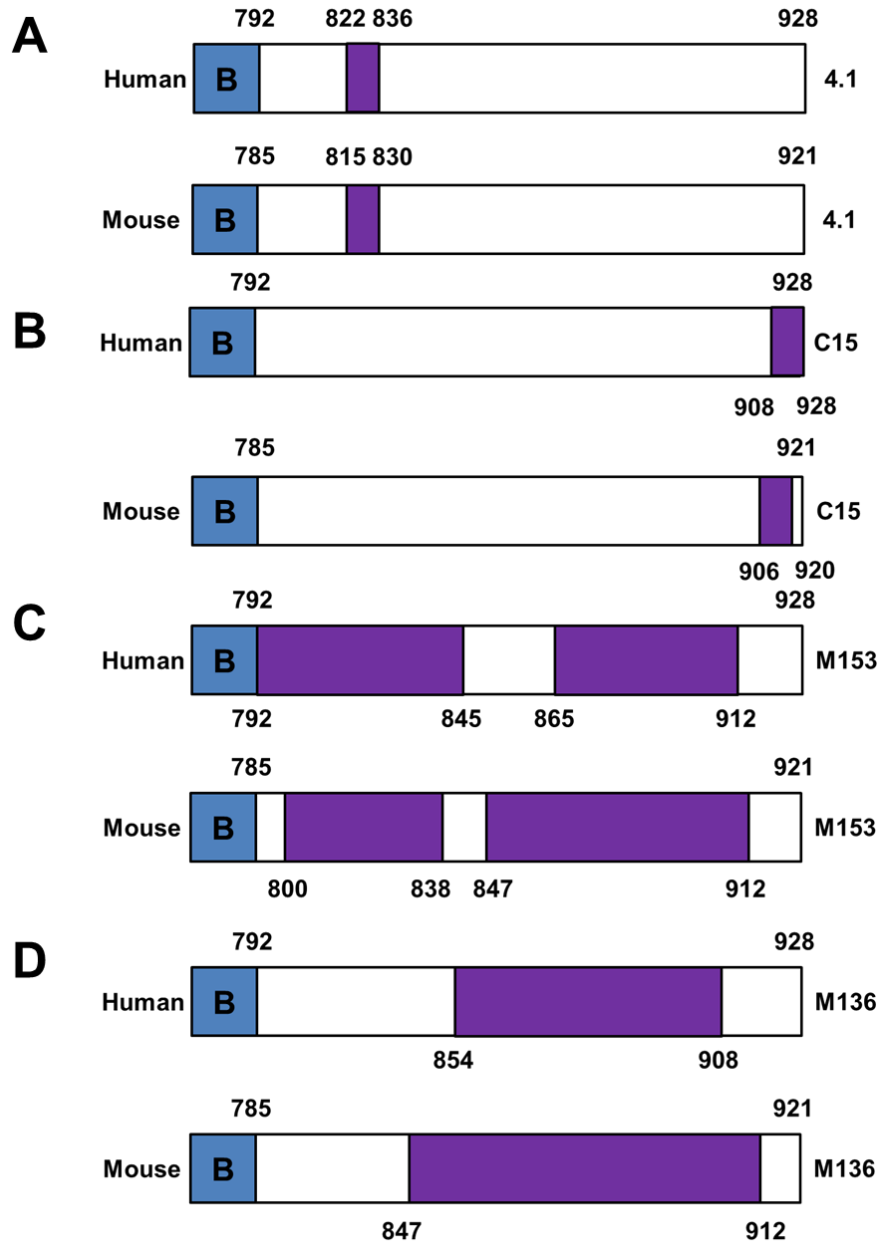
Figure 3.2: Schematic of pRB Antibody Epitopes on Human and Mouse pRB

A) Schematic detailing the regions in the pRB C-terminus that demonstrated epitopes for mouse hybridoma antibody RB4.1. **B)** Schematic detailing the epitope in the pRB human C-terminus for rabbit pRB antibody C15. **C)** Schematic detailing the epitopes in the pRB C-terminus for rabbit polyclonal antibody M153 from Santa Cruz. **D)** Schematic detailing the epitopes in the pRB C-terminus for sheep polyclonal antibody M136 developed in the Dick Lab

modifications. This process is outlined in the illustration presented in Figure 3.3A. Covance Research Products Inc. was contracted to make an antibody that was able to specifically detect acetyl-K873/K874. Two rabbits were immunized with a 15 amino acid acetylated peptide that surrounded K873/K874 (NPPKPL-Ac-K-Ac-K-LRFDIEG) following a standard 118 day protocol. The animals were subjected to a 3 week cycle of antigen boosts and test bleeds were taken 10 days following the boosts. From the initial test bleeds, Enzyme linked immunosorbent assays (ELISA) was carried out to test serum for antibody production and response against both a synthetically modified peptide versus an unmodified peptide, which are depicted in Figure 3.3B. Presented in Figure 3.4A is quantification of these experiments, where we can see that serum was able to elicit a significant response against both acetylated and unmodified peptide when compared to the negative control through the use of statistical analysis at the half maximal absorbance dilution. Through statistical analysis, no significant difference was observed for the unmodified versus the acetylated peptide. With this result, this encouraged us to continue production of serum and to begin purification and characterization of the antibody. For purification of acetyl K873/K874 pRB antibodies we used a two-step protocol of immunoaffinity column chromatography. Serum was first passed through a column that contained acetyl K873/K874 coupled peptides and I eluted under low pH conditions to isolate antibodies. To eliminate antibodies that detected non-acetylated or pRB, the elution was passed through a second column that contained unmodified coupled peptide (NPPKPL-K-K-LRFDIEG) and unbound flow through was collected for downstream use. An ELISA was then carried out to test specificity of purified antibodies towards acetylated peptide versus unmodified peptide. From this analysis, presented in Figure 3.4B, the purified antibody was able to recognize the acetylated peptide with at least 100 times higher affinity when compared to the unmodified peptide. Statistical analysis using a T-test confirmed that this difference was significant. This result suggested to us that the purified antibody was responsive to our antigen with a high order of specificity and prompted us to further examine and characterize this antibody in regards to detection of pRB K873/K874 acetylation.
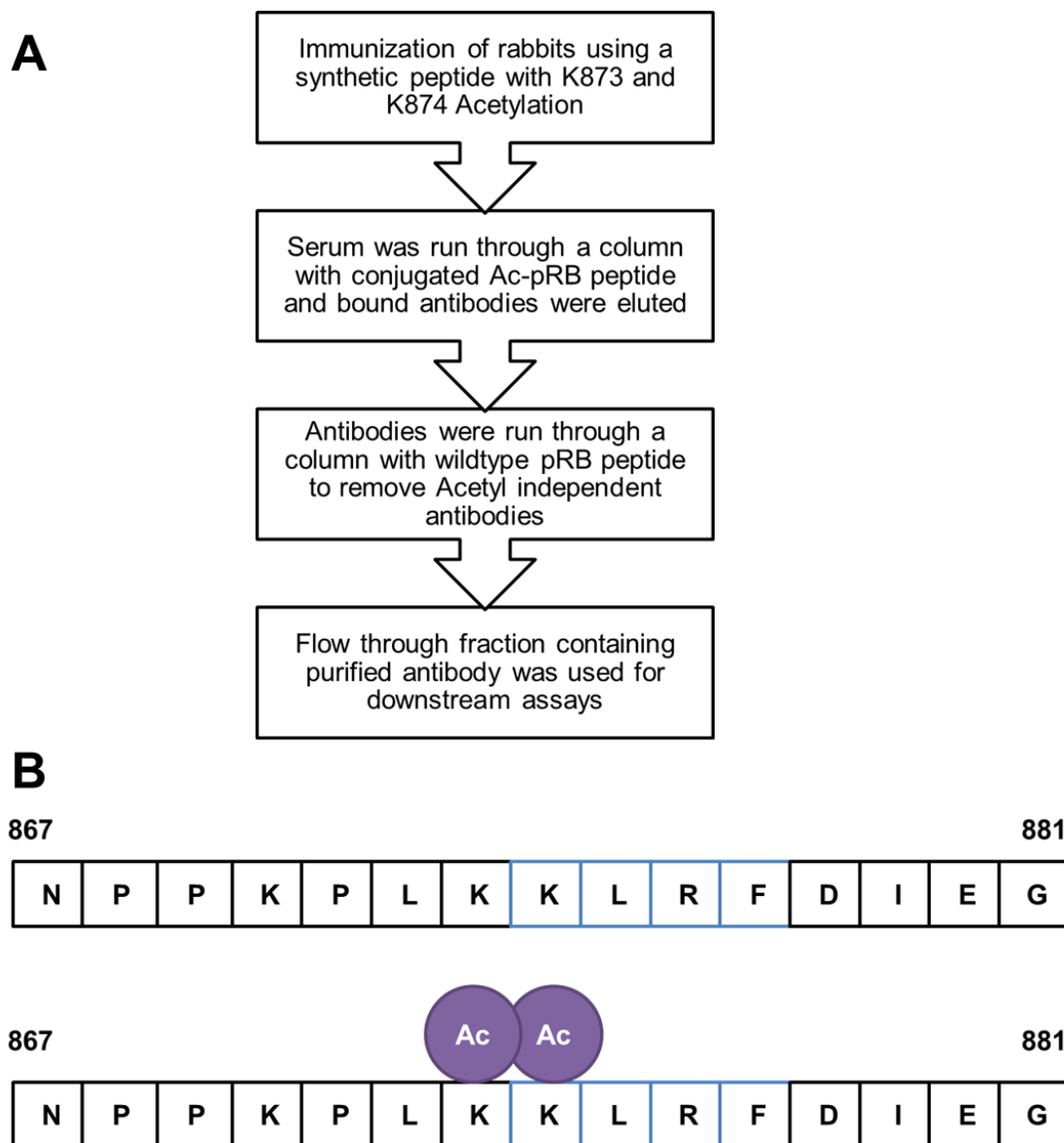
Figure 3.3: Generation of an Antibody Against Acetylated pRB

A) Schematic diagram for the generation and purification of anti-acetyl K873/K874 pRB antibodies. Serum was also used for indicated experiments where purification steps were bypassed. **B)** Schematic of the two synthetic peptides used for antibody generation and purification with mouse pRB sequence and residue numbers indicated. Synthetic acetylation's on residues K873/K874 are indicated in purple. Residues highlighted in blue are critical in the Protein phosphatase 1 docking site of pRB.

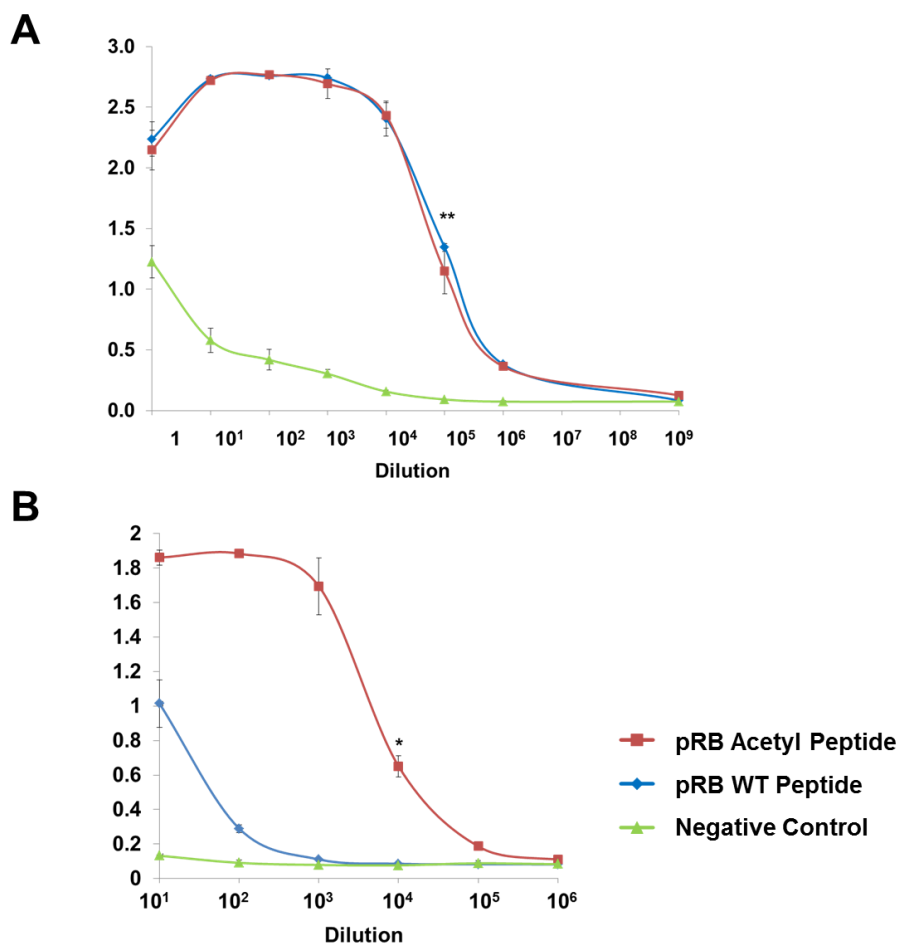**Figure 3.4: ELISA Analysis Demonstrating Specificity of Anti-Acetyl K873/K874 pRB Antibody**

ELISA experiments were carried out to analyze specificity of anti-acetyl K873/K874 pRB antibodies. Experiments were performed in triplicate on the same plate. **A)** Serum was diluted and added to wells coated with the indicated peptides. Following addition of Alkaline phosphatase secondary antibody and substrate, absorbance values were plotted against serum dilution for each respective peptide or negative control. ** $P<0.05$ for acetyl and wt peptides to negative control at half maximal absorbance dilution using a T-test. **B)** Antibodies against acetyl K873/K873 pRB were and added to wells coated with the indicated peptide or TBS as a negative control. Absorbance was determined for each peptide and plotted against the indicated dilution. * $P<0.05$ for acetyl versus wt peptide at half maximal absorbance dilution using a T-test. Error bars were plotted as standard deviations.

3.3   Acetyl K873/K874 pRB Antibody Specifically Detects Acetylated Recombinant pRB

Our initial characterization of the acetyl K873/K874 pRB antibody suggested specificity towards acetylated peptide when compared to unmodified peptide, we next wanted to examine more stringent conditions to further asses the specificity of our antibody. I set out to carry out *in vitro* acetylation assays and assess specificity by the use of western blotting. To begin these characterizations, I first set up *in vitro* acetylation assays using recombinant GST-pRB LP as substrate of the Histone Acetyl Transferase (HAT) domain of pCAF, which has been characterized in the past to acetylate pRB(Pickard et al., 2010). These assays were carried out with either recombinant GST-pRB LP or non-specific substrates GST or BSA. From the western blot presented in Figure 3.5A, we can see that when probed with acetyl K873/K874 pRB antibody, we see a strong signal for acetylated GST-pRB LP compared to GST-pRB LP lacking pCAF enzyme, as well as GST or BSA. When this blot was probed with an acetyl-Lysine antibody as observed in Figure 3.5B, we can see a specific signal for GST-pRB LP in the presence of pCAF. From these observations, we suggest that our acetyl K873/K874 pRB antibody is able to specifically recognize acetylated GST-pRB LP using *in vitro* acetylation. To further test the specificity of our acetyl K873/K874 pRB antibody, specifically towards acetylation of residues K873/K874 versus other potential acetylation sites on pRB a mutant of pRB was tested in which K873 and K874 were changed to alanine. When *in vitro* acetylation assays were carried out over a time course using this mutant of GST-pRB LP as shown in Figure 3.6, and then probed with acetyl-K873/K874 pRB antibody, we observed that wildtype GST-pRB LP shows a specific signal when compared to both unmodified wildtype GST-pRB LP as well as GST-pRB LP K873/K874A. When probed with an acetyl-lysine antibody, no discernible signal could be observed for any of the reaction conditions. Furthermore, when a Coomassie loading control was examined for the indicated reactions, it was observed that similar loading was achieved for the three different GST-pRB LP reaction groups, and although no signal was seen with the acetyl-lysine antibody western blot, the positive signal seen from the acetyl K873/K874 pRB blot suggests that the experimental conditions were successful. Thus it can be concluded that pRB acetylation on K873/K874 can be specifically detected by our acetyl-
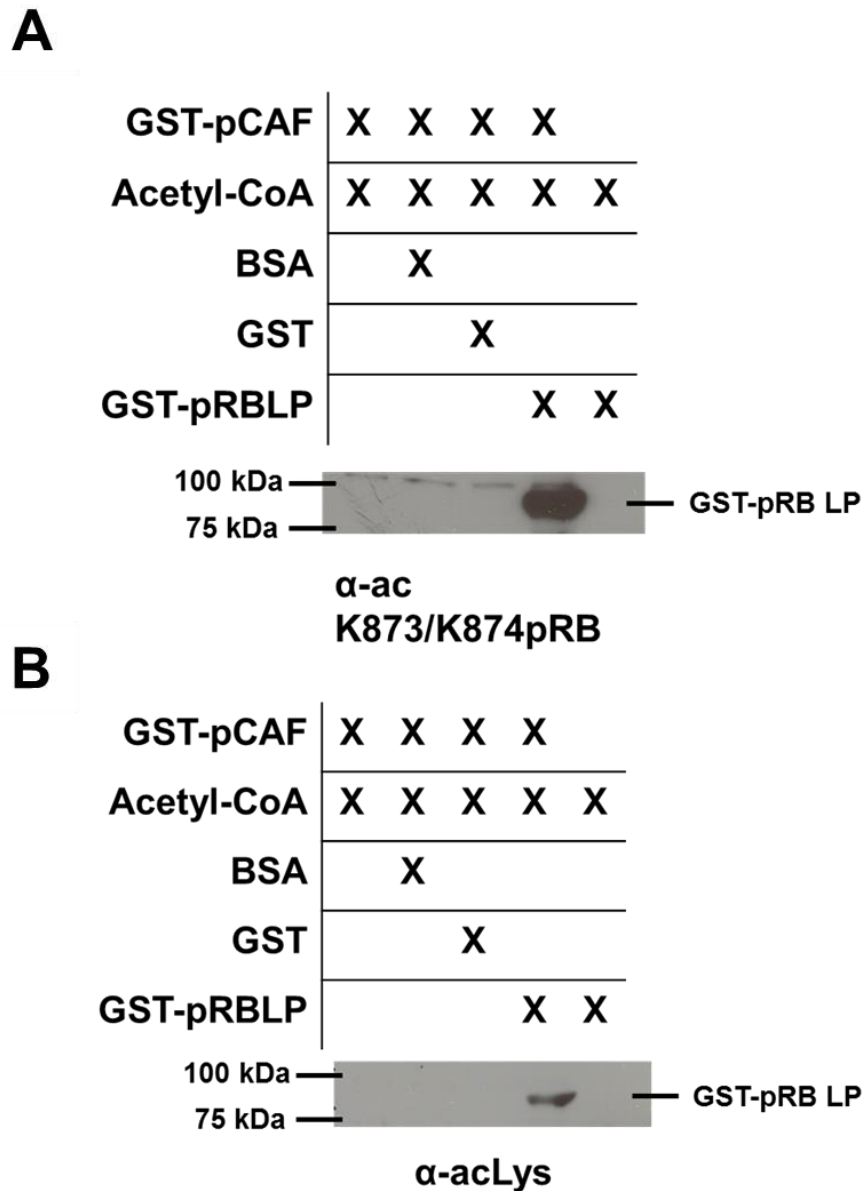
Figure 3.5: Anti-Acetyl-pRB Antibody Specifically Detects Modified GST-pRB LP

3 μg of recombinant GST-pRB LP (aa 379-928) was used as a substrate in an *in vitro* acetylation assay with recombinant pCAF. BSA and GST were used as non-specific substrates and reactions without pCAF were used to gauge specificity towards modified substrate. Following 30 min of incubation at 37°C, samples were analyzed by SDS-PAGE and western blotting. **A)** Blots as probed with anti-acetyl K873/K874 pRB antibody while in panel **B)**, samples were probed with an acetyl-lysine antibody. N=1 for both blots. N=1 for both blots presented in this figure.

	0 Mins			10 Mins			20 Mins			
pCAF	X	X		X	X	X		X	X	X		X
Acetyl-CoA	X	X	X	X	X	X	X	X	X	X	X	X
GST	X				X				X			
GST-pRBLP		X	X			X	X			X	X	
GST-pRBLP K873/874A				X				X				X

α-acetyl K873/K874 pRB

α-ac Lys

Coomassie

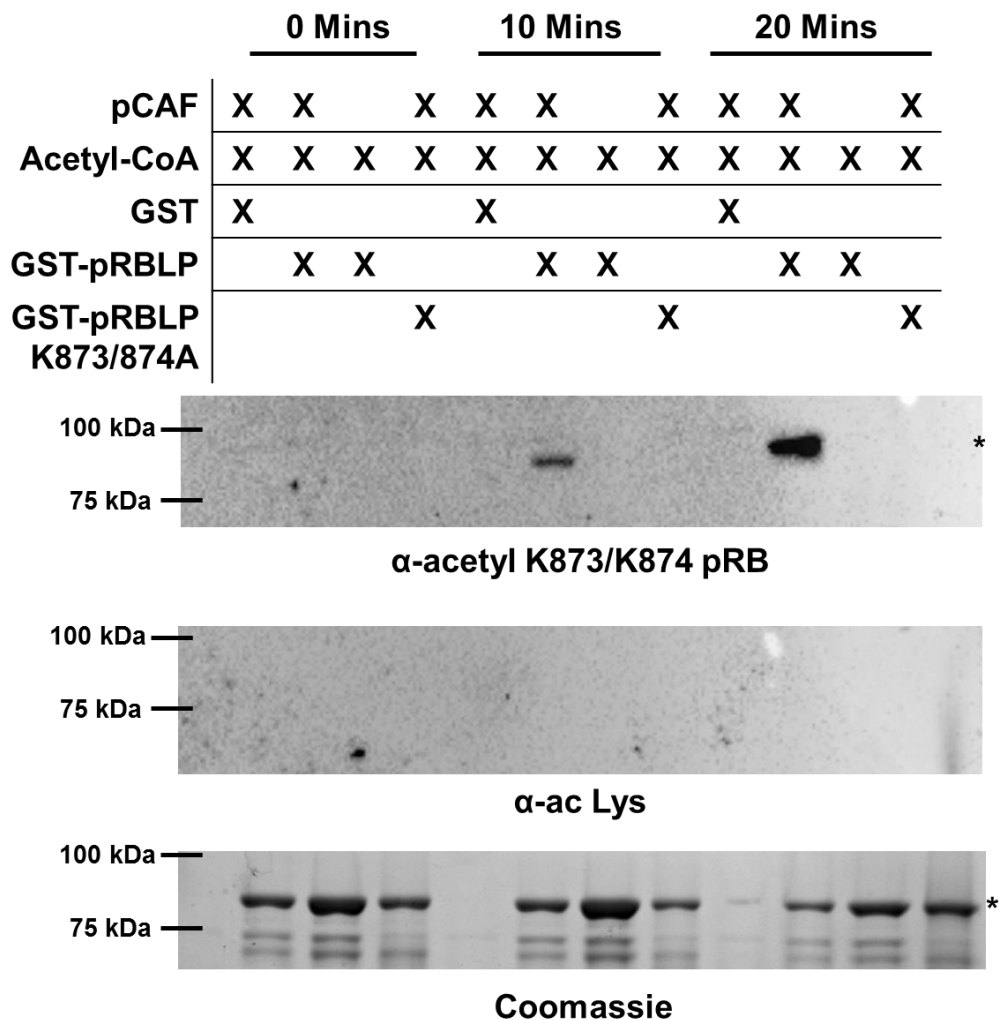**Figure 3.6: Anti-Acetyl K873/K874 pRB Antibody Specifically Detects Modified GST-pRB LP**

3 μg of recombinant GST-pRB LP or K873/K874A GST-pRB LP were used for *in vitro* acetylation by recombinant pCAF. GST was used as non-specific substrate and reactions without pCAF were used to gauge specificity of antibodies towards modified substrate. Samples were incubated for the above indicated times and then subjected to analysis by SDS-PAGE and western blotting. The top image is of a western blot using anti-acetyl K873/K874 pRB antibody, with the lower image showing Coomassie staining of GST-pRB LP protein loading for each respective sample. The middle image is a western blot using anti-acetyl-lysine-antibody. Bands indicated with an * correspond to GST-pRB LP. N=1 for both blots and Coomassie gel presented in this figure.

K873/K874 antibody and that this antibody demonstrates sufficient specificity with regards to the *in vitro* assays carried out to undertake further analysis of pRB acetylation using this reagent.

3.4 CDK Phosphorylation of pRB May Be Affected by pRB K873/K874 Acetylation

To further characterize pRB acetylation on lysine residues 873 and 874, I set out to examine CDK kinase activity towards pRB which had been acetylated on these residues through *in vitro* assays. Previous work suggests that acetylation of pRB at these residues disrupts CDK phosphorylation of pRB, though many of these studies are based on molecular modeling of the pRB C-terminus using X-ray crystallography structures (Chan et al., 2001; Lowe et al., 2002; Wallace and Ball, 2004). Thus to assess CDK phosphorylation of pRB, I carried out an *in vitro* kinase assay using CDK2. CDK2 was immunoprecipitated from proliferating C33A cells, which are pRB null, and thus will not offer endogenous pRB to confound our interpretation. Recombinant GST-pRB LP that was either modified through the use of an *in vitro* acetylation assay or unmodified acted as substrate for immunoprecipitated CDK2. As presented in Figure 3.7, we can see that the ability of pRB to act as a CDK2 substrate seemed to be unaffected by the presence of K873/K874 acetylation compared to unmodified pRB when compared to levels of recombinant GST-pRB LP present in each lane. From these observations, we questioned whether the population of GST-pRB LP that was acetylated was substantial enough to mask the effects of non-acetylated pRB, due to the high sensitivity of detection of radiolabelled substrate. With this possible caveat, I then set out to further test this using a more selective approach. To carry out this more selective approach, I established an immune-purification approach for isolating K873/K874 acetylated GST-pRB LP from the population, the outline for this experiment is detailed in Figure 3.8. For this protocol, I used either the K873/K873 acetyl pRB antibody or M136 antibody, which had been purified against the unmodified peptide of the K873/K874 antibody. This allowed for us to normalize for epitope efficiency in regards to immunoprecipitation between the two respective antibodies. Following immunoprecipitation of the indicated substrates, CDK2 kinase assays were performed to compare the incorporation of γ-^{32}P-ATP between

observed that the band intensity for incorporation of γ-^{32}P-ATP for the unmodified GST-purified K873/K874 acetylated GST-pRB LP and unmodified GST-pRB LP. From observation of Fig. 3.9A, we noticed that the acetylated K873/K874 GST-pRB LP that was immunopurified exhibited less incorporation of γ-^{32}P-ATP compared to unmodified wildtype GST-pRB LP. When densitometry was performed on the autoradiogram, I observed that the band intensity for incorporation of γ-^{32}P-ATP for the unmodified GST-pRB LP was reduced compared to the acetylated GST-pRB LP. When the loading of the samples was analyzed using Western blot analysis with the RB4.1 antibody, we can see from Fig. 3.9B that both antibodies were able to pull down similar levels of either acetyl K873/K874 pRB or unmodified pRB, suggesting the differences in CDK2 phosphorylation we observed could be attributed to the acetylation of K873 and K874. From these experiments, we have evidence to suggest that acetylation of these two indicated lysine residues in pRB may in fact regulate the ability of pRB to serve as a CDK2 substrate *in vitro*.

3.5   Acetylation of pRB at K873 and K874 is Unable to be Detected *In Vivo*

The *in vitro* results presented earlier in this chapter suggested that the antibody that we had developed for acetyl K873/K874 pRB was a highly specific reagent that could be used to examine the occurrence of these post-translational modifications in cells. To determine whether pRB could be acetylated on K873/K874 in cells that were asynchronously proliferating, I carried out immunoprecipitations for pRB from two different cell types and performed western blot analysis on IP fractions using acetyl K873/K874 pRB antibody. As demonstrated in Figure 3.10B, when probed with our antibody we were unable to detect the presence of acetyl K873/K874 pRB. This is in accordance with Western blot analysis presented in Figure 3.10A, which demonstrated the IP efficiency for pRB. Furthermore both blots were run with a positive standard, which was GST-pRB LP which had been acetylated using an *in vitro* acetylation assay. The positive signal from this standard as shown in Figure 3.10B demonstrates that our antibody was able to detect modified pRB, suggesting no technical reasons for the lack of bands corresponding to acetyl K873/K874 in the IP lanes.
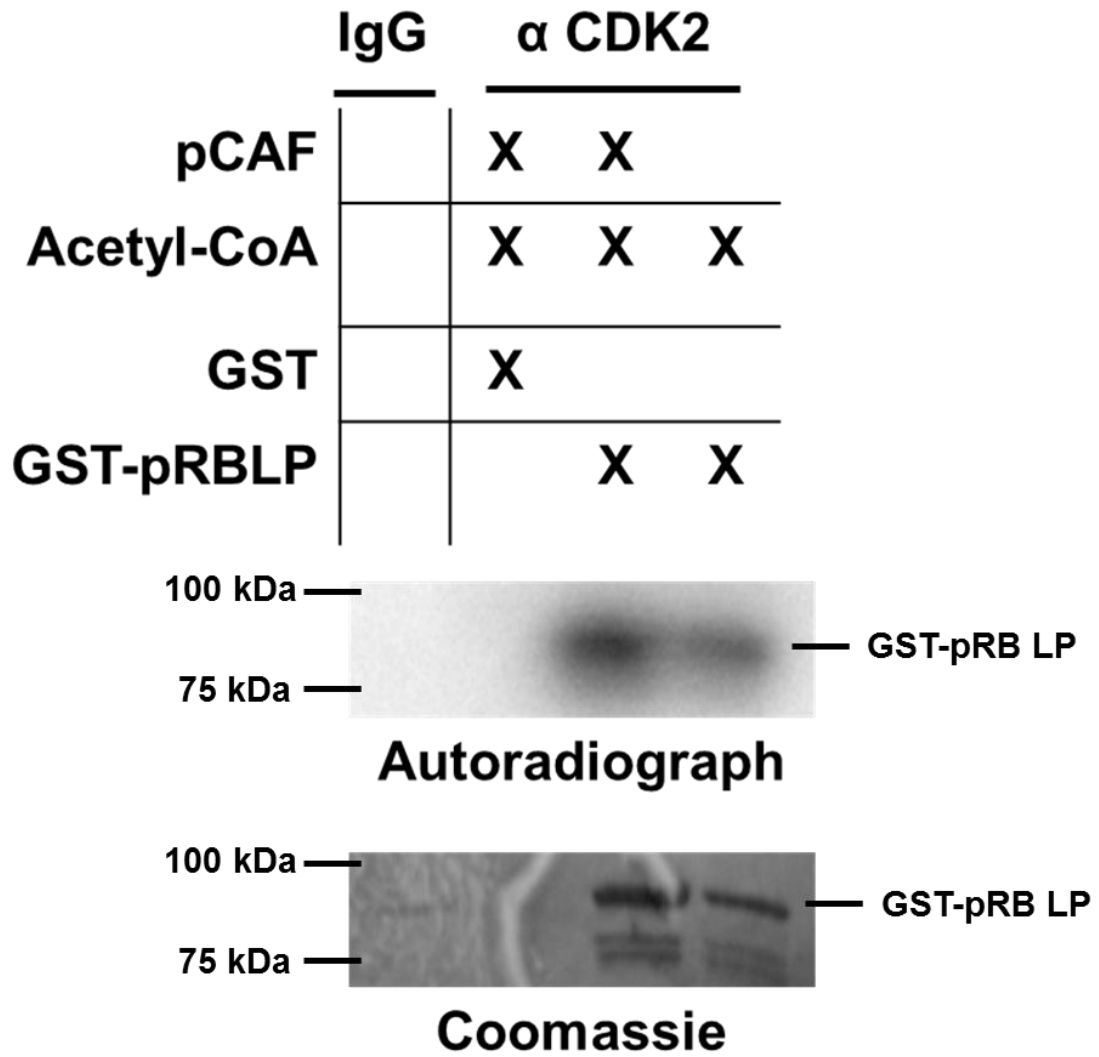
	IgG	α CDK2		
pCAF		X	X	
Acetyl-CoA		X	X	X
GST		X		
GST-pRBLP			X	X

Figure 3.7: CDK Phosphorylation of Acetylated GST-pRB LP

Kinase activity of CDK2 was determined against acetylated and unmodified GST-pRB LP substrates as described in the previous figure. 3 µg of GST-pRB LP substrates were exposed to immunoprecipitated CDK2 along with γ-^{32}P-ATP at 30°C for 20 mins. Samples were then resolved on a polyacrylamide gel. Phosphate incorporation was determined by autoradiography and relative protein levels are shown by Coomassie staining. N=1 for the autoradiograph and Coomassie for those presented in this figure.
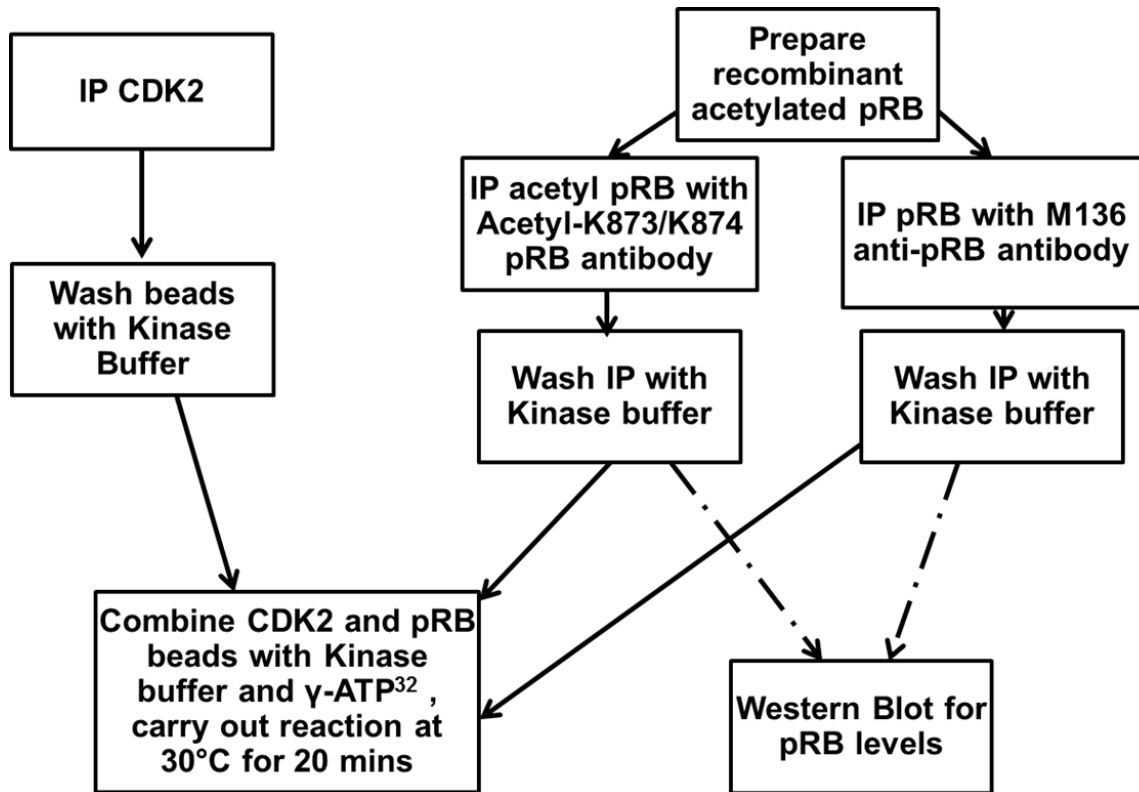
Figure 3.8: Schematic of Immunopurified Acetyl K873/K874-pRB Substrate Kinase Assay

Procedure used to immunopurifiy GST-pRB LP substrate that had undergone acetylation and its use as a substrate in a CDK2 kinase assay
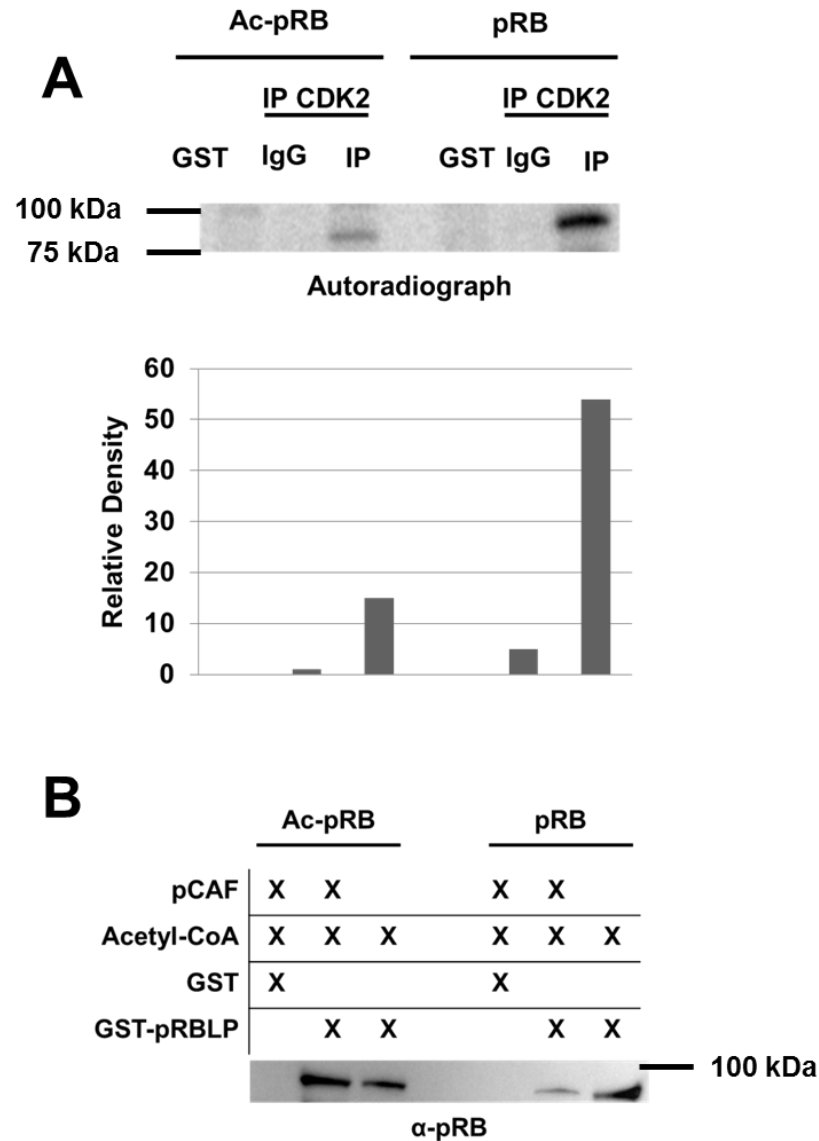
Figure 3.9: Acetylation of pRB Inhibits CDK Phosphorylation

A) Kinase activity of CDK2 was determined against acetylated and unmodified GST-pRB LP substrates as described in the previous figure. Substrates were exposed to immunoprecipitated CDK2 along with γ-^{32}P-ATP at 30°C for 20 minutes. Samples were then resolved by SDS-PAGE, a representative radiogram is shown with densitometric quantification below. **B)** Immunoprecipitation efficiency of acetylated and unmodified GST-pRB LP substrates by their respective antibodies was determined by Western blotting. Equal volumes were analyzed by SDS-PAGE followed by Western pRB with an independent antibody. N=1 for experimental data presented in this figure.

From these results, we next wanted to examine conditions from the literature that have been suggested to increase the accumulation of acetyl K873/K874 pRB. One of the conditions that have been suggested from prior work is double stranded DNA breaks induced by etoposide treatment (Carnevale et al., 2012; Markham et al., 2006). To examine DNA damage induced pRB acetylation of K873/K874, I treated ML1 cells with100 µM of Etoposide for 8 hours and performed GST-E7 pulldowns to enrich for pRB from these cells. Following GST-E7 pulldowns, Western blot analysis on the pulldown fractions of Etoposide treated and DMSO treated cells was performed and presented in Figure 3.11A. We observed that treatment of ML1 cells with Etoposide lead to an activation of p53 signaling as demonstrated by increased pS15-p53, and thus an activation of DNA damage signaling. From Western blot analysis presented in Figure 3.11B, we can observe that GST-E7 pulldown from these cells was sufficiently able to enrich for endogenous pRB. When pulldown fractions were then probed with the acetyl K873/K874 pRB antibody, we observed no detectable signal from either the input or pulldown fractions, suggesting that acetyl K873/K874 pRB was in fact not present as shown in Figure 3.11C. Further, through Western blot analysis using an acetyl-lysine antibody as depicted in Figure 3.11D, we observed a band in the input fraction that could correspond to pRB but this band was not replicated in the pulldown fraction, suggesting this band does not correspond to acetyl-pRB and agrees with the results demonstrated in Figure 3.11C. From these experiments, we can suggest that acetyl K873/K874 pRB is not enriched for in cells that have undergone DNA double strand breaks.

Based on previous work in our lab, it has been suggested that upon DNA double strand breaks induced by Etoposide, pRB and E2F1 complexes are formed that include acetylated pRB. I treated U2OS cells with 100 µM Etoposide for 8 hours and performed an immunoprecipitation for E2F1. Presented in top portion of Figure 3.12 is Western blot analysis for E2F1 demonstrating that the IP was successful while in the bottom portion of Figure 3.12 is Western blot analysis using acetyl K873/K874 pRB antibody. From this analysis, we can see that there is no significant enrichment of acetyl K873/K874 signal over IgG in either treatment group. This result suggests that under a scenario of DNA damage, acetyl K873/K874 pRB is not associating with a pRB bound E2F1 population.
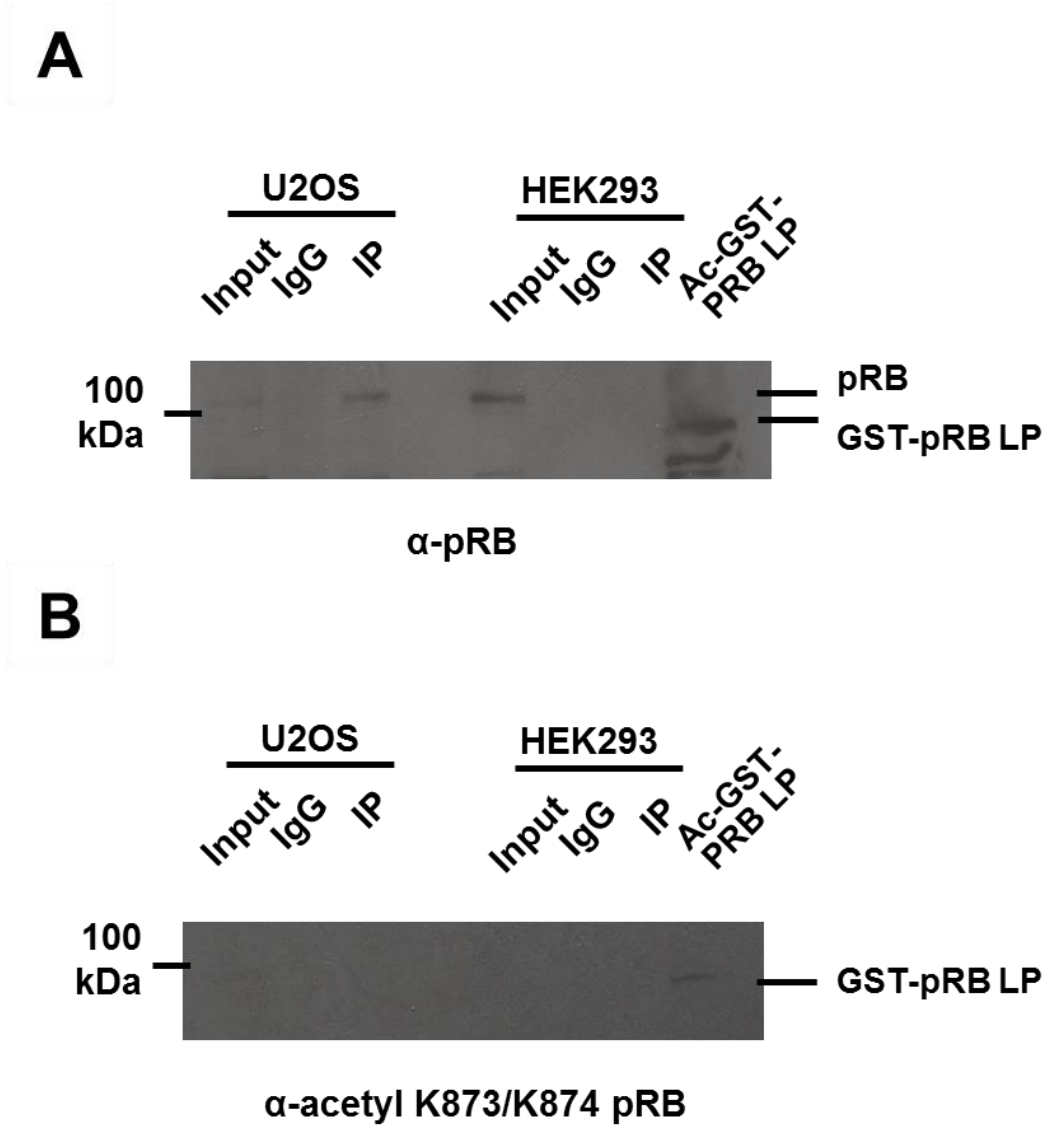
Figure 3.10: Unable to Detect Ac-pRB in Asynchronous Cells

U2OS and HEK293 nuclear extract was harvested from asynchronous proliferating cells. Immunoprecipitations were performed for pRB with 2 mg of extract from both sets and corresponding samples were resolved by SDS-PAGE with indicated western blots performed. 200 µg of nuclear extract was taken and loaded as input while 300 ng of Ac-GST-pRB LP was used as a positive control. **A)** A western blot against pRB using an antibody independent of the immunoprecipitation. **B)** Western blot with acetyl K873/K874 pRB antibody. N =1 for the blots presented in this figure.
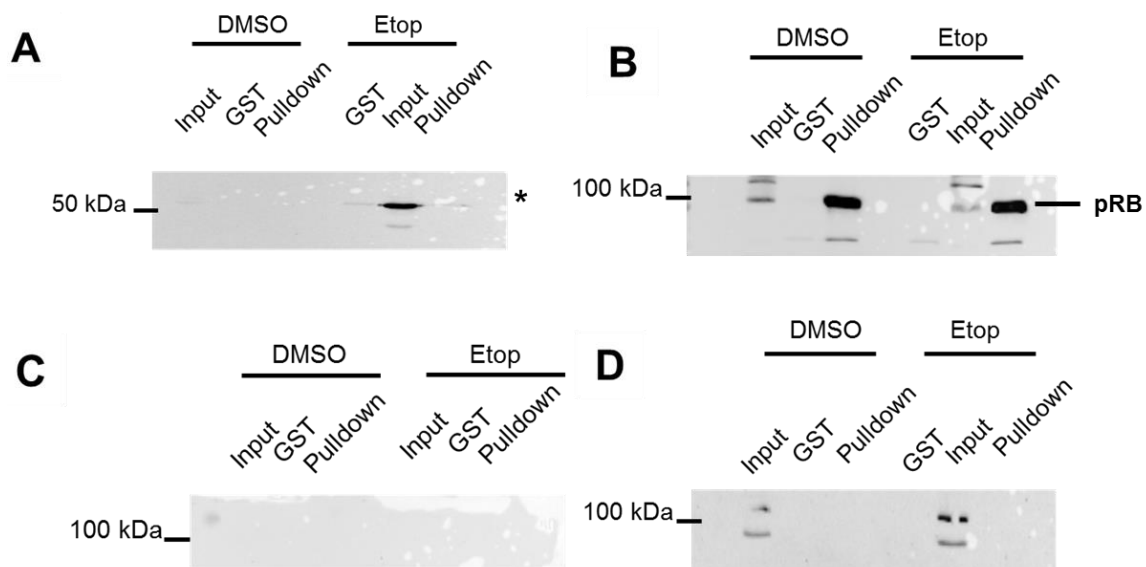
Figure 3.11: Unable to Detect Ac-K873/K874 pRB Following DNA Damage

GST pulldown binding experiment using recombinant GST-E7 with 2 mg of nuclear extract from ML1 cells treated with either 100 μM Etoposide (Etop) for 8 hrs or DMSO vehicle as control. 200 μg of nuclear extract from either DMSO or Etop were used as the corresponding input. **A)** Western blot for pS15 -p53 to demonstrate that Etoposide treatment lead to an increase in DNA damage signaling. Bands indicated with the * correspond to pS15 p53. **B)** Western blot for pRB to demonstrate that the recombinant GST-E7 was able to successfully pulldown pRB from these cells and to demonstrate similar loading between treatment groups. **C)** Western blot for ac K873/K874-pRB using purified antibody from both treatment groups. **D)** Western Blot for acetyl lysine from both treatment groups. N =1 for all the blots presented in this figure.
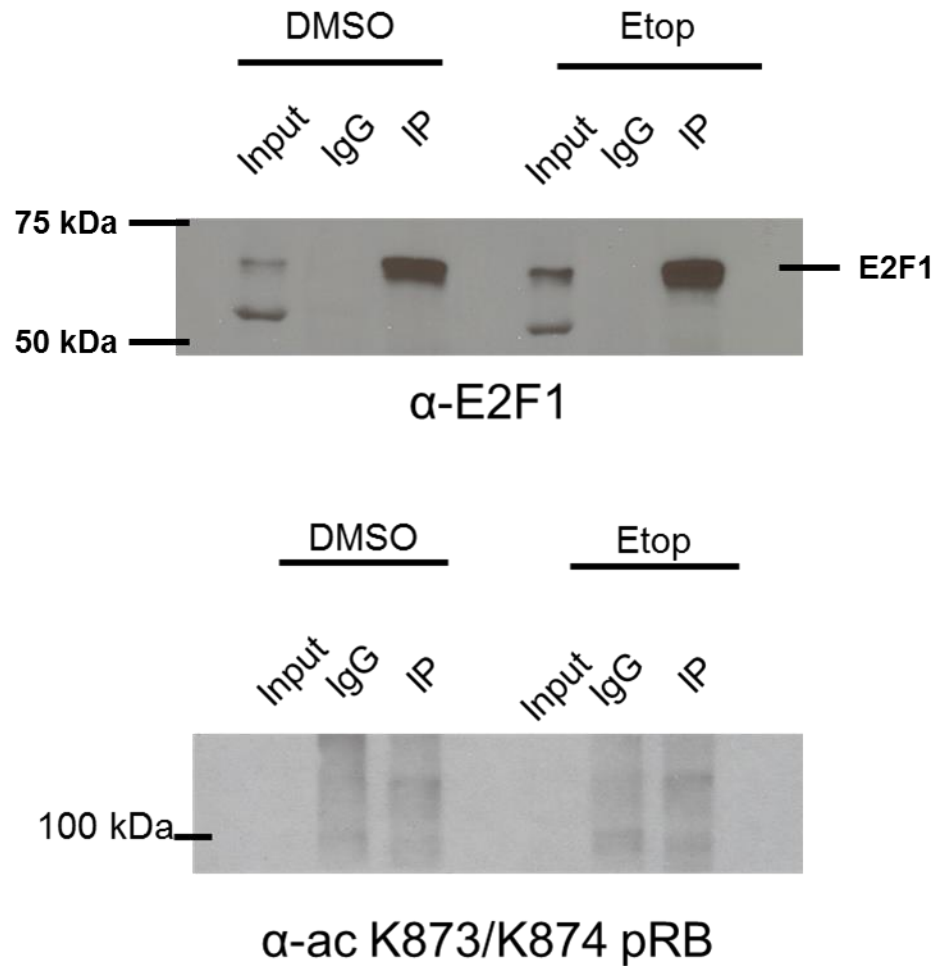
Figure 3.12: **Unable to Detect Ac-K873/K874 pRB Binding to E2F1**

E2F1 immunoprecipitations were performed from U2OS cells treated with 100 μM etoposide for 8 hrs or DMSO vehicle control. 2 mg of nuclear extract were collected from each treatment group with 200 μg of extract used for input. **A)** Western blot for E2F1 confirming immunoprecipitation from both treatment groups. **B)** Western blot for ac-K873/K874-pRB is performed on each immunoprecipitated fraction using purified antibody. N= 1 for both blots presented in this figure.

3.6　　References

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Chapter 4

4 The $Rb1^{\Delta PP1}$ Mouse

4.1 Generation of Rb1ΔPP1 Gene Targeted Mouse Model

Previous work in our lab has identified a specific mutation in the pRB C-terminus, identified as R876F/ F877R, which is able to disrupt Protein Phosphatase 1 binding while retaining CDK2 binding in the analogous region (Hirschi et al., 2010). This mutation allows for the study of PP1 binding in the pRB C-terminus and its direct function in regards to pRB while still maintaining proper CDK phosphorylation of pRB. To study this potential means of phosphorylation regulation of pRB, I set out to create a gene targeted mouse which incorporated the analogous mouse mutation R869F/F870R into the mouse *Rb1* gene and we designate this allele of *Rb1* the $Rb1^{\Delta PP1}$ mutation. As presented in the schematic in Figure 4.1B**,** this mutation in the *Rb1* allele would potentially allow for a mutant of pRb that would discretely disrupt PP1 mediated activation of pRB while maintaining CDK phosphorylation. This theoretically could alter the equilibrium between these states and force pRb towards a more hyperphosphorylated form in this gene targeted mouse model.

To generate this mouse model for $Rb1^{\Delta PP1}$, detailed in Figure 4.1A is a schematic detailing the work flow associated with generating this mouse model. The first step was the creation of the targeting vector to allow for homologous recombination of our desired mutation into the mouse *Rb1* locus. We used a previous method to target a nearby region in *Rb1* as a basis for our strategy, which is summarized in Figure 4.2 detailing the $Rb1^{\Delta PP1}$ targeting construct in relation to the *Rb1* locus (Chau et al., 2002). A 12 kb portion of the 3' end of the *Rb1* allele was used as a means to facilitate homologous recombination, allowing for integration of our mutation into the *Rb1* gene. The R869F/F870R mutation was inserted into exon 25 along with an *Eco*RI restriction enzyme digest site in intron 24. The addition of this *Eco*RI site allows for differentiation between targeted alleles and wildtype alleles when performing the Southern blot screening. A PGK-Neo cassette flanked by LoxP sites was then inserted into intron 25 to

allow for neomycin selection of mouse embryonic stem cells with integration of the targeting vector into the mouse genome. The completed targeting vector was linearized using a NotI restriction digest and provided to the London Regional Transgenic and Gene Targeting facility where this construct was electroporated into mouse embryonic stem cells with clonal expansion of the neomycin resistant cells.

4.2   Southern Blot Screening Lead to a Small Selection of Potential Targeted Clones

To determine whether mESC clones had properly incorporated the $Rb1^{\Delta PP1}$ targeting construct into the mouse genome, I performed Southern Blot screening to determine proper targeting of the *Rb1* locus. 180 mESC clones which had been expanded and selected in neomycin were used for Southern blot screening, with genomic DNA isolated from each clone. Genomic DNA for each clone was digested using the *Hin*dIII restriction enzyme and electrophoresed on an agarose gel. Following transfer onto a nitrocellulose membrane, a radiolabelled probe for Exon 24 of the mouse *Rb1* gene was used as a probe. Membranes were then washed and exposed for autoradiography and images were developed. Presented in Figure 4.3A is a representative blot of screening using the Exon 24 probe with the *Hin*dIII restriction digest and from this blot we can observe that the clones labeled 1-5F and 1-2D both show a doublet of bands. Clone 1-2D shows the expected bands at 6 kb and 9 kb indicating a properly targeted clone. Clone 1-5F had an observable pair of bands, though it appears that the bands were at higher than the expected size. This aberrant size of the observable doublet could have been due to unusual migration of digested DNA due to contaminates in the DNA, such as excess salts. Further screening was carried out and as shown in Figure 4.3B, clone 4-4A when digested with *Hin*dIII and subjected to Southern blot analysis with the Exon 24 probe, produced an observable doublet of bands at 6 and 9 kb. This doublet pattern again is representative of the correct size distribution expected from a correctly targeted *Rb1* locus. From this comprehensive screening of 180 mESC clones, two clones offered potential as correctly targeted clones. These candidate clones, along with others, were selected to be regrown from cellular stocks to be subjected to more rigorous screening to confirm the presence of the $Rb1^{\Delta PP1}$ allele.
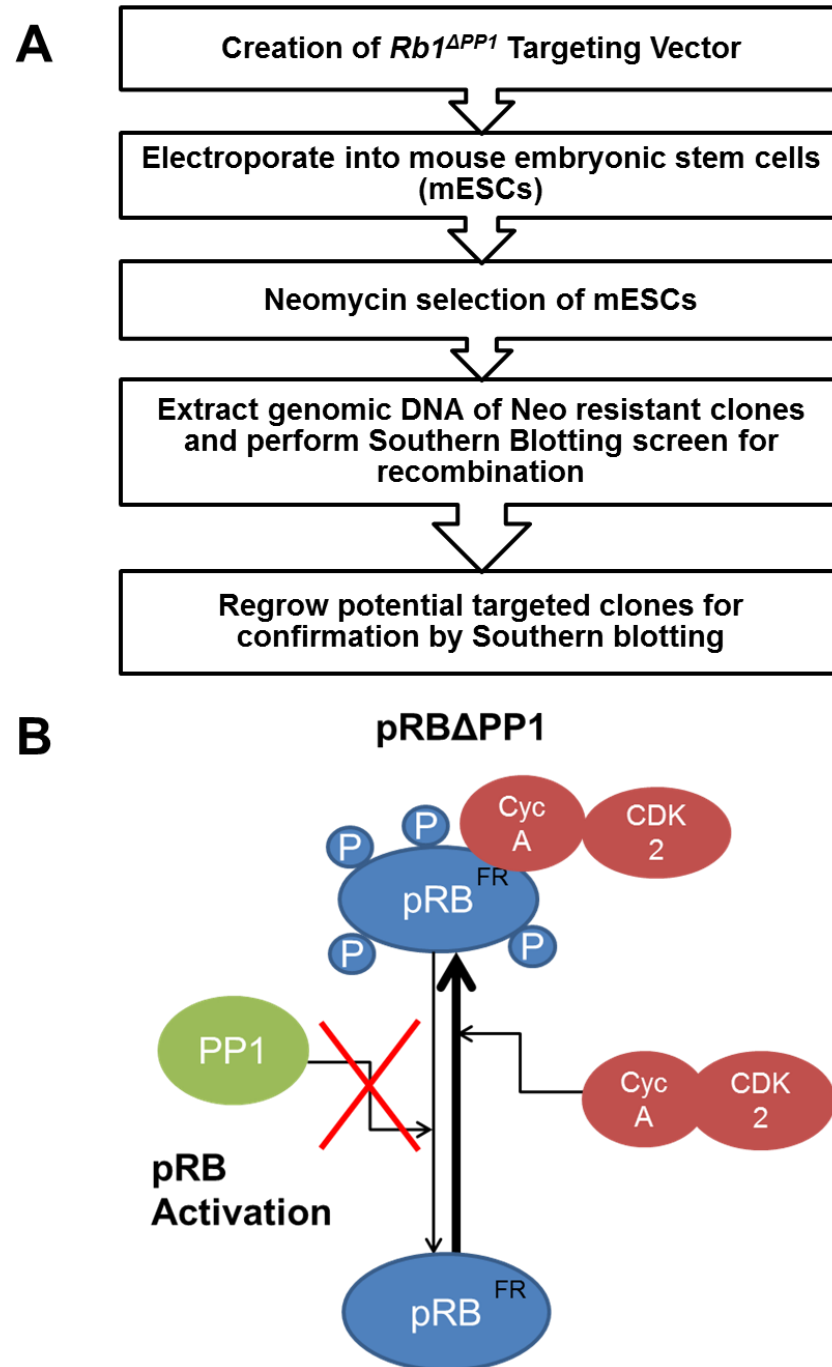
Figure 4.1: The *Rb1*^{ΔPP1} Mutation and Schematic of Targeting for *Rb1*^{ΔPP1} Gene Targeted Mice

A) General schematic for the process of generating a gene ES line that would contain the ΔPP1 mutation in the *Rb1* gene. **B)** Schematic depicting the potential molecular consequence of this mutation on the pRb protein.
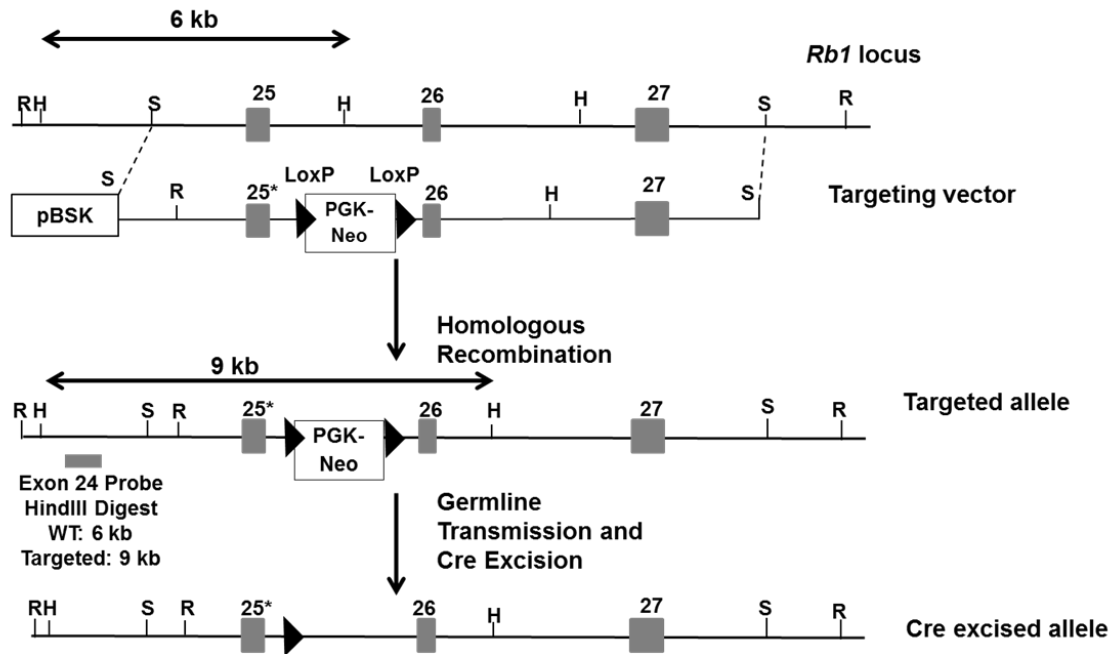
Figure 4.2: Schematic of Targeting for *Rb1*^ΔPP1 Mutation in the *Rb1* Locus

Following electroporation of 129 ES cells with the targeting construct, clones were screened by Southern blotting. Initial screening of ES clones was carried out using a HindIII digestion to give a wildtype fragment of 6 kb versus 9 kb for targeted alleles. B-*Bam*HI, H-HindIII, R-*Eco*RI, S-SacI, 25*-Exon 25 with ΔPP1 mutation, 25- Exon 25, 26-Exon 26, 27- Exon 27
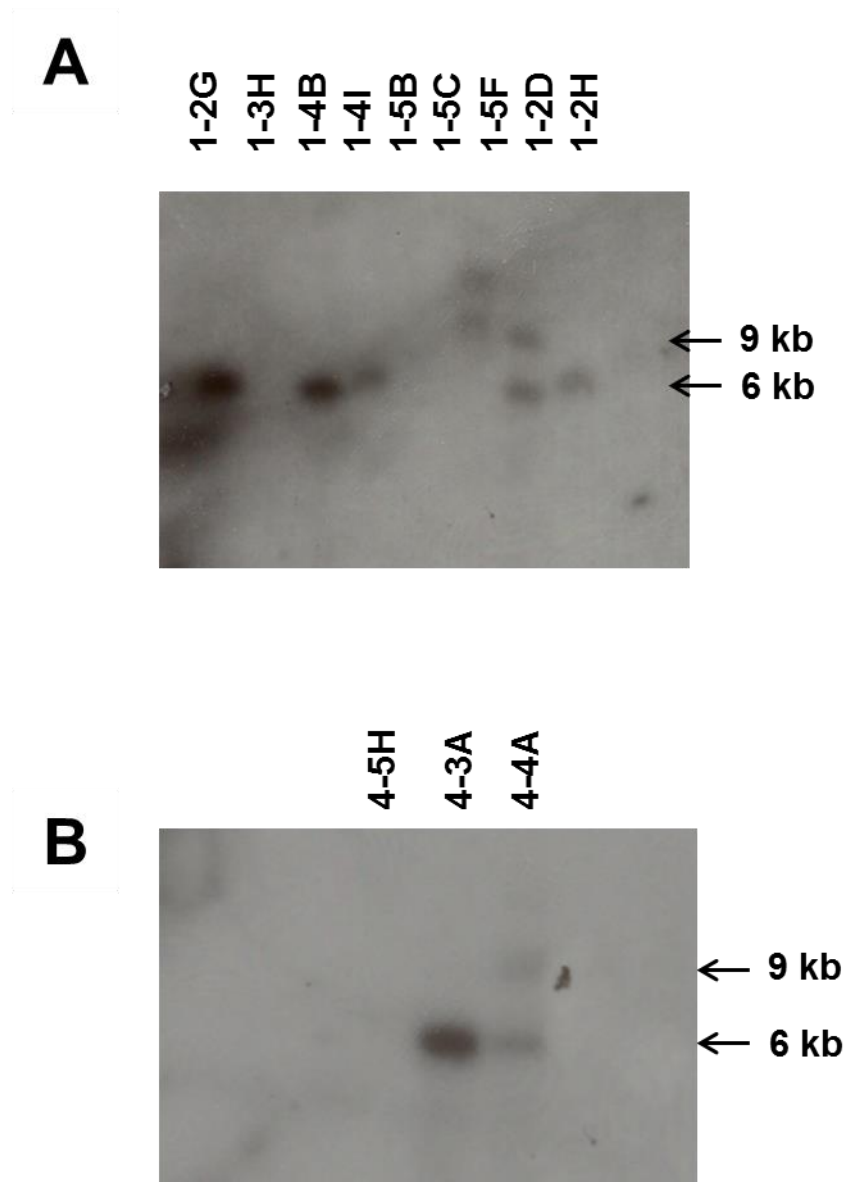
Figure 4.3: Screening of mESCs with Southern Blotting for Incorporation of the *Rb1*^ΔPP1 into the *Rb1* Locus

Incorporation of the ΔPP1 mutation into the Rb1 locus, through homologous recombination, was determined by Southern blotting. **A)** A representative image shows southern blot screening using the Exon 24 probe and a HindIII digest. **B)** A representative image shows Southern blot screening using the Exon 24 probe and a HindIII digest. N =1 for both Southern blots presented in this figure.

4.3   Confirmation Screening of Candidate mESC Clones Showed No Correctly Targeted Clones

To ensure that our candidate mESC clones were in fact targeted and usable for blastocyst injections, a more rigorous round of screening was performed to confirm our results from the mass screenings. The indicated mESC clones were regrown in neomycin selection medium and genomic DNA was extracted. Southern blot screening was carried out as a means to confirm proper single integration of the targeting construct into the mouse *Rb1* locus. Southern blots were performed using genomic DNA digested with *Hin*dIII restriction enzyme and probed using a radiolabeled probe corresponding to the neomycin resistance cassette. From this screening, we observed that the candidate clones showed single integration of the neomycin cassette into the mouse genome (Figure 4.4). With this result, I then set out to carry out the confirmation screening using the Exon 24 probe with the *Hin*dIII digest and a *Eco*RI digest. As shown in Figure 4.5A, when the *Hin*dIII digest was carried out and the radiolabelled Exon 24 probe was used in Southern Blot screening of the three indicated mESC clones, it can be observed that all three clones showed the presence of only the 6 kb band, which corresponds to the wildtype *Rb1* locus. In coordination with this screening, the *Eco*RI digest was performed and Southern blot analysis was performed using the Exon 24 probe which is presented in Figure 4.5B. From analysis of this Southern blot we observed that all three clones displayed a band at approximately 20 kb. This 20 kb fragment corresponds to the wildtype *Rb1* locus, whereas a correctly targeted clone would have a 9 kb fragment when subjected to Southern blotting with the Exon 24 probe and the *Eco*RI digestion. From these results, we can conclude that of the three candidate clones isolated from our initial mass screening of 180 mESC clones, none were in fact correctly targeted when subjected to this second round of more rigorous screening.
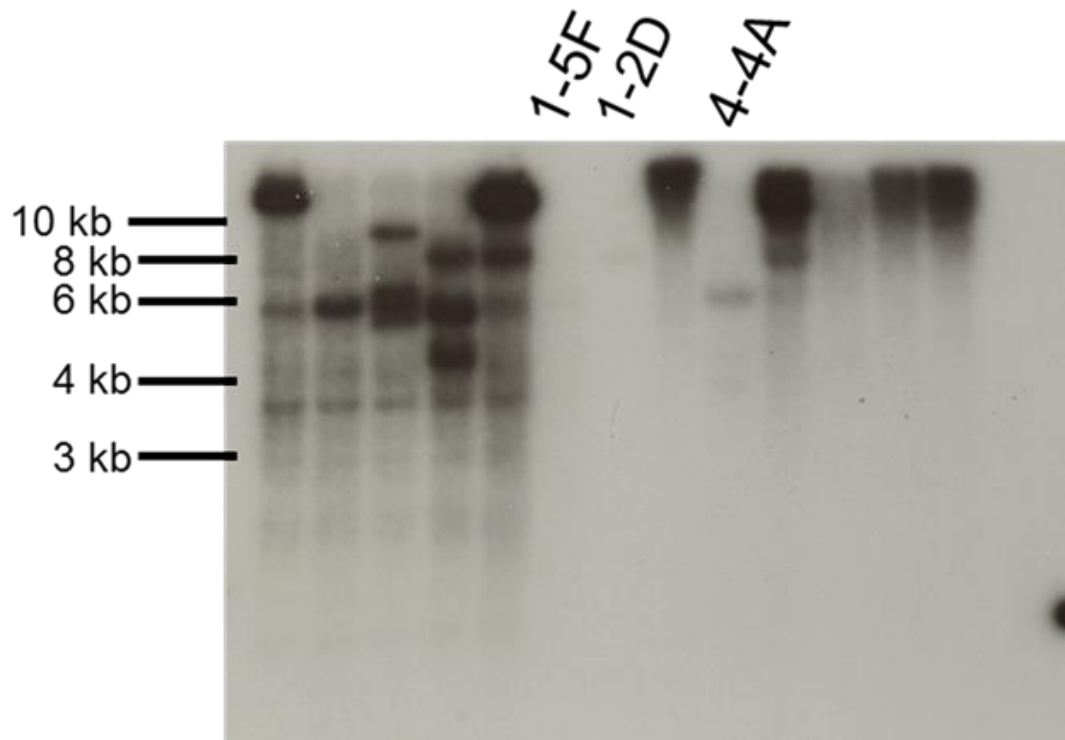
Figure 4.4: Single Integration of the Neomycin Selection Cassette in Candidate Clones

Single incorporation of the Neomycin selection cassette, through homologous recombination, was determined by Southern blotting. Southern blotting using the Neo probe following *Hin*dIII restriction digest of isolated genomic DNA was performed for mESC clones which had been selected for confirmation screening. Potential candidate clones are indicated. N = 1 for Southern blot screening presented in this figure.

A

Targeted allele —
WT allele —

| 4-4A | 1-2D | 1-5F |

—10 kb
— 8 kb
—6 kb
—5 kb

B

| 4-4A | 1-2D | 1-5F |

—WT allele

10 kb —
8 kb —

—Targeted allele

6 kb —
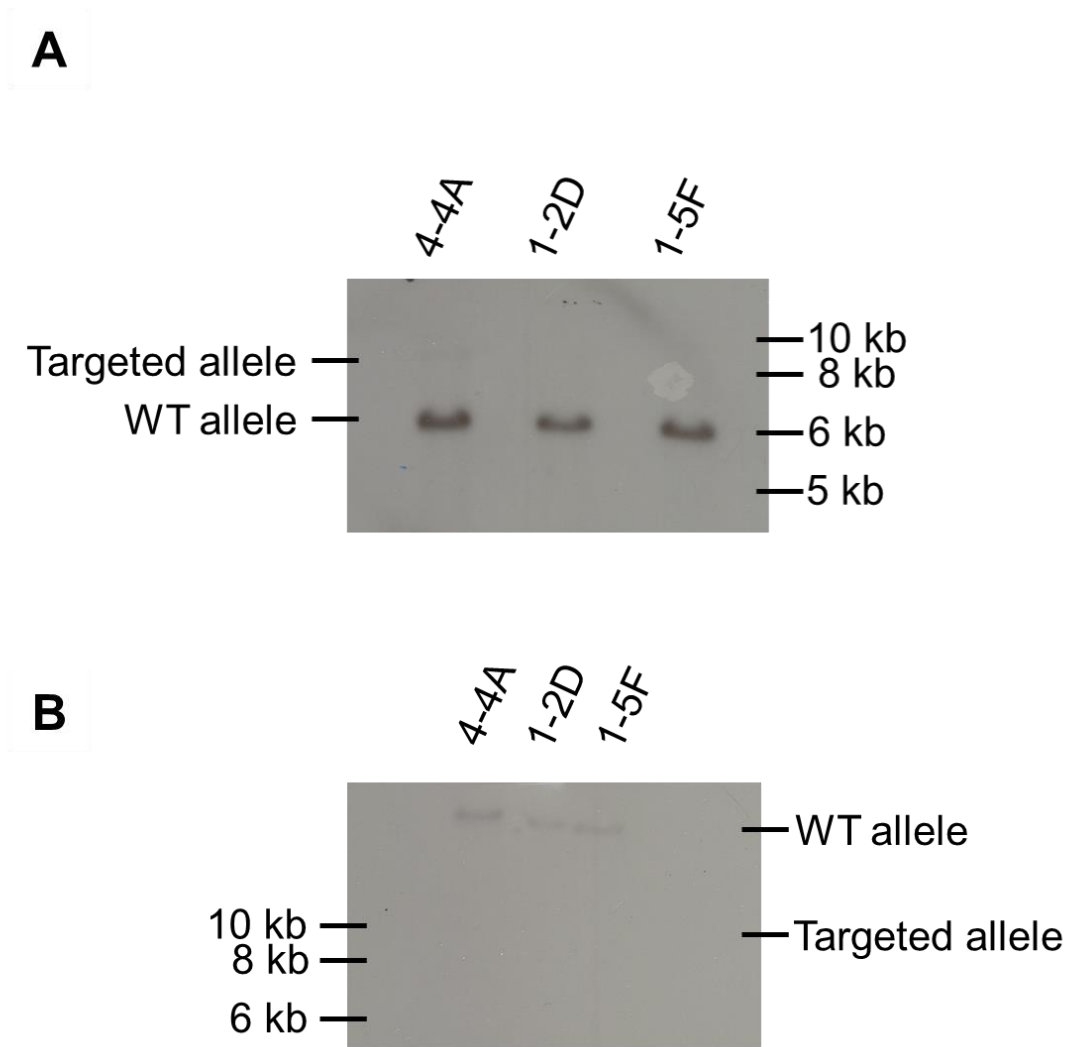
Figure 4.5: Confirmation Southern Blot of Three Potentially Targeted mESC Clones

Selected mESC clones were regrown and genomic DNA was isolated and southern blot screening was carried out to determine if potential clones were correctly targeted. **A)** The indicated mESC clones were digested with HindIII and southern blot was performed using the exon 24 probe. **B)** The indicated mESC clones were also digested with *Eco*R1 and southern blot was performed using the exon 24 probe**.** N = 1 for Southern blot screens performed in this figure.

4.4 References

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5   Discussion

5.1   Summary of Findings

The retinoblastoma protein has been extensively studied in regards to post-translational modification primarily through phosphorylation by CDKs. More recent work has begun to emerge in the field that has begun to focus on other post-translational modifications of pRB and the potential for added complexity in regards to the multitude of pRB functions. One potential means for further regulation of pRB function may lay in acetylation in the C-terminus of pRB at lysine residues 873 and 874(Chan et al., 2001). These particular lysine residues lie directly in a binding domain for both Cyclin/CDKs and PP1(Hirschi et al., 2010). I hypothesized that the regulation of pRB dephosphorylation is critical in regulating pRB function and activity and that acetylation of lysines 873 and 874 in the pRB C-terminus may play a role in the regulation of pRB dephosphorylation. From the work presented in this thesis, I have shown the generation of an acetyl K873/K874 pRB antibody which specifically detects acetylation of K873/K874 in the pRB C-terminus through the use of *in vitro* assays. Through the use of these assays I was able to demonstrate that CDK2 kinase activity towards pRB, which had been acetylated at K873/K874, was in fact reduced when compared to unmodified pRB. *In vivo* assays that were used to isolate conditions where acetyl K873/K874 pRB has been suggested to occur, such as DNA damage, were then assessed to act as positive control conditions for future work(Carnevale et al., 2012; Markham et al., 2006). From the results presented in Figures 3.9-3.11**,** we interpret that with our antibody, we were unable to detect acetylated K873/K874 pRB through enrichment of pRB or through association with E2F1 under either asynchronous or DNA damage through double stranded breaks.

Regulation of phosphorylation of the retinoblastoma protein has largely been studied by the means of regulating Cyclin/CDK phosphorylation of pRB. Recent work from our lab though has uncovered an overlapping CDK and PP1 binding domain in the C-terminus of pRB. This overlapping binding site offers a novel and compelling means of discrete regulation of pRB, by the means of competitive access between the respective kinase and phosphatase. From previous work in our lab, a mutation in this binding site was uncovered that was able to specifically disrupt PP1 binding to pRB without

disrupting CDK binding to the pRB C-terminus. This mutation in the pRB C-terminus is distinguished as the pRB$^{\Delta PP1}$ mutation and allows for discrete study of pRB dephosphorylation uncoupled from CDK phosphorylation. In this thesis, I detailed work outlining the construction and generation of a targeting construct that would allow for gene targeting of the mouse *Rb1* locus with the pRb$^{\Delta PP1}$ mutation. As presented and shown in this work, through Southern blot screening, we were able to see clones that appeared to be targeted but through screening confirmation using regrown mESC clones and southern blotting, these clones appeared to lose the targeted allele.

As mentioned previously in my introduction, the study of pRB dephosphorylation has been characterized previously in the literature but to a much lesser extent than CDK phosphorylation of pRB. This work though has typically lacked insights into how phosphatases are specifically recruited to act on pRB, in particular cellular contexts of stress distinctively highlight our lack of understanding into the mechanistic basis of pRB dephosphorylation. The work highlighted in this thesis is a novel approach in regards to studying pRB with respect to dephosphorylation, particularly through a study of post-translational modification and the PP1/CDK binding site within pRB. This study helps us to gain further insight into the process of dephosphorylation, and highlights the regulatory elements such as the PP1 C-terminal docking site and pRB acetylation that may play an important role in this process.

5.2   Acetylation of pRB

Our observations in respect to the lack of pRB acetylation at K873 and K874 *in vivo* through western blotting using our anti-acetyl K873/K874 pRB antibody was a stark contrast to the reported literature findings. A critical examination of the body of literature with respect to pRB acetylation at K873 and K874 though may provide insights in regards to our observations. Previous work including (Chan et al., 2001) regarding acetylation of pRB identified lysine residues 873 and 874 as acetylation sites through similar *in vitro* acetylation assays. The observations noted in Chapter 3 of this thesis are similar to those documented by Chan and colleagues, including the need to selectively purify acetylated pRB substrate to demonstrate biochemical effects such as reduced CDK phosphorylation. Interestingly from this work, experiments confirming that these sites

were indeed present *in vitro* are lacking substantial *in vivo* verifcation to confirm that K873 and K874 are the acetylation sites responsible for the observed effects. Most *in vivo* experiments used to confirm the presence of these modifications are reliant on the use of immunoprecipitations and western blotting using pan-acetyl-lysine antibodies, similar to those presented earlier in this work. Anecdotally, these antibodies appear to be challenging to present reliable data that can be easily interpreted by the user, which can be noted from discrepancies of Western blots presented in Figure 3.5B and Figure 3.6 with regards to western blots with anti-acetyl lysine antibodies. Most of the previously discussed work in the literature is also limited to transfection based experiments involving isolation of tagged pRB populations and western blotting with acetyl lysine antibodies. These experiments highlighted from previous reports raise the questions of how representative these experimental conditions are to more physiological experiments. As discussed in the introduction, various proteomic approaches have highlighted multiple acetylation sites throughout pRB. These acetylation sites in pRB could have been falsely attributed to acetylation at K873 and K874 primarily due to most experiments having to rely on acetyl-lysine antibodies for detection of K873/K874 acetylation. This ambiguity regarding the exact conditions of K873/K874 acetylation leads to the obvious question of whether the conditions we attempted to observe this set of modifications, specifically DNA damage signalling induced by double stranded breaks, were in fact the best choice to find this set of modifications. While the work of Markham and colleagues (Markham et al., 2006) suggest that pRB under DNA damage is acetylated on K873/K874 and promotes association with E2F, other work has shown more generalized results attributed to acetyl lysine blots or association with acetyltranferases including p/CAF (Carnevale et al., 2012; Ianari et al., 2004; Ianari et al., 2009). The later reports again leave open the possibility that in fact acetylation of pRB observed under DNA damage scenarios could be attributed to other sites in pRB or in fact other proteins involved in large protein complexes. A further layer of complexity regarding K873/K874 acetylation lies in the lack of proteomic evidence for K873 acetylation under the admittedly limited conditions investigated, as well as these studies showing no occurrence of both K873/K874 on pRB peptides analyzed. This ambiguity that underlines the prior literature draws into question

whether K873/K874 acetylation is in fact present under the conditions of DNA damage that we examined or in fact whether both lysine residues are in fact acetylated *in vivo*.

5.3   Lack of K873/K874 Acetylation of pRB In Vivo Under DNA Damage with Acetyl-K873/K874 Antibodies

The lack of commercial reagents towards K873/K874 acetylation has further hampered their study, with present knowledge suggesting one other group attempting to generate any type of antibody against this set of modifications. Prior work by Markham and colleagues (Markham et al., 2006) demonstrated the production of a similar antibody against this modification, though work using this antibody is limited to immunofluorescence and western blotting with mixed results. Our hypothesis that pRB phosphorylation could be regulated through the acetylation of K873 and K874 through acetylation motivated us to generate a specific antibody against the combination of acetylated K873/K874. Through the *in vitro* work presented to characterize our antibodies against acetylated K873/K874 demonstrated specificity for the tandem acetylation by means of Western blotting, we were unable to detect acetylation with our *in vivo* experiments. One explanation for this is technical limitations of the presented experiments in this work, either through scale of the experiment with respect to amounts of material used or limits of detection of substrate in regards to western blotting. Ideally, future experiments involving mass spectrometry using cells that have been subjected to DNA damage would be the best scenario to test for the presence of K873/K874 pRB acetylation and whether these modifications occur in tandem.

The lack of evidence for pRB acetylation at K873 through proteomic approaches compared to the more targeted approaches to studying post-translational modifications creates a conflict of whether in fact K873 is acetylated in physiological paradigms. Further analysis of K873 presents literature that acts to further confound this issue, as pRB has been shown previously to be methylated on K873 and not on K874(Munro et al., 2010). While DNA damage conditions are not specifically analyzed in this report for K873 methylation, the author's report that methylation at this site seems to be a requirement of proper *in vitro* myoblast differentiation and senescence of cells. The requirement of K873 methylation in myoblast differentiation mimics a prior report

(Nguyen et al., 2004) that states K873/K874 acetylation is required for myoblast differentiation. With similar conditions attributed to both modifications, this prompts us to speculate whether prior results of K873/K874 acetylation observed under DNA damage could in fact be K873 methylation with K874 acetylation. Thus the possibilities that under DNA damage, pRB could be acetylated at only K873, methylated at K873 or a combination of both modifications could be present on pRB in this scenario. The circumstances where different single site modifications or combinations of acetylation present obvious issues in regards to our generated antibody. At this time, we are unable to rule out whether our acetyl K873/K874 pRB antibody is able to detect instances where only K874 is acetylated, K873 is methylated or the possible instance where K873 is methylated and K874 is acetylated. To further elucidate the specificity of our generated antibody against these specific combinations of post-translational modifications, one could envision performing similar ELISA experiments highlighted in Figure 3.5, with a range of synthetically modified peptides baring the various combination of modifications to test whether our antibody is able to recognize these epitopes. The generation of a panel of antibodies against the different combination of modifications and performing similar experiments against DNA damaged cells would be worthwhile to further explore whether these combination of modifications are responsible for the previously reported studies.

The work outlined in this thesis helps us to further build an understanding of the intricate nature of post-translational modifications in regards to pRB. While historically pRB has been regarded for the general system of widespread phosphorylation by CDKs, even this paradigm has begun to be challenged. As summarized in recent review articles, work in the field has led to the emergence of a host of post-translational modifications of pRB that could act in concert to regulate the multitude of pRB functions (Macdonald and Dick, 2012; Munro et al., 2012). With the emergence of better analytical techniques and proteomic approaches to study post-translational modifications of proteins, we are beginning to see the higher order complexity involved in managing and regulating the wide cascade of post-translational modifications. This work begins to outline acetylation of pRB at K873 and K874 in the pRB C-terminus and the potential for a much more complicated system of post-translational modifications that may be context dependent.

5.4 Attempted Creation of the Rb1ΔPP1 Gene Targeted Mouse

In chapter four of this thesis, I detailed work to produce a targeting construct to generate gene targeted mice which harboured a mutation in pRB which we deem the ΔPP1 mutation. This mutation in pRB has been previously characterized in our lab to disrupt Protein Phosphatase 1 binding in the C-terminus of pRB in a region where Cyclin/CDKs have been shown to bind pRB without disrupting Cyclin/CDK binding (Hirschi et al., 2010). This mutation presented us with a unique means of studying the process and regulation of pRB dephosphorylation in an *in vivo* model system. Much of the work to date characterizing pRB dephosphorylation *in vivo* has relied on drug treatments targeting phosphatases, leaving the direct consequences of deregulated pRB dephosphorylation difficult to interpret due to the wide swath of cellular functions attributed to protein phosphatases (Moorhead et al., 2007). Thus our approach of generating a gene targeted mouse disrupting PP1 binding to pRB would allow us to study the consequences of pRB dephosphorylation in isolation with respect to other PP1 cellular functions

Through construction of our *Rb1^{ΔPP1}* targeting construct we envisioned the ability to isolate mESC clones which harboured our mutant allele of pRB. The strategy used to build our targeting construct was based off work in the Wang lab, which generated a gene targeted mouse targeting residues in exon 25, similar to our mutation (Borges et al., 2005; Chau et al., 2002). This gene targeted mouse, along with pRB gene targeted mice established in our lab had no issues with viability and are able to create viable mice, thus we hypothesized there would be no issues with the allele recombination and that these mice would be viable(Cecchini et al., 2014; Isaac et al., 2006). Through Southern blot analysis of mESC clones electroporated with our targeting construct, our initial screening identified two candidate clones which appeared to have proper targeting of the *Rb1* gene. When our confirmation screening was carried out, surprisingly we found that our three clones appeared to not have the targeted allele.

These results were surprising to us as we had expected little to no issue with generating the targeted mESC cells based on prior experiences in our lab and as well as work done by the Wang group, as outlined in the preceding paragraph. While we cannot

provide definitive reasons for the odd behaviour exhibited through our work targeting the PP1 binding site within pRB, I offer up some speculative and circumstantial reasoning why this phenomenon was observed in our hands. Recent literature that emerged during screening of our mESC clones highlights the potential regulatory role of pRB in stem cells. From this work, though mostly done in hESCs, presented the idea that inactivation of pRB forces hESCs to either undergo differentiation or have apoptotic programs activated. Furthermore, the authors suggest that a pool of hypophosphorylated or active pRB is maintained, even in systems where hESCs are still proliferating and maintaining stem-like qualities (Conklin et al., 2012; Sage, 2012). The introduction of our pRBΔPP1 mutation into mESC cells, with the effect of disrupting the dephosphorylation of pRB and subsequent activation may have a much larger role in this cell population than initially thought of by disrupting the balance of pRB activity required in these cells. The disruption of this small pool of activated pRB may have the unintended effect of forcing these cells to differentiate or in fact promoting apoptosis in these cells, hindering our ability to clonally expand and isolate correctly targeted cells.

As previously mentioned above, the targeting strategy for our allele was adopted from the method used by the Wang lab to develop mice which removed a caspase cleavage site in this region of pRB(Borges et al., 2005; Chau et al., 2002). One detail that initially was not considered when creating this construct was the orientation of the neomycin cassette used for selection of targeted cells in our system. The method used by the Wang lab when constructing their construct left the Neo cassette in the same orientation as the *Rb1* gene, allowing both the pRb transcript and the Neo transcript. This production of the pRb transcript and subsequent protein product allows for the active expression of our mutant protein in the mESC cells, whereas the alternative orientation of the Neo cassette would primarily have Neo transcript produced without mutant pRb transcript. Thus in our system, pRbΔPP1 protein could be expressed in our mESC cells during initial selection. The expression of the mutant pRb in this context could easily be envisioned to create a disruption in the balance of inactivated hyperphosphorylated pRB versus activated hypophosphorylated pRB in these mESCs that were in fact correctly targeted. This imbalance could be predicted to lead to either cells being selected for differentiation or for apoptosis. This would mirror the effects we observed where as we

continued to passage the potentially targeted mESC clones, we lost the targeted allele when genomic DNA was analyzed using Southern blots. Thus we could speculate that this loss of the targeted allele could be attributed to positively targeted cells undergoing differentiation or apoptosis, and thus being lost over subsequent passages.

From this speculation into our failed targeting of the pRbΔPP1 mutant, some identifiable caveats to our targeting have potentially emerged that could be mitigated to some degree. One potential means of allowing for easier targeting would be to reverse the orientation of the Neomycin cassette, thus restricting the ability for our mutant allele to be transcribed until later stages of the process, where targeted mice are bred with mice expressing Cre recombinase which cleaves the LoxP elements to remove the Neo cassette.

This work detailing our attempts to generate the pRbΔPP1 mouse model to study targeted disruption of PP1 binding to pRb and potentially deregulating pRb dephosphorylation offers a potentially exciting insight into this relatively understudied process in regards to pRb function. From our results, it may appear that regulation of PP1 binding in pRb by means of the C-terminal binding site within pRB may be critical to mESC and potentially all stem like cells.

5.5   Conclusions

The work presented in this thesis help to begin to elucidate potential means of regulating pRB dephosphorylation, which is a critical process in activating pRB, specifically in scenarios such as DNA damage. The work presented here in regards to acetylation of pRB at K873 and K874 which lie in a binding domain for PP1 with pRB, potentially show a much greater complexity than initially reported. While the potential post-translational modification regulatory system for the PP1 and CDK binding domain may be more complicated than initially hypothesized, the need to understand regulation of this site may be critical in regards to fully understanding PP1 mediated dephosphorylation of pRB. From our work described within this thesis to generate a gene targeted mouse model to examine disruption of the PP1 binding site in pRB, we have potentially isolated

a critical means of controlling pRb dephosphorylation and activation that when perturbed, may have deleterious effects in mESC stem cells.

5.6   References

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